Long-term cultures of testicular biopsies from boys with cryptorchidism: effect of FSH and LH on the number of germ cells

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BACKGROUND: A long-term culture system of testicular biopsies from boys with undescended testes was established to evaluate the effect of gonadotrophins on germ cell survival and growth. METHODS: Biopsies from 25 boys with cryptorchidism, aged 1.1–9.9 years, were obtained at the time of surgery. Each biopsy was subdivided into fragments and cultured for 1 or 3 weeks in a testis culture medium without gonadotrophins or supplemented with FSH (50 IU/l) or LH (5 IU/l), or a combination of FSH (50 IU/l) plus LH (5 IU/l). The survival of the germ cells was evaluated by calculating the ratio of spermatogonia and gonocytes per cross-sectioned testicular tubule, the S/T ratio. RESULTS: All cultured fragments maintained their overall morphology. A significantly reduced S/T ratio was observed in the fragments cultured in the presence of LH ($P < 0.006$), independently of the culture period. The mean tubular diameter in fragments cultured for 1 week with hormones was significantly larger than that of the controls ($P < 0.0002$). All cultured fragments had a larger tubular diameter than the uncultured fragments ($P = 0.0002$). CONCLUSIONS: This culture system supports survival of spermatogonia for at least 3 weeks. LH either alone or in combination with FSH results in a significantly reduced S/T ratio.

Key words: human/prepubertal/testis/tissue culture

Introduction

Cryptorchidism is characterized by the lack of one or both testes in the scrotum, and is associated with infertility later in adulthood. It also presents an important risk factor for the development of testicular malignancies (Chilvers et al., 1986). The risk of testicular cancer in individuals with a history of cryptorchidism is approximately four times higher than in the general population (Swerdlow et al., 1997; Cortes, 1998). The undescended testis may originally be abnormal and is exposed to a higher temperature compared with the scrotal temperature. There is progressive Leydig and Sertoli cell atrophy, and the testosterone production by the Leydig cells is reduced (Forest et al., 1974). These changes become manifest in the undescended testes during the first years of life (Hadziselimovic et al., 1960; Lala et al., 1997).

The optimal age for surgical placement of the undescended testis into the scrotum, orchidopexy, is considered to be before 15–18 months (Kogan et al., 1990; Cortes, 1998). This may stop the changes caused by the high temperature, and generally the number of spermatogonia does not decrease after the undescended testis is located into the scrotum (Cortes, 1998).

Hormonal therapy is based on the hypothesis that cryptorchidism is caused by a defect in the hypothalamic–pituitary–gonadal axis (Hadziselimovic, 1983). It is therefore suggested that treatment with GnRH or with HCG, which exhibits LH activity, induces the descent of an undescended testis. GnRH stimulates the pituitary to release LH and FSH, which stimulate hormone production by the Leydig cells and perhaps also the Sertoli cells. In adult life both FSH and LH are important regulators of spermatogenesis (Steinberger et al., 1964, 1970). Treatment with GnRH analogue, buserelin, may increase the number of germ cells significantly in boys with cryptorchidism (Hadziselimovic, 1984). However, doubts have been raised as to whether hormonal therapy of cryptorchidism is safe for the spermatogonia in boys aged 1–3 years. This is based on observations that the number of spermatogonia per tubule was higher in cryptochid patients who underwent only surgery compared with those patients who underwent GnRH or HCG treatment before surgery (Cortes et al., 2000).

Mancini et al. were the first to use the ratio between the number of gonocytes and spermatogonia per tubular cross section, the S/T ratio, in the histological description of normal and cryptorchid testes (Mancini et al., 1960). The S/T ratio has been found to correlate well with the stereological estimate.
of the number of spermatogonia in the testis (Cortes, 1990). The S/T ratio is used as an alternative method to stereology when stereological estimations cannot be made due to lack of reference parameters such as, for example, the volume of the testis (Braendgaard and Gundersen, 1986).

Long-term culturing of testis biopsies from boys with cryptorchidism may be used to evaluate and analyse the effect of gonadotrophins on gonocytes, and spermatagonia survival and differentiation in infancy and early childhood. The perspectives of such a functional culture system are multiple: the impact of hormonal exposure on spermatogonia cells can be manifested in cultures, as can other components that may influence spermatogonia survival and differentiation. In addition, this in-vitro system can be compared with similar in-vitro and in-vivo animal studies, perhaps providing the opportunity to draw parallels to the in-vivo situation in humans.

The aim of this study was to establish a long-term culturing system for testicular biopsies from boys undergoing surgery for cryptorchidism in order to evaluate the survival of spermatogonia and to evaluate the effect of the addition of FSH and LH. The S/T ratio, the diameter of the tubular cross section, and the overall histological appearance of the tissue fragments were used to evaluate the effect on gonocytes and spermatogonia under these culture conditions.

Materials and methods

Human tissue
Biopsies were obtained from 25 boys (age range: 1.1–9.9 years; mean ± SD: 4.6 ± 2.2; median: 4.2) undergoing surgery for cryptorchidism at the Department of Paediatric Surgery, The National University Hospital. The boys had not received previous hormonal therapy. When possible the biopsies were taken from identical areas in all boys. The biopsies were divided into two age groups: group I: biopsies from boys <4 years (n = 12; mean ± SD: 2.4 ± 0.8; median: 2.3 years) and group II: biopsies from boys >4 years (n = 13; mean ± SD: 5.6 ± 1.6; median: 5.1 years).

The biopsies were immediately transferred to testis culture medium (TCM) at room temperature (see culture conditions) and brought to the laboratory within 10 min for culturing or fixation. The Scientific Ethical Committee of the Municipalities of Copenhagen and Frederiksberg (KF01-374/95) approved the long-term culturing of the testicular fragments in FSH and LH. The S/T ratio, the diameter of the tubular cross section, and the overall histological appearance of the tissue fragments were used to evaluate the effect on gonocytes and spermatogonia under these culture conditions.

Culture conditions
Each biopsy measured ~1–2.5 mm³. Due to the small size of the biopsy only two time periods, 1 and 3 weeks, were tested. The biopsy was divided into nine tissue fragments under a dissection microscope. One fragment from each biopsy was fixed and processed for histology (see below) and used as an uncultured control fragment, while the remaining eight fragments were used for culturing. Some of the biopsies contained segments of testicular capsule, which were removed before further careful division into fragments of equal size. Four fragments were cultured for 1 week and four were cultured for 3 weeks in a humidified atmosphere of 5% CO₂ and 95% air, at 34°C. The TCM contained Dulbecco’s modified eagle medium/F-12 (GibcoBRL, Life Technologies, Taastrup, Denmark) supplemented with 5 µg/ml Transferrin (Sigma, Vallensbæk Strand, Denmark), 20 µg/ml insulin (GibcoBRL ), 30 mg/ml L-Glutamin (GibcoBRL ) plus 50 IU/ml penicillin and 50 µg/ml Streptomycin (GibcoBRL). The fragments were then arranged on a Nunc TC insert polycarbonate membrane (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 plasticware, Taastrup, Denmark).

Four different media were used; the control medium (TCM); TCM with 50 mIU/ml FSH (Gonal-F; Serono Nordic, Brønshøj, Denmark); TCM with 5 mIU/ml LH (LHadi, Serono Nordic); and TCM with a combination of 50 mIU/ml FSH and 5 mIU/ml LH. The media were replaced every third day.

At the end of the culturing period the fragments were processed for histological analysis in the following way. The tissue was fixed in Bouin’s fluid for 1 h and stored in 70% ethanol until all fragments in a biopsy could be processed simultaneously. The fragments were then dehydrated and embedded in paraffin. Serial sections 5 µm thick were cut and every sixth section was placed on a separate slide for later histochemical analysis, while the rest of the sections were stained with Periodic Acid Schiff (PAS) reagents and Mayer’s haematoxylin according to standard procedures.

Histological evaluation
The sections were analysed with an Olympus BH-2 microscope. The image was captured with a digital project video camera connected to an IBM Pentium computer using an S3 Trio64v2 video adapter with version 2.0 driver software.

Sections were observed using ×40 and ×100 objective lenses (apo 40, apo 100 Olympus, Japan). Two numerical parameters were used to evaluate the number of the germ cell and the overall condition of the cultured fragments: the diameter of the cross-sectioned tubulus was measured and the S/T ratio calculated (Cortes, 1998). A tubulus was defined as being cross-sectioned when the ratio between two perpendicular diameters was between 0.9 and 1.1. The two tubular diameters were measured interactively on the video screen using the ‘Computer Assisted Stereological Toolbox’ (CAST-Grid system Ver. 1.02, 1998, Olympus, Denmark) software package. To avoid bias, one of the first four sections of each biopsy fragment was randomly selected as the first section for evaluation. Because the diameter of the spermatogonium nucleus is ~7 µm (Muller and Skakkebaek, 1983), only every second section was counted in order to avoid counting the same spermatogonium twice. The gonocytes or spermatogonia in a total of 150 tubules in each fragment were counted and the S/T ratio was calculated. Simultaneously, the diameters of a total of 50 tubules were measured in each fragment. These numerical parameters were compared as described below.

Statistics
The S/T ratio and the tubular diameters of the seminiferous tubules were analysed separately. In order to avoid problems with mass significance we included observations from all four media and the two culture periods simultaneously. This allowed for formal tests of interaction (e.g. effect of culture period dependent upon media), and only if this could be assumed to be negligible did we proceed to compare the fragments cultured in different media and the effect of the culturing period. Since the data by design include multiple measurements of different fragments originating from the same biopsy under different culture conditions, the statistical model was an analysis of variance with mixed effects. TCM with or without FSH or LH, the culturing period and age group, including possible interactions between these, were of primary interest and were treated as systematic effects. Interactions between fragments and the above-mentioned factors were treated as random with an assumed normal distribution.
In order to obtain variance homogeneity and make the normal distribution assumption reasonable, we had to make a logarithmic transformation of the S/T ratio (after adding 0.01 in order to incorporate for null values). Furthermore, for the tubular diameter, the analysis showed that the variation between tubules of the same fragment was of minor importance, and subsequent analyses were therefore carried out on the average tubular diameters for each biopsy fragment. Based on theoretical calculations of the magnitude of the variance components, we concluded that the number of tubular diameters included in this average was of minor importance, as long as the number of tubules exceeded 10. The effect of age was investigated graphically (through residual plots, not shown), and by comparing the two age groups. Based on the model, we calculated the confidence intervals (CI) for the parameters of interest.

Results

Histology of the uncultured fragments

The fragments in group I contained several spermatogonia, some in mitosis. The spermatogonia had a bright nucleus and large clear cytoplasm and were located at the basement membrane (Figure 1A and A-insert). The Sertoli cell nuclei in the group I fragments were round to oval and their long axis was frequently perpendicular to the basement membrane. A few of the Sertoli cells in group I were in mitosis. Several interstitial cells were scattered around the tubules without any indication of being grouped or in mitosis and some were in the process of degenerating with condensed chromatin.

The fragments from boys in group II (Figure 1D and D-insert) exhibited only few spermatogonia located at the basement membrane and a few others closer towards the centre of the testicular tubule. The Sertoli cells had the same appearance as in group I, but their nuclei were located closer to the basement membrane. None of the interstitial cells were mitotic in these fragments. A generally higher abundance of degenerating cells was found in these fragments (Figure 1D) compared with group I.

General histological appearance of the cultured fragments

During culture, five fragments were contaminated and 14 fragments were too small to count 150 tubular cross sections. Four fragments had no tubules at all. A degeneration of cells occurred in the centre of three of the larger fragments cultured for 1 week, and in five fragments cultured for 3 weeks. The centre of these fragments contained necrotic areas including cells with pyknotic nuclei. There was a distinct border between the morphologically normal and affected areas. The size of these necrotic areas was in general <15% of the total section area. However, two fragments were excluded because these areas exceeded 15%. Taken together, 33 fragments were excluded from the analysis, leaving 167 cultured fragments and 25 uncultured fragments for analysis.

Fragments cultured in TCM

One week of culture

Spermatogonia in the group I fragments that were cultured for 1 week in TCM had a similar morphology to those that were uncultured. Only few spermatogonia were in mitosis (Figure 1B). The morphological appearance of the Sertoli cells was the same in both cultured and uncultured fragments with rounded oval nuclei. A few of the Sertoli cells were in mitosis. The interstitial cells were similar in appearance to those in the uncultured fragments; however, a few scattered cells were degenerating and exhibited condensed chromatin in the interstitium. None of the interstitial cells were in mitosis.

The morphology of the spermatogonia in the group II fragments was similar to that of the uncultured fragments (Figure 1E), with only a few mitotic spermatogonia (Figure 1E-insert). The Sertoli cells had the same appearance as in group I.

Three weeks of culture

The spermatogonia in group I fragments that were cultured for 3 weeks had the same appearance as those cultured for 1 week (Figure 1C), but a few of them were degenerating and had condensed chromatin. The Sertoli cells had the same appearance as in the fragments cultured for 1 week. None of the Sertoli cells or the interstitial cells in these fragments were in mitosis. Some interstitial cells were necrotic.

A few spermatogonia were degenerating in the fragments from group II. The morphology of the Sertoli cells and interstitial cells was similar to that of group I fragments cultured for 1 week. However, degenerating cells in the tubules and interstitium were more abundant than in group I (Figure 1F).

Fragments cultured in TCM plus FSH

There were no morphological differences between the spermatogonia in the group I fragments that were cultured for 1 week and the spermatogonia in the uncultured fragments with a few mitotic spermatogonia (Figure 1G-insert). The morphology of the Sertoli cells and the interstitial cells was similar to those in the fragments cultured for 1 week in TCM, without any mitoses (Figure 1G). There were no mitotic cells (Figure 1H) in the group II fragments.

The spermatogonia, Sertoli cells and interstitial cells in group I and group II fragments that were cultured for 3 weeks appeared to be similar to and did not differ from those in the fragments cultured for 1 week in TMC with FSH (Figure 1I). However, the larger fragments included in the study appeared to contain more degenerating interstitial cells.

Fragments cultured in TCM plus LH

The spermatogonia in biopsy fragments from age group I that were cultured for 1 week in TCM with LH had the same morphology as the spermatogonia in the uncultured fragments, although some spermatogonia were degenerating (Figure 1J). The morphology of the Sertoli cells and the interstitial cells in group I was similar to that of the Sertoli cells and interstitial cells cultured in TCM with FSH in the first week.

When cultured for 3 weeks, no morphological differences between the two groups were observed (Figure 1K). However, degenerating cells were more abundant in the fragments of group II.

Fragments cultured in TCM plus FSH and LH

There were no changes in the morphologies of the spermatogonia or the Sertoli cells in the fragments from the two groups.
compared with the respective groups cultured for 1 week in TCM. When cultured for 3 weeks, degenerating spermatogonia and Sertoli cells became more abundant. There were only few interstitial cells an increased number of degenerating cells and no mitoses (Figure 1L). Some of these fragments presented small areas of necrotic cells in the interstitium (<5% of the total area).

**Analysis of the S/T ratio and tubular diameter**

We found that the effect of the culturing period on the S/T ratio was the same under all culture conditions. The S/T ratio was reduced by 21% in the 3 week cultures as compared with the 1 week cultures (CI = 5.2–34.6%) (Figure 2). However, when LH was present, the S/T ratio was reduced by an additional 16% (5.1–26.5), which was statistically significant ($P = 0.006$) (Figure 2 and Table I).

Analysis of the tubular diameter revealed that the variation between tubular diameters in the same fragment was of a much smaller magnitude than the variation between the fragments (~25%). As a consequence, the subsequent analyses were carried out on the average tubule diameter of each fragment.

The tubular diameter of all cultured fragments was significantly larger than in the uncultured fragments ($P < 0.0001$) (Figure 3 and Table II). When cultured for 1 week with FSH, LH or a combination of FSH and LH, the tubular diameter was significantly increased compared with that in the fragments.
Table 1. S/T ratio of the uncultured and cultured fragments

<table>
<thead>
<tr>
<th>Age group</th>
<th>Uncultured</th>
<th>Weeks of culture</th>
<th>TCM (50 IU/l)</th>
<th>TCM+FSH (50 IU/l)</th>
<th>TCM+LH (5 IU/l)</th>
<th>TCM+FSH+LH (5 IU/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>0.105 ± 0.023</td>
<td>1</td>
<td>0.163 ± 0.052</td>
<td>0.131 ± 0.036</td>
<td>0.102 ± 0.032</td>
<td>0.087 ± 0.026</td>
</tr>
<tr>
<td>(n = 12)</td>
<td></td>
<td>3</td>
<td>0.126 ± 0.044</td>
<td>0.083 ± 0.035</td>
<td>0.088 ± 0.045</td>
<td>0.083 ± 0.031</td>
</tr>
<tr>
<td>Group II</td>
<td>0.113 ± 0.024</td>
<td>1</td>
<td>0.132 ± 0.039</td>
<td>0.158 ± 0.038</td>
<td>0.148 ± 0.034</td>
<td>0.127 ± 0.031</td>
</tr>
<tr>
<td>(n = 13)</td>
<td></td>
<td>3</td>
<td>0.104 ± 0.026</td>
<td>0.195 ± 0.062</td>
<td>0.123 ± 0.036</td>
<td>0.133 ± 0.041</td>
</tr>
<tr>
<td>Group I+II</td>
<td>0.109 ± 0.016</td>
<td>1</td>
<td>0.147 ± 0.032</td>
<td>0.145 ± 0.026</td>
<td>0.125 ± 0.023</td>
<td>0.108 ± 0.021</td>
</tr>
<tr>
<td>(n = 25)</td>
<td></td>
<td>3</td>
<td>0.115 ± 0.024</td>
<td>0.138 ± 0.037</td>
<td>0.106 ± 0.028</td>
<td>0.109 ± 0.026</td>
</tr>
</tbody>
</table>

Mean (S/T) ± SD.

n = number of biopsies in the group.

cultured in TCM only (P < 0.0002). No significant increase was observed after culturing for 3 weeks with or without hormones. This means that the effect on tubule diameter in the fragments cultured for 3 weeks with hormones is smaller compared with the effect on fragments cultured without hormones (P < 0.0002).

We did not find any indication of an age effect on the S/T ratio or tubular diameter after culturing the fragments for 1 or 3 weeks.

Discussion

The present results are, to our knowledge, the first that indicate that spermatogonia survive in testicular biopsies from prepubertal boys with cryptorchidism cultured at 34°C for at least 3 weeks. Our preliminary results show that long-term cultures of these biopsies have a significantly reduced S/T ratio when cultured in the presence of LH, either alone or in combination with FSH. This effect is independent of whether the tissue is cultured for 1 or 3 weeks.

Human testis biopsies have been cultured for long periods before (Steinberger et al., 1970) according to the organ culture technique originally developed for the rat testis (Steinberger et al., 1964). It was shown that testicular biopsies from adult men could maintain the overall tubular structure and viability of Sertoli cells and spermatogonia in culture for >7 weeks. Other approaches to the in-vitro culturing of human testis have recently been used, in which disintegrated testicular biopsy samples from men with obstructive azoospermia were cultured in the presence of various concentrations of FSH (10–100 IU/l) together with testosterone, in order to study the effect on spermatogenesis (Tesarik et al., 1998). However, the hypothesis that intact junctions between Sertoli cells and germ cells enhance the viability of the system, prompted us to culture whole testicular fragments.

Relatively high concentrations of exogenous gonadotrophins were used in the present study compared with the physiological levels found in normal prepubertal testis (Andersson et al., 1998). However, they do lie within the range of concentrations applied in other culture systems for human testis (Tesarik et al., 1998), rat testis with 0.1–5 µg/ml of FSH (Tres and Kierszenbaum, 1983), and mouse testis where the concentration of FSH and LH varies from 0.1–500 and 0.01–50 µg/ml respectively (Haneji and Nishimune, 1982).
The characteristic changes in gonadotrophin levels and the modulations of spermatagonia differentiation in newborn and infant boys are not fully elucidated. In the normal human testis the serum levels of FSH, LH, and testosterone are all higher at 3 months of age than during the following 3–6 months (Andersson et al., 1998). These serum levels increase continuously from the age of 4 years (Gendrel et al., 1980b; Mitamura et al., 1999). At the beginning of puberty FSH, LH, testosterone, estradiol and inhibin B significantly increase (Andersson et al., 1997). The serum LH levels (Forest et al., 1974) and testosterone concentrations (Gendrel et al., 1980a) are lower in boys with cryptorchidism and lower, compared with the LH and testosterone concentrations, in normal boys.

Our data indicate that the reduction of the S/T ratio remains the same in the testicular fragments cultured from 1 to 3 weeks. Since this effect is seen under all culture conditions, it is most likely the effect of the fragments being cultured. However, when cultured with LH either alone or in combination with FSH, the S/T ratio is additionally reduced by 16%.

The effect of HCG treatment on the spermatogonia and spermatogenesis in undescended and normal testis has been subjected to much attention. Matsumoto et al. showed that long-term administration of HCG to adult volunteers induces a suppression of spermatogenesis (Matsumoto et al., 1986). Dunkel et al. found that the apoptotic loss of spermatogonia after HCG treatment of boys with cryptorchidism was increased 4.3 fold, as estimated by the amount of low molecular weight DNA (Dunkel et al., 1997). In addition, Cortes et al. found a reduced number of spermatogonia per transverse tubule in boys with cryptorchidism at the age of 1–3 years after undergoing unsuccessful treatment with HCG (Cortes et al., 2000). However, this effect on the number of spermatogonia was not observed in boys with cryptorchidism who were 10–11 years at orchidopexy after an initially unsuccessful hormonally treatment (Cortes et al., 1996). Furthermore an inflammation-like reaction occurs in boys with cryptorchidism immediately after HCG treatment (Hjertkvist et al., 1993). This reaction disappears after 6–12 months. Similary, animal studies have shown that treatment with HCG or GnRH may have a harmful effect on the testis and lead to spermatogenic interruption, Leydig cell hyperplasia and reduced in-vitro testosterone production compared with normal levels (Kerr and Sharpe, 1986). Together with the findings in our study, the role of HCG in therapy warrants a re-evaluation of the safety of this treatment. In the first years of life the S/T ratio decreases significantly in the undescended testes and remains low during the following years (Cortes et al., 1995). It has been demonstrated that if the S/T ratio is <1% of the normal value for that particular age (i.e. roughly if no germ cells are present in the biopsy), there is a great risk of infertility later in life. The risk is ~33% in cases of unilateral cryptorchidism and 73–100% in bilateral cryptorchidism, the latter depending on whether there is a lack of germ cells in one or both testes (Cortes, 1998). The S/T ratio is therefore an important part of the evaluation of biopsies taken from cryptorchid testes.

The age of the boy with undescended testis had no effect on the number of the spermatogonia in our culture system. Long-term cultures of undescended testes can therefore be initiated at any age between 1–10 years with this in-vitro system. Our model may therefore be valuable when analysing the effect of hormones and other components on the spermatogonia in the endeavour to improve testicular descent.

The average tubular diameter in undescended testes in boys up to 15 years of age decreases during the third year of life (Mengel et al., 1974) as compared with the average tubular diameter in testes of normal boys (Stadtler and Hartmann, 1972). It remains constantly low during childhood.

In our model, the diameter of the testis tubules increased significantly in all fragments during culturing compared with the uncultured fragments. When the two culture periods were compared, the mean tubular diameters were significantly larger in the fragments cultured for 1 week with gonadotropins compared with those cultured without gonadotrophins (in TCM only). A progressive increase in testicular size from a chronological and bone age of 11 years (Burr et al., 1970; Muller and Skakkebaek, 1983) correlates with the initial increase in serum FSH and LH (Burr et al., 1970; Paniagua and Nistal, 1984). It is possible that the increase in tubule diameter observed in our model during the first week of culture might be a consequence of an in-vitro differentiation or maturation of the seminiferous tubules. However, this effect is not reflected in an increase of the S/T ratio, since an increase in S/T ratio was only seen in fragments cultured for 1 week in TCM or FSH, while fragments cultured with LH had a significantly lower S/T ratio. The effect of the gonadotrophins on the tubulus diameter may alternatively be a temporary effect of the Sertoli cells, as no significant change in the diameter was observed in the 3 week cultures with or without

### Table II. Mean diameter of the tubules of the uncultured and cultured fragments

<table>
<thead>
<tr>
<th>Age group</th>
<th>Uncultured culture</th>
<th>Weeks of (50 IU/l)</th>
<th>TCM (5 IU/l)</th>
<th>TCM+FSH (50 IU/l+ 5 IU/l)</th>
<th>TCM+LH</th>
<th>TCM+FSH+LH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>39.33 ± 1.222</td>
<td>1</td>
<td>57.43 ± 0.90</td>
<td>63.43 ± 2.32</td>
<td>62.21 ± 2.20</td>
<td>63.84 ± 2.63</td>
</tr>
<tr>
<td>(n = 12)</td>
<td></td>
<td>3</td>
<td>71.23 ± 2.42</td>
<td>67.98 ± 3.43</td>
<td>65.51 ± 3.89</td>
<td>69.00 ± 2.88</td>
</tr>
<tr>
<td>Group II</td>
<td>40.17 ± 1.998</td>
<td>1</td>
<td>58.78 ± 2.87</td>
<td>64.54 ± 3.57</td>
<td>65.99 ± 4.95</td>
<td>68.24 ± 3.83</td>
</tr>
<tr>
<td>(n = 13)</td>
<td></td>
<td>3</td>
<td>76.50 ± 3.37</td>
<td>74.77 ± 3.67</td>
<td>69.86 ± 4.10</td>
<td>76.91 ± 4.14</td>
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<tr>
<td>Group I+II</td>
<td>39.78 ± 1.195</td>
<td>1</td>
<td>58.10 ± 1.48</td>
<td>64.05 ± 2.19</td>
<td>64.10 ± 2.66</td>
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<td>3</td>
<td>74.01 ± 2.15</td>
<td>71.37 ± 2.61</td>
<td>68.05 ± 2.83</td>
<td>73.74 ± 2.85</td>
</tr>
</tbody>
</table>

Mean tubular diameter (μm) ± SD.

n = number of biopsies in the group.
gonadotrophins. The increase in mean diameter may reflect adaptation to the decreased temperature, an effect which might be accelerated by the presence of gonadotrophins. Additional studies are required to clarify these findings.

It has been shown that addition of nucleosides and mercaptoethanol to an in-vitro single cell clonogenic assay of mouse gonocyte increases the proliferation of the gonocytes (Has-thorpe et al., 1999). The presented new culture system has the potential to give valuable information on the effects on the spermatogonia when cultured in a medium with growth factors. However, care should be taken to account for the paracrine effects from the Sertoli and Leydig cells which are crucial for the establishment of long-term culture of testicular tissue.

In conclusion, we were able to culture biopsies from undescended testes of 1.1–9.9 year-old boys for 3 weeks. The number of spermatogonia, as described by the S/T ratio, was significantly inhibited when cultured with LH either alone or in combination with FSH, both after 1 and 3 weeks of culturing.

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References


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