Parental Acceptance Rate of Testicular Tissue Cryopreservation in Danish Boys with Cryptorchidism

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Keywords
Cryopreservation · Cryptorchidism · Germ cell · Male fertility · Spermatogonial stem cells · Testicular tissue

Abstract
Despite orchidopexy within the first year of life, 20–25% of boys with nonsyndromic cryptorchidism may risk infertility according to histological and hormonal data obtained during surgery. The aim of this study was to evaluate the acceptance rate of testicular tissue cryopreservation among parents of prepubertal boys with cryptorchidism. Fourteen boys with cryptorchidism and high infertility risk were offered cryopreservation as an additional procedure after orchidopexy based on abnormal histopathological findings at primary surgery, whereas 27 boys with bilateral cryptorchidism were offered cryopreservation at the initial orchidopexy. A total of 90% of parents (37/41, 13/14, and 24/27) gave consent to perform cryopreservation, despite being well-informed that the procedural efficacy is largely unproven and may only be needed in about 20% of cases. The number of germ cells per tubule cross-section was 0.03–1.70 (median 0.37) and 22 boys (54%, 22/41) had a value below the lower range. Twelve boys (29%, 12/41) had no type A dark spermatogonia in their biopsy. Cryopreservation of testicular tissue is the first step to introduce spermatogonial stem cell-based therapy into clinical male infertility treatment. At the time of orchidopexy, a testicular biopsy can be collected to ascertain the infertility risk, and it may be an option for boys with bilateral cryptorchidism to have spermatogonial stem cells frozen as a fertility reserve.

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Cryptorchidism arises from a failure in the descent of one or both testes toward the scrotum. With a high prevalence of about 1 in 40 boys, it is one of the most common genital anomalies in full-term newborns [Cortes, 1998; Barthold and Gonzalez, 2003; Cortes et al., 2008]. Cryptorchidism is associated with an elevated risk of impaired fertility. Adult men with persistent bilateral cryptorchidism have azoospermia [Cortes, 1998], whereas 18–46% of prepubertal boys with bilateral cryptorchidism had azoospermia in adulthood despite surgical treatment [Cortes and Thorup, 1991; Hadziselimovic and Herzog, 2001a]. Even when bilateral cryptorchidism was treated surgically before 2 years of age, azoospermia is found in 17% of the patients at follow-up at adult age [Engeler et al., 2000]. Regardless of treatment, 5–13% of boys with unilateral cryptorchidism will have azoospermia at adult age [Cortes et al., 1996; Hadziselimovic and Herzog, 2001a]. Paternity rates are not significantly different between men with unilateral cryptorchidism and the general population (90% vs. 93%, respectively), but are considerably lower (65%) for those with bilateral cryptorchidism when attempting conception [Lee, 2005]. Indeed, about 10–17% of infertile men have a history of surgery for cryptorchidism [Grasso et al., 1991; Olesen et al., 2017].

Histological assessment of the germ cell number per seminiferous tubule cross-section (G/T) and type A dark (Ad) spermatogonia number per seminiferous tubule cross-section (AdS/T) has been widely used, since such data are found to correlate with later spermatogenesis [Hadziselimovic et al., 1984; Cortes and Thorup, 1991; Hadziselimovic and Herzog, 1997]. In a recent study, we found that, despite early orchidopexy within the first year of life, about 20–25% of boys with nonsyndromic cryptorchidism may risk infertility according to histological and hormonal data obtained at the time of surgery [Hildorf et al., 2020]. The impact of cryptorchidism can be seen already during the 3rd trimester of gestation, where more than 20% of fetuses had a reduced number of germ cells [Cortes et al., 1995].

Currently, there are no established protocols for fertility preservation in prepubertal boys. The challenge is that storage of sperm cannot be offered. In 1994, Brinster and Avarbock transplanted murine spermatogonial stem cells into the testis of an infertile mouse and were able to restore spermatogenesis and produce healthy offspring. Subsequently, transplantation of spermatogonial stem cells has successfully restored spermatogenesis in several animal species including rats, dogs, bovines, and rhesus monkeys, and spermatogonial stem cell-derived offspring has been obtained from mice, chicken, sheep, and goats [Gassei et al., 2017]. In a study involving rhesus macaques, Fayomi et al. [2019] recently showed that sperm isolated from autografted immature testicular tissue fragments have the full potential to produce a healthy offspring after using intracytoplasmic sperm injection. Based on successful application in animal models, including non-human primates, surgical retrieval of immature testicular tissue for cryopreservation for future engraftment back into the patient in adulthood is conducted in several medical centers as an experimental procedure. Alternatively, in the future, cryopreserved immature testicular tissue could be used in humans for in vitro germ cell maturation to reach fertilization via intracytoplasmic sperm injection.

Prepubertal testicular tissues might contain gonocytes and spermatogonia, even if they do not have Ad spermatogonia. A recent study demonstrated the feasibility of long-term in vitro expansion of human spermatogonial stem cells from infant cryptorchid testes [Dong et al., 2019]. But there are still challenges in selecting the optimal spermatogonial stem cells for proper germ cell development to ensure the transplantation efficiency. To implement spermatogonial stem cell transplantation methods successfully, in vitro propagation of spermatogonial stem cells might be an essential step due to the limited number of spermatogonial stem cells in cryopreserved tissue from boys with cryptorchidism. In addition, malignant cells may be detected and dissolved during the process of propagation [Dong et al., 2019].

Cryopreservation of immature testicular tissue is the first step for the clinical introduction of spermatogonial stem cell-based therapy for treatment of male infertility. Today, only a few specialized centers worldwide have started to cryopreserve immature testicular tissues with the expectation that strategies for the use of the tissue will be developed in the near future [Goossens et al., 2013; Picton et al., 2015; Gassei and Orwig, 2016; Valli-Pulaski et al., 2019]. These centers have focused on childhood cancer patients and their parents’ acceptance rate [van den Berg et al., 2007; Ginsberg et al., 2010, 2014; Wyns et al., 2015].

To date, little attention has been paid to prepubertal boys with bilateral cryptorchidism and to their parents’ acceptability of cryopreserving a testicular biopsy. The major difference between cancer patients and boys with cryptorchidism is that the testes are likely to be normal in untreated cancer patients whereas they may be abnormal in cryptorchid testes. This is important when considering the success of restoration techniques. One center has in-
cluded boys with bilateral cryptorchidism in their series of testicular biopsies for cryopreservation [Sadri-Ardekan et al., 2016]. Cryopreservation of testicular tissue can be offered as a separate procedure or during initial bilateral orchidopexy. Importantly, the efficacy of this procedure is largely unproven, and furthermore, it may only be needed in about 20% of boys with bilateral cryptorchidism where early orchidopexy is carried out [Hadziselimovic, 2016; Hildorf et al., 2020]. In spite of this, we argue that prepubertal boys with cryptorchidism may be potential candidates for testicular tissue cryopreservation, especially since those having the biopsy taken during the initial bilateral orchidopexy do not suffer an additional surgical risk associated with testicular tissue retrieval. We hypothesize that offering testicular tissue cryopreservation to parents of prepubertal boys with bilateral cryptorchidism would be well accepted. Consequently, the aim of the present study is to report our experience with offering testicular tissue cryopreservation to parents as a fertility restoration option for their prepubertal boy with cryptorchidism relating solitary to the acceptance rate of cryopreservation as a pioneer investigation.

Material and Methods

Study Population

In total, Danish parents of 41 prepubertal boys aged 4–45 (median: 13) months old with cryptorchidism were offered the opportunity for cryopreservation of a testicular biopsy. Thirteen boys with bilateral cryptorchidism and 1 boy with unilateral cryptorchidism were offered an additional procedure involving bilateral testicular tissue cryopreservation (group A). Such patients were carefully selected for this pilot procedure given that their findings at primary surgery indicated a high risk of infertility according to the hormonal profile and the severe abnormal histopathology, which was revealed routinely at orchidopexy. Between July 2014 and January 2020, 14 boys met eligibility criteria for this study and were included 1 year post surgery. In a following setup, 27 boys with congenital bilateral cryptorchidism were offered cryopreservation as a part of initial orchidopexy to avoid another surgical procedure (group B). Since 2018, all parents of boys diagnosed with bilateral congenital cryptorchidism at first encounter, younger than 3.5 years, and clinically examined by J.T. were included. Once identified, the parents were informed that surgery alone could improve the fertility potential and that the procedure might only be indicated in about 20% of cases. Boys with retractile testes, ascending testes, or proven syndromic cryptorchidism were excluded. Ascending testes are defined as a testis that has descended into the scrotum in early infancy, but later ascends into a cryptorchid position [Hutson et al., 2016], whereas retractile testes can be manipulated satisfactorily into the scrotum and remain there without traction until the cremasteric reflex is induced [Barthold and Gonzalez, 2003]. Syndromic cryptorchidism was defined according to the guidelines of the American Urological Association and our previously published method [Kolon et al., 2014; Osterballe et al., 2017].

The same surgeon (J.T.) examined all boys and informed all parents in order to obtain a thorough and uniform clinical examination and to minimize variability in indications to offer cryopreservation. The parents received detailed information about (1) the surgical approach and risks, (2) the cryopreservation technique, (3) withdrawal of consent, (4) additional blood testing, and (5) the prospects for the cryopreserved testicular tissue. It was made clear to all the parents that, at present, no protocols were available to derive sperm from the cryopreserved immature testicular tissue. Moreover, the parents were asked for informed consent that a small part of the testicular biopsy would be separated for ongoing research in this matter, e.g., germ cell derivation and propagation. All information was given orally and in writing according to an approved protocol. No extra costs were related to this decision as cryopreservations were covered by the National Health Service. Additional blood testing for certain diseases, such as human immunodeficient virus-1 and 2, hepatitis B and C, and syphilis, were required according to the Danish National Board of Health (European directive #2004/23/EF). The parents had approximately 2 months from the time of information to the scheduled procedure. During this period, they had access to the written information form on cryopreservation and could contact the surgeon (J.T.) for clarifying information. Written approval of consent was given by both parents at the day of surgery. All the included patients underwent orchidopexy and testicular tissue retrieval for cryopreservation by the informative surgeon.

Testicular Biopsies

A testicular biopsy measuring 2–5 mm$^3$ (mean weight 7 mg) was collected from each testis. There were no surgical complications related to obtaining the testicular biopsies. From one testis, the collected testicular biopsy was divided into 2, with 1 fragment immediately immersed in McCoy’s 5A medium (modified, 22330–021, Gibco, Life Technologies, Paisley, UK) and transported to the laboratory until further preservation, and another fragment immediately fixed in Steve’s solution for histological examination. The contralateral testicular biopsy sample was divided into 3, with 2 fragments prepared as described above and the 3rd fragment prepared for research use. Consequently, 2 testicular tissue samples, 1 sample from each side, were prepared for cryopreservation. Cryopreservation took place at the Laboratory of Reproductive Biology at Copenhagen University Hospital Rigshospitalet by slow-freezing to preserve tissue integrity for long-time storage in liquid nitrogen cryotanks (−196°C). Briefly, testicular biopsies were placed in a 10 mL plastic tube containing 7 mL freezing solution comprising 0.1 mol/L sucrose (Merck, Darmstadt, Germany), 1.5 mol/L ethylene glycol (Sigma-Aldrich, Merck), and 10 mg/mL human serum albumin (200 mg/mL, CSL Behring, King of Prussia, USA) in phosphate-buffered saline. The biopsies were equilibrated in freezing solution for 20 minutes at 1–2°C (crushed ice) on a tilting table, and subsequently transferred individually to 1.8 mL cryovials (Nunc A/S, Roskilde, Denmark) each containing 1 mL of freezing solution, and cryopreserved using a programmable Planer freezer (Kryo 360–17). The following program was used: starting temperature of 1°C, then −2°C/min to −9°C, 5 min of soaking, then manual seeding for ice crystal induction, −0.3°C/min to −40°C, −10°C/min to −140°C, and directly into liquid nitrogen.
For routine histological examinations, the specimens were embedded in paraffin and cut into 2 μm sections. The histological sections were stained with hematoxylin and eosin and incubated with D2–40 (1:25, M3619, Dako, Glostrup, Denmark), CD99/MIC-2 (1:100, 12E7, Dako), and placental-like-alkaline phosphatase (1:200, PL8-F6, Biogenex, Fremont, USA). From the histological sections, the number of G/T and the number of AdS/T were measured. In measurement of G/T, all germ cell types, gonocytes, spermatogonia, and Ad spermatogonia, were included. For every patient, the mean G/T and AdS/T were calculated from at least 100 or 250 cross-sectional seminiferous tubules, respectively as previously described [Cortes and Thorup, 1991; Thorup et al., 2013; Stukenborg et al., 2018]. Normal reference for G/T was defined by Cortes [1990] and Cortes et al. [1995]. These normal autopsy specimens were collected at our hospital, underwent staining at the same laboratory, and were evaluated with the same staining protocol regarding hematoxylin and eosin and histological assessment. Ad spermatogonia were identified according to the criteria: 1) location at the basement membrane, 2) having a rarefaction-zone centrally located within the nucleus, and 3) a homogeneous deeply staining of the nucleus, as previously described [Roosen-Runge and Barlow, 1953; Huff et al., 2001] and the number was considered decreased as <0.01 based on previously published studies [Hadziselimovic et al., 2004; Verkauskas et al., 2019]. Histological data from 10 of the boys are also included in a previous publication [Thorup et al., 2018].

**Hormone Assays**

At the time of surgery, serum hormone levels of follicle stimulating hormone (FSH), luteinizing hormone (LH), testosterone, and inhibin B were obtained routinely. Blood samples were drawn between 8:00–11:00 h, centrifuged, and stored at −20°C until anal-

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**Fig. 1.** Testicular biopsy from a 9-month-old boy with bilateral cryptorchidism stained with hematoxylin and eosin (A), CD99/MIC2 (B), D2–40 (C), and PLAP (D) demonstrating germ cells (arrow) and Ad spermatogonia (arrowhead).
ysis. Serum FSH and LH levels were assessed using a time-resolved immunofluorometric assay (Autodelfia, Wallac, Turku, Finland). The assay’s limits of detection of FSH and LH were both 0.05 IU/L. Serum levels of testosterone and inhibin B were determined with DPC Coat-A-Count radioimmunoassay kit (Diagnostic Products, Los Angeles, USA) and inhibin B ELISA kit (Serotec Ltd., Oxford, UK), respectively. The detection limits of serum testosterone and inhibin B were 0.35 mmol/L and 3 pg/mL. Normal reference serum levels of FSH, LH, testosterone, and inhibin B were defined by our laboratory and data have been published [Andersson et al., 1998]. Hormonal data from 10 of the boys are also included in a previous publication [Thorup et al., 2018].

Statistical Analyses
Differences between the groups were analyzed using Mann-Whitney test and χ²-test. A significance level $p < 0.05$ was considered statistically significant. Prism GraphPad version 8.1 (GraphPad, San Diego, USA) was used for data analysis.

Results

The total acceptance rate to perform testicular tissue cryopreservation was 90% [95% confidence interval (CI) 0.77–0.97] with 37 out of 41 parents.

In 14 cases, cryopreservation of immature testicular tissue was offered as an additional procedure (group A), whereas 27 boys with bilateral cryptorchidism were offered cryopreservation as part of initial orchidopexy (group B). The acceptance rate was similar between the 2 groups (93% v. 89%, $p = 0.68$). Table 1 outlines age at or-chidopexy, hormonal and histological data obtained at orchidopexy, and parental consent among boys in group A and B, respectively. All values of testosterone were below the detection limit and are not shown. Quantification of germ cells within cross-sectional seminiferous tubules were performed (Fig. 1).

In our material, the median value of G/T was 0.37 (range: 0.03–1.70; Table 1) with significant higher G/T values in group B compared to group A (0.56 v. 0.07, $p < 0.0001$). Twenty-two boys (54%, 22/41) had a G/T value below the normal lower range (Table 1). Twelve boys (29%, 12/41) had no Ad spermatogonia present in their entire biopsy obtained at orchidopexy, 8 of these (66%, 8/12) were from group A (Table 1). The median level of inhibin B was 126 ng/mL (17–300 ng/L), whereas 12 boys (29%, 12/41) had a serum inhibin B level below the normal 2.5 percentile (Table 1).

After successful orchidopexy, parents of 1 boy with unilateral and 12 boys with bilateral cryptorchidism (93%, 13/14) gave their consent of a testicular biopsy for cryopreservation as an additional procedure (Table 1, group A). Serum hormonal values, G/T, and AdS/T at primary surgery used for selection are shown in Table 1, and data obtained at the additional procedure for group A patients are shown in Table 2. One boy with unilateral cryptorchidism was eligible with low serum inhibin (44 pg/mL), G/T-value of 0.03, and lack of Ad spermatogonia (Table 1). Collection for cryopreservation revealed G/T impairment in both testes and hormonal data indicating a bilateral disease affecting both testes (Table 2).

In group A, the median G/T value was 0.07 (range: 0.03–0.37), so in all cases G/T was below the normal range, whereas 11 boys (79%, 11/14) had reduced AdS/T value (Table 1; Figs. 2, 3). The serum level of inhibin B was median 109 pg/mL (range: 17–300 pg/mL) (Fig. 4). Five boys from group A had all 3 parameters reduced (G/T, AdS/T, and serum inhibin B level; Table 1).

In group B, parents of 24 boys with bilateral congenital cryptorchidism (89%, 24/27) decided to cryopreserve a testicular biopsy as a part of their sons’ initial orchidopexy (Table 1). Of these, 8 boys (29%, 8/27) had a G/T value below the normal lower range (G/T median: 0.56, range: 0.03–1.70; Table 1; Fig. 2). Moreover, 9 boys (33%, 9/27) had reduced AdS/T value (Table 1; Fig. 3). The median level of serum inhibin B was 136 pg/mL (range: 44–249 pg/mL) and 26% (7/27) had a value below the normal 2.5 percentile (Fig. 4).
### Table 1. Characteristics of 41 prepubertal boys with cryptorchidism who were offered immature testicular tissue cryopreservation.

| Patients | Age at orchidopexy, months | Primary orchidopexy | Parental consent to cryopreservation |
|----------|----------------------------|---------------------|-------------------------------------|
|          |                            | serum FSH, U/L       | serum LH, U/I                       | serum inhibin B, pg/mL | G/T | AdS/T |                             |
|          |                            | 0.6                  | 0.1                                 | 80                      | 0.24 | 0.005 | Yes                           |
|          |                            | 0.8                  | 0.2                                 | 158                     | 0.23 | 0.015 | Yes                           |
|          |                            | 0.6                  | 0.1                                 | 165                     | 0.06 | 0      | Yes                           |
|          |                            | 1.4                  | 0.1                                 | 126                     | 0.07 | 0      | Yes                           |
|          |                            | 0.6                  | 0.1                                 | 56                      | 0.07 | 0      | Yes                           |
|          |                            | 0.6                  | 0.1                                 | 17                      | 0.35 | 0.004 | Yes                           |
|          |                            | 0.7                  | 0.1                                 | 116                     | 0.31 | 0.014 | No                            |
|          |                            | 0.7                  | 0.1                                 | 118                     | 0.06 | 0      | Yes                           |
|          |                            | 0.8                  | 0.1                                 | 102                     | 0.37 | 0.014 | Yes                           |
|          |                            | 1.2                  | 0.4                                 | 68                      | 0.06 | 0      | Yes                           |
|          |                            | 0.8                  | 0.1                                 | 300                     | 0.07 | 0      | Yes                           |
|          |                            | 0.4                  | 0.3                                 | 150                     | 0.19 | 0      | Yes                           |
|          |                            | 13                   | 0.07                                | 44                      | 0.03 | 0      | Yes                           |
|          |                            | 1.01                 | 0.05                                | 69                      | 0.05 | 0      | Yes                           |
|          |                            | 0.47                 | 0.14                                | 72                      | 0.42 | 0.054 | Yes                           |
|          |                            | 0.35                 | 0.18                                | 139                     | 1.21 | 0.012 | Yes                           |
|          |                            | 0.58                 | 0.2                                 | 149                     | 1.37 | 0.134 | Yes                           |
|          |                            | 0.75                 | 0.7                                 | 186                     | 0.50 | 0.043 | Yes                           |
|          |                            | 0.91                 | 0.46                                | 150                     | 1.31 | 0.015 | Yes                           |
|          |                            | 0.52                 | 0.13                                | 189                     | 0.76 | 0.014 | Yes                           |
|          |                            | 0.68                 | 0.27                                | 85                      | 0.50 | 0.030 | Yes                           |
|          |                            | 0.46                 | 0.12                                | 96                      | 0.74 | 0.039 | Yes                           |
|          |                            | 0.69                 | 0.43                                | 66                      | 0.18 | 0      | Yes                           |
|          |                            | 1.64                 | 0.28                                | 106                     | 0.04 | 0      | Yes                           |
|          |                            | 0.40                 | 0.07                                | 134                     | 0.44 | 0.014 | Yes                           |
|          |                            | 1.09                 | 0.2                                 | 131                     | 0.27 | 0.009 | Yes                           |
|          |                            | 0.56                 | 0.65                                | 249                     | 0.75 | 0.025 | Yes                           |
|          |                            | 0.77                 | 0.17                                | 87                      | 0.52 | 0.003 | No                            |
|          |                            | 0.64                 | 0.14                                | 76                      | 0.28 | 0      | Yes                           |
|          |                            | 2.50                 | 0.38                                | 44                      | 0.26 | 0      | Yes                           |
|          |                            | 1.82                 | 3.86                                | 224                     | 0.94 | 0.012 | Yes                           |
|          |                            | 0.57                 | 0.35                                | 222                     | 1.55 | 0.017 | Yes                           |
|          |                            | 0.56                 | 0.05                                | 57                      | 0.57 | 0.035 | Yes                           |
|          |                            | 1.60                 | 0.37                                | 147                     | 1.70 | 0.019 | Yes                           |
|          |                            | 0.81                 | 0.08                                | 136                     | 1.14 | 0.019 | Yes                           |
|          |                            | 0.27                 | 0.13                                | 138                     | 0.33 | 0.020 | No                            |
|          |                            | 0.64                 | 0.21                                | 158                     | 0.28 | 0.006 | Yes                           |
|          |                            | 0.53                 | 0.06                                | 117                     | 0.49 | 0      | Yes                           |
|          |                            | 1.50                 | 1.61                                | 182                     | 1.33 | 0.018 | Yes                           |
|          |                            | 0.45                 | 0.60                                | 207                     | 0.95 | 0.003 | Yes                           |
|          |                            | 0.68                 | 0.20                                | 66                      | 0.94 | 0.06  | No                            |

The patients were divided into 2 subgroups: group A patients were offered cryopreservation as an additional procedure and group B patients were offered cryopreservation as a part of initial orchidopexy. The hormonal and histological data were obtained at primary orchidopexy. Bold numbers represent reduced values: serum inhibin B levels <2.5 percentile according to our hospital laboratory references [Andersson et al., 1998], G/T values below the lower value of the normal range according to our hospital references and literature [Cortes, 1990; Cortes et al., 1995], and AdS/T value estimated to be below the normal range [Hadziselimovic et al., 2004; Verkauskas et al., 2019]. Patients without parental consent for cryopreservation are shown in italics.

*Boy with unilateral cryptorchidism.
Discussion

To our knowledge, we are the first to report parental acceptability towards cryopreservation as an additional procedure for a fertility preservation option for boys with bilateral cryptorchidism based on primary histopathological and hormonal evaluation. Furthermore, we have investigated the acceptance rate of cryopreservation in boys with statistically lower infertility risk as part of the initial bilateral orchidopexy. Our study shows that the
vast majority of parents, 90% (95% CI: 0.77–0.97) accept
cryopreserving a biopsy during or even after their sons’
orchidopexy, despite being well-informed of the unproven
efficacy of fertility restoration in adult life.

It is important to clarify that our center has a unique
possibility to explore the field of pathology in cryptorchid
boys, as obtaining a small testicular biopsy has been a rou-
tine clinical practice part of all procedures for undescend-
ed testes since 1971. Testicular biopsy is not a standard
for prognostic/diagnostic purposes by all pediatric sur-
gons.

It is unknown what criteria should be fulfilled to iden-
tify patients most likely to benefit from testicular tissue
cryopreservation as a treatment for later infertility. The
rationale for including patients based on their histologi-
cal data and/or low inhibin B levels is based on well-doc-
umented findings [Thorup et al., 2013; Hildorf et al.,
2019]. A meta-analysis established a reference G/T relat-
ed to age based on testicular biopsies obtained from 334
normal prepubertal boys towards puberty [Masliukaite et
al., 2016], in which our normal references were included
and were found in the lower part of the assortment. This
means that our present values below lower value of nor-
mal range are definitely low when compared to the litera-
ture.

While unfavorable histology and/or low inhibin B may
be perceived as more robust and convincing than low in-
hibin B alone, the latter inclusion benefits from not need-

ing surgical interventions.

In group A, patients with G/T below the normal range
in testicular biopsies at the time of orchidopexy were of-
fered a second surgery for the option of testicular cryo-
preservation. According to follow-up studies, these pa-
tients in group A will risk further spermatogonial stem
cell loss and most likely experience infertility in adult-
hood [Cortes and Thorup, 1991].

If the findings at primary surgery indicate a high risk
of later infertility, the patient will need to undergo an ad-
tional surgery which involves further inconvenience
(surgical complications, extra hospital costs, psychologi-
cal aspects, etc.). Therefore, we aimed to investigate the
acceptance of parents regarding cryopreservation of a
part of the testicular biopsy obtained routinely at time of
orchidopexy for histopathological evaluation of the fertili-

potential.

Ginsberg et al. [2010] investigated parents’ approach
to cryopreservation of testicular biopsies from boys fac-
ing gonadotoxic oncological treatment. The 76% rate of
acceptance demonstrated the willingness of these families
to participate in testicular cryopreservation. The authors’
observations indicated that when initially informed of
their child’s cancer diagnosis, the majority of parents did
have an understanding that the anticancer treatment
might affect fertility. A total of 68% of parents who agreed
to the testicular biopsy felt that the possibility of freezing
tissue for future use was ‘a great idea for my son’, where-
as all of those who declined the biopsy indicated ‘not sure
if this is right’. A larger percentage of those who did not
choose to have the biopsy endorsed the idea that parents
are too overwhelmed at diagnosis to hear about testicular
tissue cryopreservation (80% vs. 31%). Those who chose
biopsy indicated that, although it can be overwhelming,
the option needed to be discussed and, whereas there may
never be the ideal time, as early as possible is best. Perhaps
unexpectedly, the experimental nature of the cryopreser-
vation process did not play a significant role in the deci-
dion-making process. However, the fact that frozen tes-
ticular tissue has not yet been used for successful human
pregnancies was considered by a larger percentage of par-
ents who opted against the biopsy than those who agreed.
Of those who agreed to the biopsy, all endorsed the con-
cept that ‘fertility is important to preserve, even though
no guarantees were given regarding the ultimate out-
come’. It should be stressed that in our study we did not
explore the reasons for declining participation.

The significantly higher rate of parents’ acceptance in
our study (90%) may be explained by the fact that parents
are focused on the fertility preserving indication for bilat-
oral orchidopexy. Another explanation for the high ac-
ceptance rate could be that for parents of boys with crypt-
orchidism, the situation is less stressful than in the oncol-
ogy situation. Decision-making can be allowed to take
more time, information can be iterated, and surgery can
be scheduled in accordance to parental preferences. Sa-
dri-Ardekani et al. [2016] investigated the acceptance
rates towards cryopreservation among parents of boys
with cancer and 19 with bilateral cryptorchidism and
found similar acceptance rates (78.6% vs. 78.9%). The
median age of the cryptorchid boys was 5 years (8
months–11 years), and 63% of the boys were older than 4
years, indicating a high proportion of late referrals.

Another ethical aspect to emphasize is that early cryo-
preservation implies that patients cannot decide for
themselves. The assumption that procedures will not be
equally successful if performed after puberty is not suffi-
ciently established and therefore speculative. In this era
of patient autonomy, one can argue that the patients
should be able to decide for themselves. However, given
the high presence of spermatogonial stem cell in early in-
fancy, the unknown degree of future testicular damage
and that the final decision to use the tissue is theirs to make, collection prepubertally during an already invasive procedure can be discussed.

The prepubertal testis contains spermatogonial stem cells, which will under the appropriate conditions differentiate into haploid germ cells (spermatozoa) upon onset of puberty. It has clearly been demonstrated that the number of germ cells is reduced in testes from cryptorchid boys [Cortes et al., 1995]. The deterioration of germ cell number progresses if the testes are not placed in the scrotum within the first years of life [Cortes et al., 1995; Hadziselimovic and Herzog, 2001a; Park et al., 2007; Allin et al., 2018]. Studies have demonstrated that later infertility correlates with a lack of germ cells, including Ad spermatogonia, in immature testicular biopsies of unde-scended testes [Cortes, 1998, 2012; Hadziselimovic and Herzog, 2001a, b; Kraft et al., 2012]. This has contributed to the Nordic consensus that surgery for congenital cryptorchid testes is recommended within the first year of life [Ritzen et al., 2007]. Consequently, early and successful surgical repositioning of the testis will improve the testicular growth percentage [Kollin et al., 2007; Tseng et al., 2017] and the fertility potential [Thorup et al., 2012, 2015]. Still, it has been reported that early and successful operation is not sufficient to preserve fertility in 22–36% of the cases [Cortes et al., 1995, 2003; Hadziselimovic and Herzog, 2001a], indicating the testes may be abnormal, which is relevant for subsequent restoration strategies. The histopathological and hormonal findings of the group B of boys who had biopsies for cryopreservation taken at the initial orchidopexy in this study are in agreement with previous findings [Hildorf et al., 2020]. Today, it is possible to a certain extent to distinguish between cases of cryptorchidism wherein the condition is part of a congenital defect or caused by a transient prepubertal hypothalamo-pituitary-gonadal hypofunction assessing hormonal and histological parameters. In the latter case, early operative treatment is not sufficient to prevent infertility, since the underlying endocrinopathy is not cured by surgery. Adjuvant hormonal treatment may stimulate germ cell maturation and gonocyte transformation into Ad spermatogonia, which are important for future fertility [Hadziselimovic, 2016; Thorup et al., 2018]. However, larger studies are needed to fully discern to what extent the germ cell improvement is seen in hormone-treated patients. Cryopreservation should be an option in case of treatment failure of adjuvant hormonal treatment [Hadziselimovic, 2016; Thorup et al., 2018]. Despite the relatively low risk of infertility, 20% makes up a large number of boys due to the high prevalence of cryptorchidism. Consequently, cryptorchidism represents the most common etiologic factor of non-obstructive azoospermia among infertile men [Fedder, 2011; Olesen et al., 2017]. Further research is needed to clarify the expectations that transplantation of previously cryopreserved tissue would successfully result in spermatogenesis when endogenous tissue does not have spermatogenesis.

In order to provide the opportunity for patients with cryptorchidism to retain fertility later in life by spermatogonial stem cell-based therapy, several advances are needed. In girls, one case has been reported on birth using cryopreserved ovarian tissue that was collected prepuber-tally [Matthews et al., 2018]. However, females are physio-logically different as they are born with a reserve of differen-tiated germ cells (oocytes) and are not dependent upon differentiation of germ cells upon onset of puberty. Since the ability of spermatogonial stem cells to complete spermatogenesis in vivo after germ cell transplantation was first demonstrated [Brinster and Avarbock, 1994], other strategies have been investigated. Most recently, it has been shown that cryopreserved immature testicular tissue from rhesus macaque was capable of spermatogenesis when autologously grafted under the back or scrotal skin [Faure et al., 2016]. Consequently, the testis-blood barrier has not developed until then. However, the biopsy on prepubertal testes is not associated with anti-sperm antibody formation as spermatids, which are poten-tially antigenic, do not appear until about 10 years of age [Faure et al., 2016]. Consequently, the testis-blood barrier has not developed until then. However, the biopsy taken in our study comprises a very small amount of tissue for clinical storage. The future potential of such a small-sized biopsy was encouraged by our preliminary findings of propagation of spermatogonial stem cell-like...
cells from such tissue samples [Dong et al., 2019]. But we did also not risk taking larger biopsies than our routine, as there is no proven efficacy of fertility restoration in adult life.

Another limitation of the study is the fact that the normal reference for G/T was based only on hematoxylin and eosin staining of autopsy histological slides. This means that counting G/T in the normal material probably is underestimated compared to counting of G/T in the study material based on mean counting of hematoxylin and eosin, D2–40, CD99/MIC-2, and placental-like-alkaline phosphatase stained histological slides because of the higher quality of the study material. On the other hand, it also means that when G/T is found below the lower value of the normal range – then it is definitely low.

In a European survey [Picton et al., 2015], none of the centers or hospitals offered cryopreservation of immature testicular tissue to prepubertal boys diagnosed with cryptorchidism. In order to avoid another surgical procedure, Thorup et al. [2018] have suggested offering boys with bilateral cryptorchidism cryopreservation of immature testicular tissue during initial bilateral orchidopexy. Furthermore, other potential candidates such as patients with syndromic cryptorchidism or boys with unilateral cryptorchidism with a disease affecting both testes could be discussed. Overall, our study reports a very high acceptance rate of parents, who were willing to pursue testicular tissue cryopreservation and gave parental consent for tissue collection from their son during or even after orchidopexy. These parents were informed and understood the nature of the undocumented procedure, surgical risks (with additional surgery), and a weak indication.

**Conclusion**

At least 20% of boys with bilateral cryptorchidism have a high infertility risk despite early orchidopexy. Today, cryopreservation of testicular tissue for fertility reserve is a possible option for these boys. The parents’ acceptance of such a strategy was 90% even though no guarantees were given regarding the ultimate outcome.

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**Statement of Ethics**

The study was conducted according to Helsinki II declaration and informed consent for retrieving blood samples and testicular biopsies during surgery was obtained from the parents. Testicular tissue collection for cryopreservation and research use was approved by the Regional Ethics Committee of Copenhagen (No. H-2–2012–060.anm.37655). Permission to re-evaluate histology slides and look up blood-samples from electronic patient files was given by Regional Ethics Committee of Copenhagen (#H-18063061).

**Conflict of Interest Statement**

The authors have no conflict of interest to declare

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**Author Contributions**

S.H. and J.T. performed clinical evaluation of patients and conducted the surgeries. S.H., J.T., and D.C. conceived and designed the study, collected data, performed data analysis, drafted the manuscript, conducted critical revision and approved the final manuscript. E.C.L. performed histological evaluation, supervision and approved the final manuscript. S.G.K. and C.Y.A. reviewed the manuscript, with special input on cryopreservation techniques. M.G., L.D., S.G.K., C.F.S.J., J.F., C.Y.A., E.R.H., J.S., and M.F. contributed to conception, critical discussions on fertility preservation, conducted a critical review of the manuscript and approved the final manuscript.

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