Cryopreservation of intact testicular tissue from boys with cryptorchidism

K.Kvist1,2, J.Thorup2, A.G.Byskov1, P.E.Høyer3, K.Møllgård3 and C.Yding Andersen1,4

1Laboratory of Reproductive Biology, Section 5712, 2Department of Paediatric Surgery, Section 4072, University Hospital of Copenhagen, Rigshospitalet, DK-2100 Copenhagen and 3Department of Medical Anatomy, University of Copenhagen, DK-2200 Copenhagen, Denmark

4To whom correspondence should be addressed. E-mail: yding@rh.dk

BACKGROUND: Boys with cryptorchidism often face fertility problems in adult life despite having orchiopexy performed at a very young age. During this operation, a biopsy of the testis is normally taken in order to evaluate their infertility potential and the presence of malignant cells. This study evaluated the morphology and functional capacity of cryopreserved testes biopsies and their possible use in fertility preservation. METHODS: Biopsies from 11 testes (eight boys) were obtained. Each biopsy was subdivided into six pieces and two pieces were frozen in each of two different cryoprotectants. One fresh and two cryopreserved pieces were cultured for 2 weeks. All pieces were prepared for histology. Used culture media were analysed for testosterone and inhibin B concentrations. RESULTS: The morphology of the fresh and frozen–thawed samples was similar, with well-preserved seminiferous tubules and interstitial cells. A similar picture appeared after 2 weeks of culture, but a few of the cultured biopsies contained small necrotic areas. The presence of spermatogonia was verified by c-kit-positive immunostaining. Production of testosterone and inhibin B (ng/mm³ testis tissue) in the frozen–thawed pieces was on average similar to that of the fresh samples. CONCLUSIONS: Intact testicular tissue from young boys with non-descended testes tolerates cryopreservation with surviving spermatogonia and without significant loss of the ability to produce testis-specific hormones in vitro. It may be an option to freeze part of the testis biopsy, which is routinely removed during the operation for cryptorchidism, for fertility preservation in adult life.

Key words: c-kit/cryopreservation/cryptorchidism/human testes tissue/spermatogonia

Introduction

Around 2.5% of all boys in the Western world have an orchiopexy performed because of cryptorchidism. The disease is associated with an increased risk of infertility in adult life, and up to 20% of boys with unilateral cryptorchidism experience fertility problems. This figure increases to 70% for boys with bilateral cryptorchidism, which occurs in 25% of all cases, and this group, therefore, comprises ~0.8% of all boys (Cortes, 1998). Testes of normal boys are located in the scrotum at birth and contain both gonocytes and spermatogonia. The total number of germ cells increases during the first 3 month of life, after which it decreases until ~3 years of age. Transformation of spermatogonia to primary spermatocytes usually starts at around the age of 3 years, and the testes of most 4-year-old boys contain primary spermatocytes. In contrast, boys with cryptorchidism are born with fewer gonocytes and spermatogonia, and the transformation of gonocytes to spermatogonia occurs less efficiently and the number of spermatogonia is reduced in almost all non-descended testes in 1-year-old boys (Cortes et al., 1995). Further, formation of primary spermatocytes also seems to be impaired, already indicating at this stage future fertility problems.

The recommended treatment of cryptorchidism is surgical placement of the non-descended testis into the scrotum. Efforts are now made to perform this operation before the age of 18 months in order to reduce the time that the testis remains at body temperature and reduce the accompanying loss of germ cells and Sertoli cells. At this early age, the cryptorchid testes normally harbour a substantial number of spermatogonia, although at a lower level compared with normal boys. At the time of orchiopexy, a testicular biopsy is routinely taken in order to search for intratubular germ cell neoplasia and to evaluate the morphology and the numbers of germ cells present (Cortes et al., 2001). Since the number of boys with cryptorchid testes who are likely to face fertility problems later on in life is substantial, the aim of the present study was to develop a method that could preserve their fertility by cryopreserving part of the testis biopsy, which was taken anyway during the operation. Injecting purified spermatogonial stem cells into a testis results in renewed spermatogenesis and production of spermatozoa that result in production of progeny in rodents (Brinster and Zimmerman, 1994; Avarbock et al., 1996). Although this technique is not yet available to men, a period of
−20 years is likely to elapse before young boys with cryptorchidism may wish to father their own children. This obviously leaves a considerable amount of time for refinements of the techniques and to make it applicable to humans. Such a method may, in addition, also be applied to other groups of patients such as young boys suffering from cancer where adequate treatment may include cytotoxic drugs that often carry the risk of destroying germ cells and making them sterile.

Contrary to the position of boys with cancer, boys with cryptorchid testes are at less risk of reintroducing malignant cells via the cryopreserved tissue. However, the testes of boys that experienced cryptorchidism in childhood may not be able to sustain spermatogenesis and production of spermatozoa in adult life even if techniques of transferring spermatogonia do become available to humans. The testes may have been permanently damaged due to a diminished surge of gonadotrophins in the first 6 months of life. Also a malfunctioning testis unable to support spermatogenesis may be part of the clinical picture associated with cryptorchidism. Therefore, it was also our aim to develop a cryopreservation method in which intact testis tissue remained functional and preserved germ cells after a period of cryopreservation. In addition to the option of purifying spermatogonia for injection into the testis, cryopreservation of intact testis tissue may allow culture of the tissue and the opportunity to generate haploid gametes in vitro in the future. Another option may be to utilize the tissue for the production of haploid gametes in connection with ectopic grafting or perhaps xenografting. In combination with ICSI, cryopreservation of intact testis tissue may therefore represent a future way of allowing these men to father their own children.

Materials and methods

Human tissue
From August 2003 to December 2003, 11 biopsies were obtained from 8 boys (age 12–66 months, mean 29 months) undergoing surgery for cryptorchidism (three bilaterally and five unilaterally) at the Department of Paediatric Surgery, University Hospital of Copenhagen. In all cases, the testes were located inguinally, none were intra-abdominal, and none of the boys had received hormone therapy prior to the procedure. One boy had Downs’ syndrome. As a standard procedure in connection with this operation, a testis biopsy was taken in order to evaluate the morphology of the testis, the number of spermatozoa and to the procedure. One boy had Downs’ syndrome. As a standard procedure in connection with this operation, a testis biopsy was taken in order to evaluate the morphology of the testis, the number of spermatozoa and the opportunity to generate haploid gametes in vitro in the future. Another option may be to utilize the tissue for the production of haploid gametes in connection with ectopic grafting or perhaps xenografting. In combination with ICSI, cryopreservation of intact testis tissue may therefore represent a future way of allowing these men to father their own children.

Freezing media and protocol
Two different types of cryoprotectants were used. Cryomedium I consisted of Leibovitz L-15 medium (Gibco-BRL, Paisley, UK) supplemented with 1.5 mol/l ethylene glycol, 0.1 mol/l sucrose and 10 mg/ml human serum albumin (HSA). In cryomedium II, Leibovitz medium was exchanged with phosphate-buffered saline (PBS), whereas the other substances remained the same.

Two pieces of tissue were equilibrated in each of the cryomedia by placing a vial with 10 ml of medium on a tilting table at 2°C for 10 min. Each tissue piece was stored in 1.8 ml cryovials (NUNC A/S, Roskilde, Denmark) each containing 1.0 ml of cryoprotectant and cryopreserved using a programmable Planar freezer (Planar K10, Planar Products Ltd, Sudbury-on-Thames, Middlesex, UK). The following programme was used: start at 1°C, 2°C/min to −9°C, 5 min of soaking, then manual seeding for ice crystal nucleation, 0.3°C/min to −40°C, 10°C/min to −140°C, at which temperature the samples were plunged into liquid nitrogen at −196°C (Newton et al., 1996; Schmidt et al., 2003).

Samples were thawed rapidly in a 37°C water bath. The cryomedium was removed in a three-step procedure. The tissue was left for 5 min at each step in 0.75 mol/l ethylene glycol, 0.1 mol/l sucrose, then moved to 0.1 mol/l sucrose and finally to PBS without cryoprotectants.

Culture conditions and experimental set-up
After freezing, the cryovials were left for 1 h in the Dewar storage with liquid nitrogen. Thereafter, they were thawed as described above, one tissue piece from each cryomedium was fixed in Bouin’s fixative and one piece was immediately cultured using the same dish that contained the fresh tissue but a different well. Thereby the fresh and the frozen–thawed tissue experienced exactly the same culture conditions.

The biopsies were cultured on Nunc TC insert polycarbonate membranes (Life Technologies, CM-lab, Vordingborg, Denmark) with a pore size of 3.0 μm, and placed on a Techno Plast Products (TPP) tissue culture test plate (NUNC A/S) with 1.5 ml of TC medium in a humidified atmosphere of 5% CO₂ and 95% air at 34°C. TC medium contained Dulbecco’s modified Eagle’s medium/F-12 (DMEM/F-12) (Gibco-BRL, Life Technologies, Tastrup, Denmark) supplemented with 5 μg/ml transferrin (Sigma, Vallensbaek Strand, Denmark), 20 μg/ml insulin (Gibco-BRL), 30 mg/ml l-glutamine (Gibco-BRL), 50 IU/ml penicillin and 50 μg/ml streptomycin. The entire medium of each well was changed every third day for 2 weeks, and the used media were stored at −20°C until hormone measurements. At the end of the second week, the biopsies were harvested, fixed in Bouin’s fixative and stored in 70% ethanol until processed for histology.
Histological processing and evaluation

The fragments were dehydrated and embedded in paraffin. Serial sections of 30 μm thickness were collected from most biopsies and stained with periodic acid–Schiff (PAS) reagent and Mayer’s haematoxylin according to standard procedures. All biopsies from a 29-month-old boy and two cultured biopsies from two other boys were cut at 5 μm in order to evaluate the presence of germ cells by histochemical detection of c-kit. A total of 12 sections were evaluated immunohistochemically for the detection of spermatogonia.

The area of the 30 μm thick sections was measured using the ‘Computer Assisted Stereological Toolbox’ software package (CAST-Grid system Ver. 1.02, 1998, Olympus, Denmark), and was used to calculate the volume of each fragment. On every histological section, the area and the height of the section was measured. The volume was calculated by addition of all volumes from each individual section.

The diameter of 20 cross-sectioned tubules was measured microscopically for each individual fragment. A tubule was defined as cross-sectioned when the ratio between the longest diameter and the diameter perpendicular to the longest one was between 0.9 and 1.1.

For immunohistochemistry, the paraffin sections were dewaxed, rehydrated and washed in Tris-buffered saline (TBS; 0.05 mol/l Tris, pH 7.6, 0.15 mol/l NaCl) with 0.01% Nonidet P-40 (TBS/Nonidet). Antigen retrieval was performed with a microwave oven using TEG buffer, pH 9 (1.211 g of Tris base + 0.190 g of EGTA, E 437 Sigma, + 1000 ml of distilled H2O2), and a boiling time of 10 min. After heat treatment, the sections rested for 20 min at room temperature. They were incubated in 0.45% H2O2 in TBS/Nonidet for 15 min to block endogenous peroxidase activity, and then in 10% normal goat serum. According to the supplier, binding. All sections were incubated overnight at 4°C with the primary antibody, polyclonal rabbit anti-human CD 117 (c-kit, K4065, used according to the manufacturer’s recommendation. As a positive control, sections were incubated with rabbit IgG (X0903 DakoCytomation) instead of the primary antibody. A4502 DakoCyтомation,1:100/1:300), was raised against a peptide corresponding to amino acids 963–976 at the cytoplasmic C-terminal part of c-kit. The primary antibody was detected using the DakoCyto- mation EnVision+DualLink System, Peroxidase (DAB+), code K4065, used according to the manufacturer’s recommendation. As a negative control, sections were incubated with rabbit IgG (X0903 DakoCytomation) instead of the primary antibody. As a positive control, sections from tissues with known occurrence of the antigen were investigated.

Analysis of testosterone and inhibin B

The production of testosterone and inhibin B was analysed by combining the culture media from the second and third media change (i.e. media collected on days 6 and 9) and comprise the production of hormones for 1 week. The media collected after the first 3 days in culture were not included in this analysis in order to eliminate the slight time difference that elapsed from the time the fresh to frozen–thawed tissue pieces were placed in culture.

Testosterone was measured using a commercially available radioimmunoassay kit [Diagnostic Laboratories System (DSL-4100), Kingo Diagnostics, Praestø, Denmark].

Inhibin B was measured using a commercially available enzyme-linked immunoassorbent assay (ELISA) kit (The Oxford Bio-innovation kit; Biotech-IgG, Copenhagen, Denmark).

Statistics

Hormone production by the fresh, cryomedium I and cryomedium II was evaluated by the Kruskal–Wallis test. A P-value of <0.05 was considered statistically significant.

Results

The size of the biopsies used for experiments ranged from 0.05 to 0.98 mm3 (mean 0.34 mm3). Tissue from nine of the 11 testes produced measurable amounts of hormones during the culture period. The two testes that did not produce measurable amounts of hormone during culture were derived from one boy with Down’s syndrome and bilateral cryptorchidism. Further, each of the cultured pieces from these two testes was <0.1 mm3 in size. Medium from one harvest from one of the wells from testis number 9 was accidentally lost.

Morphology of the fresh and cryopreserved testis biopsies

Freshly fixed tissues and tissue fixed after cryopreservation often had limited necrotic areas that seemed to be related to the cut edges or handling (Figure 2A–C). Healthy looking areas of fresh and cryopreserved tissues generally exhibited a similar morphology, except that the frozen–thawed tissues sometimes contained necrotic cells confined to the centre of the testicular cords that was not seen in the freshly fixed tissues (Figure 3). All biopsies consisted of well-defined testicular cords delineated with a basement membrane, with Sertoli cells and a few spermatogonia. Mitotic figures were present in all biopsies albeit with low frequency. Interstitial tissue was sparse, with cells containing elongated nuclei and a non-distinct cytoplasm. In the non-cultured biopsies, the nuclei of the Sertoli cells were elongated or spherical and in some cases were positioned close to the basement membrane. Spermatogonia often contained two or more mostly spherical nuclei and were frequently associated with the basement membrane (Figure 3D–F).

Hormone production of the fresh and cryopreserved testis biopsies cultured for 2 weeks

The Leydig cell function of the testis tissue was evaluated as testosterone production per mm3 tissue and expressed relative to the production by the fresh tissue. The production of testosterone in each of the two cryoprotectants by each testis is shown in Table I. In general, there was no pronounced difference between the two cryoprotectants. The frozen–thawed tissue secreted on average more testosterone than was seen in the fresh unfrozen sample (Figure 4), but this difference did not reach statistical significance. The average testosterone production by each of the two frozen–thawed tissues was similar. The relative production of testosterone in each of the two cryoprotectants by each of the testes is shown in Figure 4.

The inhibin B secretion of the fresh and frozen–thawed tissue was used to evaluate the Sertoli cell function. The production of inhibin B in each of the two cryoprotectants by each testis is shown in Table I. The two cryoprotectants were equally effective in preserving Sertoli cell function. On average, the inhibin B production was 86 and 79% of that observed in the fresh tissue for the cryoprotectant containing Leibovitz and PBS, respectively, although this reduction did not reach a statistically significant difference.
Morphology of the fresh and cryopreserved testis biopsies cultured for 2 weeks

No differences in morphology between the three types of cultures were noticed. After culture, the biopsies had grown considerably and the diameter of the testicular tubules had increased significantly (Figure 3, Table II). However, the average tubular diameter was similar for the fresh and the frozen–thawed fragments. Also, the fresh cultured fragments showed average tubular diameters similar to that of the frozen–thawed cultured fragments (Table II). Most nuclei of cells enclosed in the testicular tubules had rounded up, and it was difficult to distinguish Sertoli cells from spermatogonia. However, some cells of the testicular tubules exhibited a positive plasma membrane staining with c-kit and were considered to be spermatogonia (Figure 3G). Testicular sections from an 11-week-old legal abortion served as positive controls for the c-kit specificity and showed that the plasma membrane of pre-spermatogonia was stained for c-kit (Figure 3H). No other cells within the testicular cords were stained by c-kit, whereas endothelial cells stained among the interstitial tissue. The interstitial cells resembled those of the uncultured biopsies. A few mitotic figures were seen in all cultured tissues.

Discussion

To the best of our knowledge, this study demonstrates for the first time that cryopreserved intact human testicular tissue from young boys maintains structural and functional characteristics equal to fresh testicular tissues. The overall morphology of the frozen–thawed tissue did not reveal any freeze injuries either immediately after thawing or after a 2 week culture period. The fresh and frozen–thawed tissue demonstrated well-preserved testis tubules and interstitial cells, and no obvious differences between fresh and cryopreserved samples were observable. The function of the somatic cells evaluated as testosterone production by the Leydig cells and inhibin B production by the Sertoli cells during culture post-thawing remained similar to that of the unfrozen control tissue. C-kit-positive spermatogonial stem cells were localized in the fresh and frozen–thawed tissue after a 2 week culture period by immunohistochemical staining, suggesting that cryopreservation of testis tissue also allows the survival of germ cells. The diameter of cross-sectioned testis tubuli increased during culture, but remained similar between the fresh and the frozen–thawed fragments before and after culture. This further supports that cryopreservation has a minor effect on the functional integrity of human testis tissue.
Taken together, cryopreservation of intact human testis tissue from young boys is now possible and allows the development of a whole spectrum of new techniques to circumvent fertility problems, which these young boys and various other groups of patients may experience in adulthood. The protocol is straightforward, and many centres performing assisted reproduction would be able to undertake the procedure.

The present study extends recent studies showing the feasibility of cryopreserving testis tissue from monkeys and other species to include the human testis (Schlatt et al., 2003; Wistuba et al., 2004). Thus, human testis tissue seems to behave similarly to tissue from other species. The presence of spermatogonia in the tissue after a 2 week culture period indicates that the fertility options that have been observed in animal models may also be applicable to humans.

In the past, preserving fertility in young boys prior to production of spermatozoa has mainly focused on cancer patients. Removal of testicular tissue prior to a potential...
**Table I.** Production of testosterone and inhibin B related to the individual size of pieces of fresh and frozen–thawed human testis tissue during culture

<table>
<thead>
<tr>
<th>Biopsy no.</th>
<th>Age (months)</th>
<th>Bilateral /unilateral</th>
<th>Testosterone (ng/mm³)</th>
<th>Inhibin B (ng/mm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Fresh</td>
<td>Cryo I</td>
</tr>
<tr>
<td>1</td>
<td>29</td>
<td>Unilateral</td>
<td>1.73</td>
<td>0.66</td>
</tr>
<tr>
<td>2</td>
<td>27</td>
<td>Unilateral</td>
<td>12.22</td>
<td>19.74</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
<td>Unilateral</td>
<td>15.65</td>
<td>20.00</td>
</tr>
<tr>
<td>4</td>
<td>12</td>
<td>Unilateral</td>
<td>11.43</td>
<td>15.56</td>
</tr>
<tr>
<td>5</td>
<td>66</td>
<td>Unilateral</td>
<td>20.00</td>
<td>33.75</td>
</tr>
<tr>
<td>6</td>
<td>29</td>
<td>Bilateral</td>
<td>46.67</td>
<td>78.33</td>
</tr>
<tr>
<td>7</td>
<td>29</td>
<td>Bilateral</td>
<td>46.60</td>
<td>53.02</td>
</tr>
<tr>
<td>8</td>
<td>14</td>
<td>Bilateral</td>
<td>12.69</td>
<td>19.93</td>
</tr>
<tr>
<td>9</td>
<td>14</td>
<td>Bilateral</td>
<td>16.40</td>
<td>22.14</td>
</tr>
</tbody>
</table>

Bilateral/unilateral refers to whether the tissue originated in a boy with cryptorchidism of either both or one testis.

Cryo I: 1.5 mol/l ethylene glycol, 0.1 mol/l sucrose and 10 mg/ml HSA in Leibovitz L-15 medium.

Cryo II: 1.5 mol/l ethylene glycol, 0.1 mol/l sucrose and 10 mg/ml HSA in phosphate-buffered saline.

**Figure 4.** Production of testosterone and Inhibin-B by human testis tissue during culture. Data expresses the mean hormone production (ng/mm³) of the frozen/thawed tissue in relation to that of fresh. There were no statistical differences observed.
The design of the present experiments allowed a direct comparison of the fresh and frozen–thawed tissue. The period in which the frozen tissue was submerged in the storage Dewar at −196°C was just 1 h. Thereby, it was anticipated that the degenerative effect on the tissue during the storage period was negligible and that prolonged storage would be without effect on the tissue once thawed. With this limitation in mind, the hormone production of the fresh and the frozen–thawed tissue was quite similar, even during conditions in which the culture medium contained no hormones such as FSH, LH or androgens, and resembles those published by Larsen et al. (2002), who found a continued hormone production during a 3 week culture period using similar tissue. Refinements of culture conditions may allow for more extended culture periods of the tissue and may allow the generation of haploid gametes (Larsen et al., 2002). The testosterone production by each individual testis tissue varies considerably, probably reflecting the quite heterogeneous causes that give rise to cryptorchidism. There is a tendency—although not statistically significant—for an enhanced testosterone production by the frozen–thawed fragments which may indicate alterations induced by the cryopreservation procedure. A more detailed picture is likely to be gained in future studies evaluating the LH-induced testosterone production of such tissue. There is no specific information to explain the relative testosterone production seen in case number 1.

The present study also evaluated the efficacy of two different cryopreservation protocols. Both protocols used a combination of cryoprotectants, which have been applied successfully in connection with cryopreservation of ovarian cortex (Newton et al., 1996; Schmidt et al., 2003). One of the protocols (i.e. cryomedium I) used Leibovitz medium in combination with ethylene glycol, sucrose and HSA, which has also been used in connection with cryopreservation of human spermatogonial stem cells (Brock et al., 2001), whereas cryomedium II exchanged Leibovitz medium for PBS, the latter being the cryoprotectant we found most effective for ovarian freezing (Schmidt et al., 2003).

Due to the very limited availability of tissue and our encouraging results with ovarian cryopreservation, it was decided to test the protocol we used for ovarian cryopreservation against a similar protocol in which the salt solution was exchanged with Leibovitz medium. This of course does not exclude that other cryoprotectants such as dimethylsulfoxide or propanediol will actually perform better in connection with cryopreservation of testis tissue.

However, the two protocols performed remarkably similarly and, despite some variation in hormone production in relation to the fresh sample, the two protocols resulted in a very similar hormone production in nearly all instances. Although the number of cases in this study is too small to reveal significant differences, it does demonstrate that the differences between the two protocols are likely to be small and probably will be without clinical importance.

The size of the individual pieces of testis tissue cryopreserved in this study was ∼0.5–1.0 mm² and the small variation in hormone production of tissue cryopreserved with either one of the two protocols may reflect that the testis tissue of this size

table

| Table II. Average tubule diameter in fresh and frozen–thawed human testis tissue before and after culture for 2 weeks (μm, mean ± SD) |
|-----------------|-----------------|-----------------|
|                 | Fresh           | Cryo I          | Cryo II         |
| Uncultured      | 39 ± 2.6        | 36 ± 2.6        | 35 ± 4.6        |
| Cultured for 2 weeks | 65 ± 6.3      | 62 ± 6.2        | 63 ± 9.1        |

A significant increase in average diameter was observed when comparing the unculured and the cultured fragments. P < 0.05 in each case, respectively. For the composition of cryo I and cryo II, see footnotes to Table I.

gonadotoxic treatment and subsequent retransplantation after recovery could at the very best result in restitution of spermatogenesis and natural production of spermatozoa. Reconstitution of spermatogenesis by injection of spermatogonia either directly into the seminiferous tubules or into the rete testis has been performed successfully in several animal species including monkey (Brinster and Avarbock, 1994; Brinster and Zimmerman, 1994; Schlatt et al., 1999). This technique has also been applied in vitro to human testes, suggesting that clinically applicable procedures may be developed (Brock et al., 2001). However, testes from boys with cancer may potentially harbour malignant cells, highlighting the necessity to use purified suspensions of spermatogonia. This has led to effective protocols of purifying spermatogonial cells using either magnetic immunobeads (von Schönfeldt et al., 1999) and/or density gradient centrifugation (Brock et al., 1997). Previously there has only been little focus on boys with cryptorchidism in connection with fertility preservation, although this group of patients numerically represent a larger group than those who acquire cancer as young boys. A substantial number of these boys will invariably face fertility problems in adult life. In some aspects, boys with cryptorchidism may be more likely candidates to undergo cryopreservation of testicular tissue than boys with cancer for the following reasons: (i) evaluation for the presence of malignant cells in the biopsy taken at the time of operation indicates whether the biopsy can be transplanted without further precaution or whether other methods need to be employed; and (ii) the testes may have been permanently damaged and will be unable to sustain spermatogenesis although the testis has been replaced in the scrotum, or its possible smaller size may render injection of spermatogonia technically difficult. Therefore, at least a proportion of the boys who experienced cryptorchidism in childhood, and who face fertility problems in adult life, are likely to be unable to benefit from the injection of a purified suspension of spermatogonia. This group of boys may require methods to be developed that generate haploid gametes in vitro in order to benefit from cryopreservation of testis tissue. If the tissue is without malignant cells, it may also be envisaged that similar techniques to those applied to mice, in which the intact testis tissue is transplanted under the skin and produces haploid gametes leading to progeny, may also work in humans (Schlatt et al., 2003). As a more remote possibility, xenografting may represent a future alternative (Honaramooz et al., 2002, 2004; Oatley et al., 2004). Taken together, cryopreservation of intact testis tissue seems to be an option for future use in restoring fertility.
is relatively robust and resistant to damage by cryopreservation. In addition, the cryopreservation protocol reduced the time of exposure to the cryoprotectant from 30 to 10 min. Further, the present observations also support an earlier study that showed similar survival rates following cryopreservation of partly purified single cell suspensions of human spermatogonia using different cryoprotectants (Brock et al., 2001).

The present study did not attempt to count and quantitate the number of spermatogonia present in fresh and frozen–thawed tissue, but did show surviving c-kit-positive germ cells in both types of tissue. The individual tissue pieces were rather small and, after the culture period, the number of germ cells might be reduced further, thereby leaving the numbers of germ cells too low to allow comparison between the fresh samples and the two types of cryoprotectants. However, future studies should include a direct comparison of surviving germ cells either after purification or by counting histological sections.

In conclusion, the present study demonstrates that human testis tissue from young boys under the age of 5 or 6 years tolerates cryopreservation without significant loss of its ability to produce testis-specific hormones in vitro and sustain survival of spermatogonia. These observations open up new possibilities for fertility preservation in young boys and especially in boys with non-descended testes which do not contain malignant cells that otherwise may prevent autologous transplantation.

Acknowledgements
The expert technical assistance of T.Roed, I.Husum, H.Hadberg, P.S.Thomsen and H.Nguyen is gratefully acknowledged. We thank the Vera and Carl Johan Michaelssens Legat and Professor J.Christoffersen Foundation for financial support.

References

Submitted on January 31, 2005; resubmitted on September 7, 2005; accepted on September 12, 2005