Differentiation of spermatogenic cells during in-vitro culture of testicular biopsy samples from patients with obstructive azoospermia: effect of recombinant follicle stimulating hormone

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In-vitro differentiation of spermatogenic cells is a potential approach to the treatment of male sterility due to spermatogenic arrest. This is a pilot study evaluating meiotic, morphogenetic and cytoplasmic maturation of spermatogenic cells from 18 patients with obstructive azoospermia, during in-vitro culture of partly disintegrated testicular biopsy samples in the presence or absence of recombinant follicle stimulating hormone (rFSH). Meiotic progression was detectable only in the presence of rFSH in culture medium. FSH-dependent condensation, peripheral migration and protrusion of spermatid nuclei, together with FSH-independent flagellar growth, were the main events indicating post-meiotic sperm cell differentiation. rFSH also promoted the progression of spermatid cytoplasmic maturation, reflected by acceleration of acrosomal development. These differentiation events appeared to be mediated by humoral activity of Sertoli cells, without the need for a direct Sertoli–sperm cell contact. These findings provide a background for similar studies in patients with non-obstructive azoospermia. If reproducible in the latter group, transmeiotic in-vitro differentiation of primary spermatocytes may be useful in cases of complete maturation arrest, whereas the development of culture-specific forms may help select viable spermatids in cases of complete spermiogenesis failure.

Key words: in-vitro spermiogenesis/recombinant FSH/spermatids/spermatocytes/spermiogenesis

Introduction

In spite of the reported births of normal babies after uterine transfer of embryos obtained by intra-oocyte injection of round (Tesarik et al., 1995, 1996; Antinori et al., 1997; Vanderzwalmen et al., 1997) and elongated (Fishel et al., 1995, 1996; Mansour et al., 1996; Araki et al., 1997; Vanderzwalmen et al., 1997) spermatids from patients with non-obstructive azoospermia, the success rates of spermatid conception in humans remains highly variable from case to case (Tesarik and Mendoza, 1996). The cause of this variability is unknown and suspected to be related to the underlying testicular pathology. In fact, patients in whom even a limited production of late elongated spermatids or spermatozoas has been detected, have a better prognosis than those with a complete arrest early in spermiogenesis (Amer et al., 1997; Vanderzwalmen et al., 1997). However, many other factors, such as immaturity of spermatic centrosomes, incompleteness of nuclear protein transition and deficiency of oocyte-activating substances, may be responsible for spermatid conception failure. Such factors may result from a general cytological unripeness of spermatids from a given patient; this cytological unripeness (occurring independently of the achievement of meiotic maturity) can be a sequel of abnormal function of the testis as an organ (e.g. abnormalities of Sertoli cells and Leydig cells, disturbances of microcirculation) rather than abnormal function of the spermatid as a cell. If this is the case, spermatids explanted from the testicular environment and maintained under appropriate conditions in vitro may undergo maturational changes that would be hardly possible in vivo. Moreover, in-vitro culture of some samples lacking spermatids in the fresh state might lead to transmeiotic differentiation of earlier spermatogenic cells and ensuing spermatid production.

Several culture systems for mammalian spermatogenesis in vitro have been described, mostly involving long-term co-culture of previously isolated spermatogenic cell lineages and purified Sertoli cell populations (reviewed in Kierszenbaum, 1994). Completion of meiosis and initiation of early spermiogenesis in vitro were achieved initially by incubating whole spermatogenic stage-specific seminiferous tubular segments (Parvinen et al., 1983; Toppari and Parvinen, 1985) and, later, by short-term co-cultures of purified pachytene spermatocytes and Sertoli cells (Le Magueresse-Battistoni et al., 1991; Weiss et al., 1997). Experience with human spermatogenesis in vitro is very limited (Tres et al., 1989, 1991). Nevertheless, distinctive growth patterns, similar to those observed in the intact seminiferous epithelium, were displayed by human spermatogonia and spermatocytes co-cultured with Sertoli cells (Tres et al., 1989), and round spermatids grew a flagellum containing the characteristic axonemal configuration (Tres et al., 1991).

The aim of this study was to examine the feasibility of in-vitro progression of spermatogenesis by incubating partly disintegrated testicular biopsy samples from patients with obstructive azoospermia and to evaluate the effect of adding recombinant follicle stimulating hormone (rFSH) to the culture medium. This pilot study, performed with samples showing ongoing complete in-vivo spermatogenesis, was undertaken as
a prerequisite for further studies dealing with patients suffering from non-obstructive azoospermia due to different kinds of spermatogenic disorders.

Materials and methods

Experimental design

The aim of this study was to determine what changes occur in human germ cells from testicular biopsy samples after mechanical disintegration of the tissue (such as the procedure used for sperm recovery for assisted reproduction) during subsequent in-vitro culture. Although most spermatogenic cells are released as single cells or small syncytial complexes by this kind of preparation, some cells remain embedded in large Sertoli–germ cell clusters. Changes occurring in isolated germ cells and in cells remaining within the clusters are not necessarily the same. Therefore, we used two different kinds of cell preparation in two consecutive series of cases.

In series 1 (12 patients) no attempt was made to disintegrate the Sertoli–germ cell clusters remaining in samples after the original mechanical disintegration. Thus, it was impossible to evaluate the eventual changes occurring in cells within the clusters in this series, and only released germ cells were taken into account. The advantage of series 1 was that the same cell populations could be evaluated longitudinally at sequential time-points.

In series 2 (six patients) all persisting Sertoli–germ cell clusters were disintegrated enzymatically (see below) shortly before the final microscopic examination. The advantage of this series was that all germ cells within each sample could be evaluated. On the other hand, the need for the destruction of cell–cell contacts before each observation was incompatible with repeated observations of the same cell populations maintained under the same conditions. Thus, different aliquots of each sample had to be prepared for observation at each time-point.

Source and preparation of testicular biopsy samples

Samples (~3×5 mm) of testicular tissue were obtained at the Centre of Reproductive Medicine by open testicular biopsy from 18 men with obstructive azoospermia, in order to recover spermatozoa for assisted reproduction attempts. All men had normal testicular size and normal values of serum FSH and testosterone.

Tissue samples were placed in Gamete-100 medium (Scandinavian IVF Science AB, Gothenburg, Sweden). Within 5 min after biopsy, they were disintegrated by stretching between two microscope slides followed by repeated aspiration with a 1 ml sterile plastic syringe. Spermatozoa were identified in all samples; fractions containing sufficient numbers of spermatozoa for the planned assisted reproduction attempts were set aside. The remainder were allocated to this study after having obtained informed consent from the patients. Samples were treated differently in each of the two series as follows.

In series 1, samples were allowed to sediment, resuspended in 1 ml Gamete-100 medium and dispatched in five drops of 0.2 ml, ~1.5 cm in diameter, on individual Falcon 3802 tissue culture dishes (Becton Dickinson Labware, Lincoln Park, NJ, USA). The drops were covered immediately with embryo-tested light mineral oil (Sigma, St Louis, MO, USA) to prevent evaporation.

In series 2, mechanically homogenized samples were divided into three aliquots; two were used for in-vitro culture while the third was subjected to enzymatic treatment to completely disintegrate the Sertoli–germ cell clusters that persisted after mechanical disintegration of testicular tissues.

Enzymatic digestion and subsequent cell analysis

Just before microscopic observation, samples were incubated for 1 h at 37°C with collagenase I (1000 units/ml) and elastase (10 units/ml) (Sigma). After washing with two cycles of centrifugation (500 g, 10 min) and resuspension in Gamete-100 medium, the enzyme-digested specimens were further divided into aliquots to determine the total cell number and viability, and the percentage of post-zygotene cells and individual stages of spermatogenesis. Total cell number was determined by DNA staining with Hoechst dye 33342 (Sigma), and viability was evaluated by eosin exclusion (World Health Organization, 1992). The percentage of post-zygotene germ cells was determined by immunofluorescence with 4D4 anti-proacrosin monoclonal antibody (BioMérieux, Marcy-l’Etoile, France) as described by Mendoza et al. (1996). For the determination of the percentages of individual stages of spermatogenesis, cell suspensions were divided into Falcon 3802 tissue culture dishes (Becton Dickinson), covered with light, embryo-tested mineral oil and inspected under an inverted microscope equipped with Hoffman modulation contrast optics and a ×40 objective.

In-vitro culture

The culture medium Gamete-100 medium containing partly disintegrated testicular tissue (see previous section) were maintained in culture at 30°C for either 48 h (series 1) or 24 h (series 2). Of the five original drops prepared from each sample, two drops received 25 mlU/ml of rFSH (Pegureon; Organon, Oss, The Netherlands), and two others were supplemented with solvent alone to serve as controls. The fifth drop was maintained without any further addition at room temperature. Cell viability was assessed by eosin or Trypan Blue exclusion and consistently was >90% at the beginning of culture and >80% at the end of culture. At different time intervals, cells in cultures were observed in the native state, by using an inverted microscope equipped with Hoffman modulation contrast optics.

Immunocytochemical visualization of proacrosin

Samples (5 µl) of cultured cell suspensions were taken at the 24 h and 48 h intervals. The cells were smeared on microscope slides, fixed by dipping the slides in 100% methanol for 20 s and processed for immunocytochemistry with 4D4 anti-proacrosin monoclonal antibody (BioMérieux) as described by Mendoza et al. (1996). Proacrosin immunoreactivity was visualized by using fluorescein-labelled anti-mouse IgG (Sigma), and cell nuclei were counterstained with ethidium bromide. Cellular distribution of proacrosin immunoreactivity was analysed by using a ×100 oil-immersion objective with a Leica DMRXA research fluorescence microscope equipped with a Q550CW cyto genetic workstat (Leica France, Rueil-Malmaison, France) and printed with a Kodak XLS 8600 colour printer.

Spermatogenic cell type identification

Spermatogenic cells were classified as primary spermatocytes, secondary spermatocytes and spermatids at different stages of spermiogenesis. Spermatogonia mostly remained embedded in aggregates with Sertoli cells and were not taken into account in this study. For the distinction of different stages of spermiogenesis, the terminology suggested by de Kretser and Kerr (1988) was used. In addition to stages representing normal morphogenetic differentiation of round (Sa), elongating (Sh1, Sh2, Sc) and elongated (Sd) spermatids (de Kretser and Kerr, 1988), three stages (Saf, Sbp, Scp) representing abnormal spermatid morphogenesis were defined (Figure 1). The Saf (Sa with flagellum) stage only differed from Sa by the presence of a flagellum, whereas the cell did not show any signs of elongation, and the nucleus was still round, centrally located and without signs of condensation. The Sbp (Sb pathological) stage was a pathological form of the Sb stage. As with Sh1, Sbp spermatids still retained the round shape, but the nucleus was condensed, elongated and protruding at one pole of the cell, like the Sb2 stage
but without the corresponding cell elongation. Scp (Sc pathological) spermatids possessed a flagellum like Sc spermatids, but they still retained the round cell shape. Scp spermatids also had a protruding, condensed and elongated nucleus as Sbp spermatids from which they only differed by the presence of a flagellum (Figure 1).

Distinction between primary (15 µm diameter) and secondary spermatocytes (10 µm diameter) is easy because of the difference in cell size (Sousa et al., 1996). Sa round spermatids were identified by using previously described criteria (Tesarik and Mendoza, 1996), whereas elongating and elongated spermatids, both normal and abnormal, were distinguished by differences in their morphological features (Figure 1).

In cell smears immunostained for proacrosin with 4D4 monoclonal antibody, germ cells are marked specifically from the pachytene spermatocyte stage onwards (Escalier et al., 1991; Mendoza et al., 1996). Individual stages of germ cells showing proacrosin immunoreactivity were distinguished according to their size and morphology (Mendoza et al., 1996).

**Quantitative evaluation**

In series 1, quantification of differentiating germ cells at different times of in-vitro culture was performed with unstained, living cells. The analysis was done by counting those cells lying dispersed on the bottom of the culture dish (see above). Floating cells, and those in multicellular aggregates, which were difficult to identify, were excluded from the analysis. Individual stages of germ cells were expressed as a percentage of the total number counted (>1000) for each patient and each experiment.

In series 2, quantification of germ cells at different times of in-vitro culture was performed with both living (as above) and immunostained cells. For immunostained preparations, 200 germ cells were analysed on each slide. Each proacrosin-positive cell was evaluated for the stage of acrosomal development. Three stages, referred to as reticulovesicular stage, vesicular stage and cap stage, were distinguished according to the morphological appearance of cell structures showing proacrosin immunoreactivity. The reticulovesicular stage was characterized by an extensive reticular network (corresponding to the Golgi apparatus) attached to a small nascent acrosomal vesicle, contrasting with the vesicular stage at which most, if not all, of the immunostained area was represented by a vesicular structure (Figure 2). For intermediate forms, spermatids with a vesicle <2 µm were classified as reticulovesicular, and ≥2 µm as the vesicular stage. The cap stage was denoted by a definitive acrosome, that appeared as a characteristically cap-like form at one pole of the nucleus (Figure 2).

**Statistical analysis**

Differences between treatment groups in the percentage germ cells and acrosome stages were evaluated by $\chi^2$ and Kruskal–Wallis tests.

**Results**

**Changes in the occurrence of normal and abnormal forms of germ cells during in-vitro culture**

Besides a few larger clusters consisting of Sertoli and spermatogenic cells, in which individual cell types could not be identified with certainty, most spermatogenic cells were seen as isolated single cells. Some free spermatogenic cells had formed pairs of closely associated, equal-sized cells, apparently resulting from previous cell division. Pairs of secondary spermatocytes, each pair having arisen from a single primary spermatocyte through the first meiotic division, and pairs of Sa round spermatids, each pair having arisen from a single secondary spermatocyte through the second meiotic division, were infrequent in freshly prepared testicular samples. Complexes of three or more spermatogenic cells, outside of the large Sertoli–germ cell aggregates, were extremely rare.

No significant disaggregation of the large cell clusters occurred during in-vitro culture. This was shown in series 1 by two types of observation. Firstly, the total number of cells counted in the same culture drop (only free cells outside of the clusters were included in these counts) did not differ at different time points indicating that there was no release of spermatogenic cells from the clusters. Second, photographs of the same cell clusters taken at different times of incubation did not show any apparent changes in cluster size and shape. These observations suggest that most of the spermatogenic cell syncytia resulting from in-vivo meiotic divisions were either retained in the persisting large cell clusters or dis-integrated to single cells during tissue preparation.

In series 1 (no enzymatic digestion of Sertoli–germ cell clusters before analysis), no increase in the percentages of pairs of secondary spermatocytes and round spermatids was detected after 24 h of culture without the addition of rFSH. However, these percentages showed a marked increase when the samples were incubated for 24 h in the presence of 25 IU/ml of rFSH (Figure 3A), suggesting a reactivation of meiotic divisions in vitro in the presence of the hormone. In series 2 (enzymatic disintegration of Sertoli–germ cell clusters just before analysis), similar data were obtained (Figure 3B). In contrast, the percentage of cell pairs formed by two primary spermatocytes did not increase during culture (data not shown).

Quantitative changes in the representation of individual stages of spermatid development were also observed during in-vitro culture both in series 1 (Table I) and series 2 (Table II), illustrating the continuation of morphogenetic processes characterizing human spermiogenesis. In series 1, one of the most salient observations was the increase in three abnormal forms that were absent (Saf) or very scarce (Sbp and Scp) in fresh samples. In the absence of rFSH, Saf (Figure 4A) was by far the most prominent of the three abnormal spermatid forms after 24 h of culture, reaching as many as 9.6% of all spermatogenic cells evaluated (Table I). The concomitant decrease in the percentage of Sa spermatids (Table I), supports...
Figure 2. Different stages of acrosomal morphogenesis in human spermatogenic cells as distinguished by immunocytochemical visualization of proacrosin using 4D4 monoclonal antibody and fluorescein-labelled antimouse IgG. Cell nuclei are counterstained with ethidium bromide. (A) Reticulovesicular stage (large arrow) in a primary spermatocyte, with abundant labelled reticular component attached to a small developing acrosomal vesicle; vesicular stage (small arrow) in a round spermatid, with most of proacrosin immunoreactivity concentrated in a vesicular structure. (B) Cap stage (arrow) in an early elongating spermatid, showing a characteristic cap-like form of the acrosome similar to the fully developed organelle. Original magnification, ×3000. Scale bar = 10 µm.

the possibility that Saf spermatids develop in culture from Sa spermatids by isolated growth of the flagellum without the concomitant changes in the cell and nuclear shape characterizing normal spermiogenesis.

In the presence of rFSH, Saf spermatids were also present, but the two other abnormal forms, Sbp (Figure 4B) and Scp (Figure 4C) prevailed (Table I). Also in this condition, these abnormal forms appeared to have developed from Sa spermatids whose percentage decreased as compared with fresh samples (Table I). After an additional 24 h of culture, Sbp and Scp spermatids became more frequent in the absence of rFSH in culture medium. On the other hand, in the presence of rFSH, Sbp spermatids were greatly reduced after 48 h of culture, whereas the percentage of Scp spermatids was further increased as compared with the 24 h time-point (Table I). Scp spermatids occurred in pairs (Figure 4C) in many cases.

As to the normal forms of spermiogenesis, a progressive, rFSH-independent decrease in the percentage of the normal
Sb2 elongated spermatids during in-vitro culture was the most conspicuous finding, besides the aforementioned decrease in the percentage of Sa round spermatids in all experimental groups. The percentage of the other stages of normal spermatogenesis remained unchanged (Table I).

As compared with series 1, quite similar data were obtained in series 2 in which, however, in-vitro culture was not prolonged beyond 24 h (Table II).

In the presence of rFSH, some primary and secondary spermatocytes underwent morphological changes similar to those observed in spermatids. In particular, some spermatocytes developed one (Figure 5A) or two flagella, while others showed nuclear condensation, peripheral migration and protrusion (Figure 5B). Such spermatocytes were distinguished from binucleated spermatids, in which assembly of a flagellum would not be unexpected, by staining with Hoechst 33342.

**Cytoplasmic maturation of spermatids during in-vitro culture**

As suggested previously (Mendoza et al., 1996), progression of acrosomal development, reflected by a progressive decrease in the percentage of cells with reticulovesicular acrosomes and an increase in the incidence of the vesicular and cap stages of acrosomal development (Figure 2), was evaluated to assess spermatid cytoplasmic maturation. In fresh biopsies and those samples cultured for 24 h without rFSH, most round spermatids had acrosomes at the vesicular stage (Figure 6). By comparison, in the presence of 25 mIU rFSH/ml, a marked increase in the percentage of round spermatids showing the cap stage of acrosomal development was the most salient observation (Figure 6). The percentage of round spermatids possessing a cap-stage acrosome was significantly higher after incubation with rFSH as compared with fresh samples or with samples incubated in the absence of the hormone (P < 0.01; \( \chi^2 \) and Kruskal–Wallis tests). These data show that cytoplasmic maturation also progresses in cultured spermatogenic cells and is accelerated in the presence of rFSH.

**Discussion**

The results of this study have shown a significant increase in the percentage of secondary spermatocytes and round spermatids

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**Table I. Quantitative representation of individual spermatids in fresh testicular biopsy samples and in samples cultured in the absence or in the presence of rFSH.** Data from undigested samples (series 1) in which cells remaining within Sertoli–germ cell clusters were not taken into account.

<table>
<thead>
<tr>
<th>Percent spermatogenic cells</th>
<th>Time of incubation (h)</th>
<th>rFSH</th>
<th>Normal spermatids</th>
<th>Abnormal spermatids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sa</td>
<td>Sb1</td>
<td>Sb2</td>
<td>Sc</td>
</tr>
<tr>
<td>0</td>
<td>–</td>
<td>8.2 ± 1.5b</td>
<td>0.5 ± 0.1b</td>
<td>4.1 ± 0.9b</td>
</tr>
<tr>
<td>24</td>
<td>Absent</td>
<td>3.9 ± 0.8c</td>
<td>0.5 ± 0.2b</td>
<td>1.2 ± 0.3c</td>
</tr>
<tr>
<td>24</td>
<td>Present</td>
<td>3.7 ± 0.7c</td>
<td>0.3 ± 0.1b</td>
<td>1.1 ± 0.2c</td>
</tr>
<tr>
<td>48</td>
<td>Absent</td>
<td>2.0 ± 0.4c</td>
<td>0.3 ± 0.1b</td>
<td>0.9 ± 0.3c</td>
</tr>
<tr>
<td>48</td>
<td>Present</td>
<td>1.8 ± 0.4c</td>
<td>0.4 ± 0.1b</td>
<td>1.4 ± 0.4c</td>
</tr>
</tbody>
</table>

*Values are mean ± SD of 12 testicular biopsy samples. At least 1000 spermatogenic cells were counted in each sample at each incubation period.*

b,c Values with different superscript letters within each column are significantly different (P < 0.05) from each other (\( \chi^2 \) and Kruskal–Wallis tests).

Sa = round; Sb1, Sb2, Sc = elongating; Sd = elongated; Saf = Sa with flagella; Sbp = Sb pathological; Scp = Sc pathological; further details are shown in Figure 1 and described in the text.
forming pairs of closely associated cells during 24 h of culture in the presence of FSH as compared with both fresh samples and samples cultured in the absence of the hormone. Because the cell pairs presumably represent dividing cells, these findings are interpreted as a consequence of a stimulatory effect of FSH on in-vitro meiosis. Even though cell associations can also arise during in-vitro culture by a simple adherence, there is no apparent reason why adherence of secondary spermatocytes and round spermatids should be promoted by FSH. If this were the case, it is also unlikely that the adherence process would result in a selective formation of cell pairs rather than larger cell aggregates. Yet, formation of aggregates consisting of three or more germ cells at the same stage of development was not observed. Thus, the above interpretation, considering the cells forming each pair as daughter cells resulting from a recent meiotic division of a single germ cell, remains the most consistent explanation, although it needs to be confirmed by a more specific methodological approach. Further study of the effect of FSH on in-vitro meiosis of human germ cells, using fluorescent in-situ hybridization, is currently in progress in our laboratories.

Similarly, care must be taken in the interpretation of nuclear changes in spermatids as spermiogenesis-related phenomena, since nuclear condensation can also be a sign of apoptosis. However, this is unlikely to be the case in this study where the nuclear condensation was accompanied by a typical nuclear protrusion, characterizing the normal process of spermiogenesis, and by the growth of a flagellum. Moreover, FSH-dependent progression of acrosomal biogenesis, documented in this study with the use of a proacrosin-specific monoclonal antibody, was clear evidence of a stimulatory effect of FSH on spermiogenesis. In fact, our preliminary data show that the above morphological changes only occur in non-apoptotic spermatids and can thus be used to select healthy cells from populations with high frequencies of cells carrying apoptotic DNA damage (J.Tesarik, unpublished observations).

It is generally accepted that the presence of Sertoli cells is required to maintain germ cell viability during in-vitro culture (Jutte et al., 1985; Cameron et al., 1987). Different culture systems, combining Sertoli and germ cells, have been used to study in-vitro differentiation of mammalian germ cells. Completion of meiosis and initiation of spermiogenesis in vitro were first achieved by incubation of whole segments of rat seminiferous tubules (Parvinen et al., 1983). Other studies used co-culture of more homogeneous fractions of Sertoli and germ cells after previous disintegration of seminiferous tubules by mechanical forces and enzymatic treatments (Tres and Kierszenbaum, 1983); these systems have been improved by the development of polarized Sertoli cell cultures using extracellular matrix-coated and uncoated permeable substrates and perifusion techniques (Hadley et al., 1985, 1987; Janecki et al., 1987; Kierszenbaum and Tres, 1987; Ueda et al., 1988). This study did not involve any cell purification step. Thus, all types of testis cells, including Leydig cells, were present. This was why rFSH was given in preference to urinary FSH preparations whose luteinizing hormone contamination might stimulate testosterone production by Leydig cells, which would complicate the interpretation of the observed FSH effects on in-vitro spermatogenesis.

In addition to the demonstration of developmental changes occurring in human germ cells during in-vitro culture, these data also suggest that such changes can occur in free germ cells lacking a direct physical contact with Sertoli cells. Thus, the only way by which Sertoli cells could influence these germ cells was through a non-contact action of humoral mediators. In addition to trophic effects, of which the production by Sertoli cells of lactate (Robinson and Fritz, 1981; Mita et al., 1982; Nakamura et al., 1984) is one example, the non-contact action of Sertoli cells upon germ cells may be mediated by paracrine regulatory molecules such as Steel factor or members of the cytokine family (Dym, 1994; Albanesi et al., 1996; Cudicini et al., 1997). Conversely, humoral factors secreted by spermatogenic cells in culture may support the viability of Sertoli cells, since cultured rat round spermatids were able to rescue Sertoli cell viability by secreting a precursor of nerve growth factor (Chen et al., 1997). Hence, in our culture conditions, Sertoli cells, stimulated by a direct contact with spermatogenic cells in the large multicellular clusters, may have secreted trophic and regulatory humoral substances that influenced the viability and differentiation of isolated spermatogenic cells in the same culture drop. Because the crude testicular samples subjected to in-vitro culture in this study contained all testicular cells, not just Sertoli and germ cells, altered production of mediators by non-Sertoli somatic

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**Table II. Quantitative representation of individual spermatids in fresh testicular biopsy samples and in samples cultured in the absence or in the presence of rFSH.** Data from samples digested with collagenase I and elastase (series 2) in which all cells could be evaluated.

<table>
<thead>
<tr>
<th>Time of incubation (h)</th>
<th>rFSH</th>
<th>Normal spermatids&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Abnormal spermatids&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Sa</td>
<td>Sb1</td>
</tr>
<tr>
<td>0 – 8.0</td>
<td>–</td>
<td>8.0 ± 1.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.5 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>24 Absent</td>
<td>3.6 ± 0.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.4 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.1 ± 0.3&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>24 Present</td>
<td>3.7 ± 0.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.4 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.3 ± 0.3&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Values are mean ± SD of six testicular biopsy samples. At least 1000 spermatogenic cells were counted in each sample at each incubation period. The percentage of viable cells did not show significant differences between groups (χ² and Kruskal–Wallis tests) and was consistently >80%.

<sup>b</sup>Values with different superscript letters within each column are significantly different (P < 0.05) from each other (χ² and Kruskal–Wallis tests).

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<sup>d</sup>See Table I for abbreviations.
Figure 4. Normal and abnormal forms of spermatids observed in the native state in culture drops after incubation in the absence and in the presence of rFSH. (A) Saf and Sb1 spermatids, along with a red blood cell (Rb), after 24 h of culture in the absence of rFSH. (B) Sbp spermatid and a red blood cell (Rb) after 24 h of culture in the presence of rFSH. (C) Syncytium of two Scp spermatids after 24 h of culture in the presence of rFSH. Original magnification, ×1000. Scale bar = 10 µm.

Figure 5. Abnormal forms of spermatocytes observed in the native state in culture drops after 24 h of incubation in the presence of rFSH. (A) Secondary spermatocyte having developed a flagellum (arrow) besides a red blood cell (Rb). (B) Primary (above) and secondary (below) spermatocyte with prematurely condensed nuclei (arrows). The nucleus of the primary spermatocyte is protruding outside of the cell, whereas that of the secondary spermatocyte shows an abnormal peripheral localization. Original magnification, ×1000. Scale bar = 10 µm.

Figure 6. The frequency of round spermatids showing individual stages of acrosomal development at the beginning of in-vitro culture (open bars) and after 24 h of culture in the absence (shaded bars) and presence (full bars) of rFSH. These data were obtained in series 2 of patients (n = 6) and are represented as percentage (mean ± SD) of all spermatogenic cells identified in culture drops. Round spermatids were identified on the basis of the nuclear size and shape. Thus, no distinction was made between the Sa and Saf stages in this part of the study.
particularly evident for the progression of meiosis in primary and secondary spermatocytes. Against the background of the ongoing debate about the respective roles of FSH and testosterone in mammalian spermiogenesis (Matsumoto and Bremner, 1989; Sun et al., 1989; Tapanainen et al., 1997), this finding suggests that FSH does support meiosis of human germ cells. The beneficial effect of FSH may be more pronounced under in-vitro conditions as compared with the situation in vivo, where the action of this hormone may be substituted more easily by alternative regulatory mechanisms. Redundancy of the mechanisms that control spermiogenesis in vitro is a generally recognized phenomenon. This redundancy may also explain the recent surprising observation that men homozygous for an inactivating mutation of the FSH receptor gene do not suffer from azoospermia or absolute infertility, although they have variable degrees of spermatogenic failure (Tapanainen et al., 1997). On the other hand, the availability of alternative spermiogenesis controlling mechanisms may be more restricted in vitro, which would explain the effects of FSH described in this study.

The non-contact effects of rFSH on spermatid differentiation in vitro described in this study are even more surprising because spermiogenesis was believed to be controlled by testosterone, with only marginal—if any—contribution of FSH (Huang et al., 1987; Sun et al., 1989, 1990; Awoniyi et al., 1992; McLachlan et al., 1994; O’Donnell et al., 1994, 1996). It is known that, once detached from their initial contact with Sertoli cells, spermatids do not differentiate easily even if they manage to bind to Sertoli cells again when the Sertoli–spermatogenic cell binding is stimulated by FSH and testosterone (Cameron and Muffly, 1991). Our data show that specific nuclear changes, similar to those occurring during the normal spermiogenesis process and characterized by nuclear condensation, peripheral migration and protrusion, can be stimulated by FSH in human spermatids in vitro. In the presence of rFSH, similar nuclear changes were even found to occur in primary and secondary spermatocytes. On the other hand, FSH-independent flagellar growth was observed in cultured round spermatids and, occasionally, also in primary and secondary spermatocytes in which two flagella sometimes developed.

It needs to be stressed that the progression of human spermiogenesis in vitro, observed in this study in such a short period of culture as 24 h, is quite an unexpected finding in view of the timing of human spermiogenesis in vivo which has been shown to occupy ~ 20–22 days (Heller and Clermont, 1964). We actually do not have any definitive explanation for this unbelievable velocity. It is possible that the long duration of spermiogenesis in vivo is due to the action of multiple checking mechanisms whose role is to coordinate individual morphogenetic and molecular transformations. In our in-vitro culture system, spermiogenesis was not coordinated correctly and mostly led to the development of abnormal spermatid forms. If the coordination, not the changes themselves, is the time-limiting step in spermiogenesis, the abnormal course of spermiogenesis in vitro may explain its unusual velocity.

To the best of our knowledge, this is the first demonstration of the progression of isolated spermiogenesis-related processes in human spermatids lacking a direct contact with Sertoli cells, although the coordination of individual morphogenetic events was mostly abnormal. The unexpected differentiation of free spermatids in culture may be explained by the simultaneous presence of relatively intact groups of Sertoli cells which may have secreted, into culture medium, factors required for spermiogenesis. In fact, the condition of Sertoli cells within the large Sertoli–spermatogenic cell clusters appears to approach the ‘enclosed’ character of the seminiferous epithelium in vivo, where stretches of coexisting spermatogenic cell lineages are assigned to specific topographical regions of functionally cycling Sertoli cells. Requirements for a closed environmental system, which may be one restricted to a relatively small unit, are supported by the observation of a relatively intact early spermiogenesis within excised seminiferous tubule segments (Parvinen et al., 1983; Toppari and Parvinen, 1985) as opposed to the absence of morphogenetic progression of round spermatids in contact with a Sertoli cell monolayer (Cameron and Muffly, 1991), which is an example of an ‘open’ culture system. It remains to be elucidated whether, and by what mechanism, the differentiation of isolated spermatids can be influenced by humoral factors emanating from the cell clusters retaining the normal Sertoli–spermatogenic cell relationship. In a preliminary series of experiments, we were unable to detect any signs of ongoing spermiogenesis in pure spermatid cultures without the presence of Sertoli cells (J. Tesarik, unpublished observations). In addition to meiotic progression and morphological differentiation, this study has also shown that human spermatids can undergo FSH-promoted cytoplasmic maturation in vitro, heralded by the progression of acrosomal development.

From the clinical viewpoint, two conclusions can be drawn from the present data; both of these conclusions can serve as a starting point for further focused research. Firstly, FSH stimulates both early and late spermatogenic processes, probably via Sertoli cells. Because the proximity of Sertoli cells, rather than a direct contact, is a prerequisite for at least some of these processes, spermatogenic arrest may be caused by a pathological ‘distancing’ of both cell types. An experimental model for this condition is the stage-specific detachment of spermatids produced in the rat seminiferous epithelium by testosterone withdrawal. In cases of human non-obstructive azoospermia, detachment of spermatids and also primary and secondary spermatocytes often occurs, as witnessed by the frequent detection of these spermatogenic cells in the patients’ ejaculates (Mendoza et al., 1996). In the absence of adequate Sertoli cell-mediated stimuli, the detached spermatogenic cells are likely to be prone to apoptosis. Studies aimed at the rescue of such cells from apoptosis by incubation in appropriate in-vitro culture systems are warranted.

Second, the in-vitro development of culture-specific abnormal forms of spermiogenesis that are not seen in fresh samples may be useful for the selection of spermatids with the optimal viability and developmental potential for eventual use in assisted reproduction. The culture period by itself may allow additional cytoplasmic maturation in adequately stimulated spermatids. The fact that the present findings were obtained with a relatively crude culture system is encouraging.
and suggests that the development of human germ cells in vitro can be further improved by refinement of the technique. It remains to be determined whether differentiation processes similar to those observed in this study in spermatogenic cells from patients with obstructive azoospermia, and thus with a relatively intact spermatogenesis, also occur in cells from patients with non-obstructive azoospermia in whom spermatogenesis is disturbed in spite of the fact that serum FSH concentrations are often elevated.

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References


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