Spermatogonial stem cell preservation in boys with Klinefelter syndrome: to bank or not to bank, that's the question

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Although early development of testis appears normal in boys with Klinefelter syndrome (KS), spermatogonial stem cell (SSC) depletion occurs in midpuberty, leading to infertility. Therefore, freezing of semen samples or testicular tissue sampling could be offered to boys with KS at onset of puberty. However, only in about half of patients with KS, adult or prepubertal, spermatozoa or SSCs can be observed, and to date, no clinical parameters are available to detect patients who might benefit from these techniques. Furthermore, strategies for the further use of the cryopreserved material are still under investigation. Retrieval of spermatogonial cells in prepubertal boys with KS should therefore still be viewed as experimental and patients and their parents must be counseled accordingly. (Fertil Steril® 2012;98:284–9. ©2012 by American Society for Reproductive Medicine.)

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Klinefelter syndrome (KS) is characterized by a phenotype including gynecomastia, small testes, elevated gonadotropins, and azoospermia (1). Approximately 80% of patients have a numerical chromosome aberration, 47, XXY; the remaining 20% have higher-grade chromosome aneuploidies or mosaicisms (2). Klinefelter syndrome is diagnosed in one of 600 male newborns, and in 11% of azoospermic men (1, 3, 4). This disorder is thus the most frequent karyotypic abnormality reported in infertile men (3). However, only 25% of the expected number of Klinefelter patients receive a diagnosis postnatally and less than 3% before puberty (5, 6).

Boys with KS progressively lose their spermatogenic capacity. From early puberty to midpuberty, there is a histologic change starting with relatively normal seminiferous tubules, reduced germ cells, and normal Sertoli/Leydig cells to the adult condition showing extensive fibrosis and hyalinization of the seminiferous tubules. As a result of Sertoli cell dysfunction, virtually all men with KS are azoospermic or severely oligozoospermic by their late adolescence or early adulthood. This progressive gonadal failure is associated with increasing FSH and decreasing inhibin B and antimüllerian hormone levels over time (7, 8).

Although previously patients with KS with a nonmosaic 47,XXY karyotype were considered sterile, the finding of focal spermatogenesis and the recovery of spermatozoa for intracytoplasmic sperm injection (ICSI) has changed their outcome dramatically (9). In couples with men with KS undergoing testicular sperm extraction (TESE) and ICSI, sperm can be recovered in about half of the TESE procedures (10, 11). The only predictive factor for successful sperm recovery is testicular histopathology (11).

Because germ cell depletion starts with the onset of puberty, freezing of semen samples containing low numbers of spermatozoa from boys with KS in early puberty has been proposed, this before initiating testosterone substitution. Early testicular tissue sampling, before starting testosterone supplementation,

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might offer an even greater chance of retrieving gametogenic cells than in adulthood (12–14). However, only limited data exist on this approach and several unresolved questions still remain. Therefore, this article discusses the current knowledge of early germ cell retrieval in boys with KS together with its benefits and pitfalls.

GERM CELL LOSS HYPOTHESES

The development of the XXY mice in 1991 provided a tool to investigate spermatogenesis in greater detail (15). In these animal models, it was observed that normal numbers of primordial germ cells migrated to the genital ridges in the XXY embryo, and that impairment of the mitotic proliferation became evident only after the differentiation of the testis started (16).

The germ cell loss in XXY mice seems to start at a time when germ cells in normal mice reinitiate mitosis and before the onset of germ cell meiosis. During this period, spermatogonia are required to migrate from the central location to reach the periphery of the seminiferous cord to initiate proliferation. At this new location, spermatogonia are exposed to extracellular matrix factors from the basal lamina and are thought to mature into the first generation of type A spermatogonia. Any germ cells that fail to move away from the laminal region of the seminiferous cord eventually degenerate. In a study by Lue et al. (17), it was shown that most of the degenerated germ cells were located near the central region of the seminiferous cord, suggesting a defect in spermatogonial migration during the postnatal period in XXY mice. It is not known whether the defect in the XXY testis is intrinsic to germ cells or whether it is due to the inability of the Sertoli cells to support normal germ cell development in XXY testis.

In humans, some reports suggest that the degeneration of germ cells already starts early in infancy, leading to a significantly reduced number of germ cells even before puberty (18–22). The reduced number of germ cells has been observed in testicular biopsies on fetuses aborted at midtrimester (19, 23). Coerdt et al. (21) observed only half of the number of spermatogonia in fetuses with KS at 19–22 weeks' gestation compared with normal XY fetuses. However, two other authors have reported normal testicular histology in 47,XXY fetuses aborted at 17 and 20 weeks (24, 25).

As seen in the mouse model, also in boys with KS, germ cell differentiation seems to be arrested at the spermatogonium or primary spermatocyte stage. At the onset of puberty, spermatogonia go into apoptosis instead of entering meiosis (26). Furthermore, Wikström et al. (12) demonstrated that immature Sertoli cells are incapable of transforming into the adult mature cell type.

Genetic features of the X chromosome appear to play a role in modulating KS phenotypes. The supernumerary X chromosome is inherited paternally in 40%–60% and maternally in 40%–60% of KS cases (27).

Skewed X chromosome inactivation, frequently observed in females, and defined as greater than 80% preferential inactivation of one of the two X chromosomes, also occurs in KS (28). X-reactivation occurs during germ cell development in the XXY mouse, and it is assumed that for the survival of germ cells in the mature testis the proper X chromosome dose is crucial. Hence, molecular mechanisms induced by an altered dose of X-encoded genes in testicular cells may, during puberty, initiate the degeneration process in the testes of boys with KS (26).

The androgen receptor (AR) gene on the X chromosome may play a particular role in differences in the KS phenotype. The AR gene contains a polymorphic stretch of CAG repeats in exon 1. The length of this stretch is inversely related to receptor activity (29) and influences physiological responses to androgens. No data exist on the role of AR in germ cell numbers.

STEM CELL BANKING IN CANCER PATIENTS

Nowadays, semen banking before any gonadotoxic treatment in adults and adolescents is considered as a valuable preventive measure in combination with techniques of assisted reproduction. Recommendations are made to universally bank sperm for all males at Tanner stage III and above with newly diagnosed malignancies, regardless of the planned treatment (30). Cryopreservation of human sperm has been reported for more than 25 years, and this without apparent loss of capacity for fertilization (31).

For boys unable to produce a sample by masturbation, alternative procedures to collect sperm cells can be used, such as penile vibro- or electrostimulation and TESE (32).

When no sperm cells can be detected, testicular tissue cryopreservation may be offered for eventual restoration of spermatogenesis from spermatogonial stem cells (SSCs) after completion of cancer treatment. One of the difficulties is to store enough spermatogonial stem cells to restore fertility. Moreover, contaminating cancer cells must be detected and removed from testicular biopsy samples. After chemo- or radiotherapy, the frozen-thawed SSCs could be reintroduced in the patient's own testis by SSC transplantation.

ARE THERE STEM CELLS TO BANK IN BOYS WITH KS?

Sperm has been found in approximately 8% of ejaculated semen samples of nonmosaic adult Klinefelter patients (6, 33). However, Aksglaede et al. (34) showed the absence of sperm in the ejaculate of 13 nonmosaic men with KS younger than age 20 years.

Several authors demonstrated spermatogenic foci in the testis of many of the patients with KS (35–40). Foresta et al. (4) performed FISH on testicular tissue of 10 nonmosaic men with KS and found residual spermatogenesis in 2 patients, and Sertoli cells were cytologically identified in all 10.

Several authors tried to retrieve spermatozoa by TESE at an early stage for cryopreservation and future utilization (41, 42). Wikström et al. (12) found that only 50% of 14 boys with KS aged 10–14 years who had undergone biopsy had germ cells in their testis, indicating severely impaired fertility potential even in the peripubertal period. All of the seven boys in whom stem cells were found were younger than 12 years and had prepubertal-sized testicular volumes and normal serum inhibin B and FSH concentrations. The same authors found that the number of adult dark spermatogonia was markedly

reduced in adolescents with KS, again indicating a severely impaired fertility potential even before puberty.

In contrast, Müller et al. (22) could not find any germ cells in 11 boys with KS beyond the age of 2 years. However, in this study, all boys had cryptorchidism. Damani et al. (41) also reported spermatogonial stem cells in the testicular tissue of a 15-year-old boy with KS with testicular volume of 10 mL, and presenting with elevated FSH concentration. Aksglaede et al. (43) described the loss of germ cells from the age of 10 years in an observational retrospective study with 29 testicular cell biopsies from variable ages.

Our own data show that spermatogonia were observed in 18% of adult men with KS in whom no testicular spermatozoa were retrieved, whereas spermatogenesis up to the spermatocyte level was observed in 14% of them (44). Furthermore, we biopsied seven adolescent patients with KS, aged 10–16 years, and observed spermatogonia in approximately half of them but failed to find any meiotic germ cells in these boys. In none of them were we able to retrieve spermatozoa (13, 14).

WHEN TO BANK?

Our own data (13, 14) and those of Wikström et al. (12) show that, for an optimal preservation of SSCs, testicular tissue preservation should preferentially be proposed before hyalinization occurs. To offer an optimal preservation of SSCs, an early detection of the syndrome, that is, before adolescence, is thus necessary.

Serum inhibin B levels during prepuberty and early puberty are normal (45–47). This suggests that during early puberty, serum inhibin B levels in boys with KS reflect the integrity or number of Sertoli cells or both. However, in the study of Wikström et al. (12) normal inhibin B levels and low testosterone levels were inconsistent with the presence of spermatogonia. Also antimüllerian hormone, whose levels rise during prepuberty and early puberty in boys with KS and decline thereafter, could not serve as an indicator of spermatogenetic activity in boys with KS (7, 12). Insulin-like factor 3 (INSL3) levels, as a marker of Leydig cell function, were found to be normal in both infants with KS (48) and early pubertal boys with KS (49) and decrease to very low levels in adult men with KS (50, 51). No clear correlation with germ cell numbers exists.

Testicular tissue preservation should thus preferentially be proposed before any decline in serum inhibin B is observed whenever optimal preservation of spermatogonial stem cells is anticipated. On the other hand, it is unknown whether in adolescents with KS in whom spermatogonia are detected, focal spermatogenesis might persist until adulthood. It is tempting to speculate that the potential of maintaining spermatogonial stem cells and focal spermatogenesis is already programmed in utero. This would explain the finding of spermatogonial stem cells in half of patients with KS at different ages. In fetuses with KS at 19–22 weeks of gestation, spermatogonia were found to be reduced to approximately half of the number observed in normal XY fetuses (21). It is striking that although spermatogonia are found in half of the adolescent patients in whom a biopsy was performed, successful recovery of spermatozoa by TESE in adults is also around 50% (9, 11). At present, it is not clear whether these two populations showing either spermatogonia in adolescence or spermatozoa in adulthood in their testes represent the same or a different subpopulation of patients with KS. However, given the limited number of adolescents with KS investigated to date and the lack of reliable longitudinal histologic data, we cannot exclude the possibility that in some of these adolescents the hyalinization process might progress very rapidly, making it unlikely to find residual spermatozoa when sampling is performed in adulthood. Certainly, cryopreservation of semen samples that contain even minuscule numbers of spermatozoa could be offered to all adolescents with KS who are interested in their future fertility.

HOW TO BANK?

Our data show that spermaturia was absent in 7 boys with KS older than 12 years (14). Also Ratcliffe et al. (20) could not find spermatozoa in the morning urine in a group of 12 pubertal boys with KS older than 16 years. Conventional semen cryopreservation is thus a nonissue. Banking testicular germ cell, however, remains a possibility.

Spermatogonial stem cells can be cryopreserved as a cell suspension or as intact tissue. Cell suspensions are regarded as easier to cryopreserve but preparation of cell suspensions requires mechanical or enzymatic digestion of tissue, which can compromise cell survival (52). For human testicular cell suspensions, a post-thaw viability of up to 60% was achieved, regardless of the cryoprotective agent. Alternatively, cryopreservation of testicular tissue pieces can be used and employed in testicular autografting, or enzymatically dispersed. Cryopreservation of testicular tissue maintains cell-to-cell contacts between germ cells and thus preserves the stem cell niche necessary for their survival and subsequent maturation. Cryoprotection with ethylene glycol and dimethyl sulfoxide using slow-programmed freezing have been applied for cryopreservation of testicular tissue (53, 54). Dimethyl sulfoxide, rather than ethylene glycol, propanediol, or glycerol, better preserves structures within human testicular tissue (55) and better maintains tissue capacity to initiate spermatogenesis in nonhuman primate tissue (56). Furthermore, slow-programmed freezing seems to better protect spermatogonial morphology. Further modification of this protocol with addition of sucrose has shown to abolish the spermatogonial and Sertoli cell loss during freezing–thawing, and spermatogonia have been able to proliferate after orthotopic xenografting (57).

An important point for cryopreservation of testicular material is the removal of sufficient, but not extensive, amount of testicular tissue. In prepubertal testes, the absence of differentiating germ cells creates a relative enrichment of spermatogonial stem cells compared with adult testes. Pediatric patients may therefore require a smaller amount of tissue for fertility preservation than adults. There are no human or nonhuman primate studies revealing how many spermatogenic stem cells can be retrieved from the prepubertal testis. According to morphological studies, it is estimated that one testis of a 10-year-old prepubertal healthy boy contains approximately 83×10^6 germ cells (58); however, there are no data on patients with KS. It is likely that spermatogonial

stem cell transplantation is not clinically applicable without a method to expand spermatogenic stem cells or to increase their colonization capacity. It was estimated that a 1,300-fold expansion of SSCs would be necessary to entirely colonize the adult testis. Recently, a culture system was developed for prepubertal or adult human spermatogonia enabling this high expansion (59, 60).

HOW TO USE THE BANKED MATERIAL?

The possible future use for infertility treatments using cryopreserved testicular samples containing spermatogonia but no mature germ cells would require in vitro maturation of spermatogonia into mature spermatozoa or at least into late/elongated spermatids. Human testicular tissue can be cultured for at least up to 3 weeks without essential loss of spermatogonia (61). Meiosis and spermatogenesis also seem to resume under culture conditions, yielding normal spermatids with some fertilization potential (61).

Several potential strategies are currently under investigation for the further use of cryopreserved spermatogonial stem cells from prepubertal boys (62, 63).

Germ cells could be transplanted using a cryopreserved single-cell suspension infused back into the seminiferous tubules of the infertile patient. However, because KS testes are characterized by extensive fibrosis and hyalinization of the seminiferous tubules, the ultimate use of the frozen tissue may be different. Yet, Lue et al. (64) demonstrated that the transplantation of euploid germ cells into germ cell-deficient XXY mice could restore spermatogenesis, indicating that the somatic niche of the XXY testis is capable of supporting germ cell development in the mouse.

As an alternative, spermatozoa could be generated from spermatogonial stem cells via in vitro differentiation. A three-dimensional soft agar culture system has been developed for mouse SSCs that allows the in vitro differentiation of spermatogonial stem cells into postmeiotic spermatozoa (65). Future studies are required to determine whether sperm generated in vitro maintains DNA integrity or whether no epigenetic changes occur, which might have long-term implications for the health of any resulting offspring or even in subsequent generations (66).

HOW ETHICAL IS IT TO BANK GIVEN THESE DOUBTS?

The primary question in the ethical evaluation is whether collecting, freezing, and possible later usage of the tissue serves the best interests of the child. This means that the costs (e.g., testicular biopsy, storage, and financial costs) should be offset by the chance to realize a good fulfillment of a future child wish (67). An important factor in this evaluation is the utilization rate. When material is stored but later on not used, the balance is negative. Utilization rate is determined first by the feasibility and safety of the procedure and second by the number of men with KS wanting children when reaching adulthood.

Offering the collection and freezing of testicular tissue as part of innovative treatment seems hard to justify given the many uncertainties regarding the future use of the banked material. The experimental nature of the whole procedure, combined with the need for surgical intervention on minors, strongly urges a cautious approach. The absence of a proof of principle is important at this point but one success would not change the situation drastically. Present research should focus on the parameters that could help identify which boys with KS would most likely benefit from testicular tissue freezing.

A recent study of Maiburg et al. (68) showed that 70% of men with KS and 74% of their partners would opt for TESE-ICSI in order to father a child. Ninety-five percent of the parents estimated this treatment a valuable option for their sons. In addition, Vernaeve et al. (69) reported that 69% of the KS couples, after counseling by a psychologist and gynecologist, wanted to try new techniques like ICSI, TESE, or preimplantation genetic diagnosis. So, assuming that the technical problems will be solved, there is a willingness to use assisted reproduction to realize a child wish. However, the utilization rate of frozen tissue will also be determined by alternatives and need: in approximately 55% of men spermatozoa can be recovered at adult age, only about 50 percent of men with KS had a partner at the age of 40 (68).

The general position on whether or not to offer storage of testicular tissue to all boys with KS will depend on arguments such as the importance of genetic parenthood and the optimism regarding the future evolution of science and medicine (70). These beliefs are in the present context of uncertainty more important to decide whether the best interests of the boy with KS are served by an intervention than purely technical aspects.

CONCLUSION

In about half of patients with KS, adult or prepubertal, respectively, spermatozoa or spermatogonial stem cells can be observed and eventually cryopreserved for future fertility. However, to date, it is not clear whether in the remaining patients earlier retrieval for spermatogonial stem cells could improve this retrieval rate, because uterine programming of the number of germ cells might come into play, leading to only half of patients with KS having germ cells left after birth. Furthermore, no clinical parameters are available to detect patients who might benefit from testicular tissue banking. To be able to perform long-term studies on younger patients to solve this question, earlier diagnosis of KS is necessary.

To date, patients must be counseled that fertility preservation is an emerging field, but with many unanswered questions remaining. Retrieval of spermatogonial cells in prepubertal boys with KS should be viewed as experimental and patients and their parents must be counseled accordingly.

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