

Developmental potential of human spermatogenic cells co-cultured with Sertoli cells

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BACKGROUND: Development of an in-vitro culture system capable of supporting human early germ cell differentiation would be important for treatment of azoospermic patients. **METHODS:** Sertoli cells, spermatogonia and spermatocytes were isolated from testicular biopsies of 61 non-obstructive azoospermic patients, and co-cultured using Vero cell conditioned medium only or supplemented with recombinant (r)FSH or rFSH plus testosterone. Germ cell purity was checked by fluorescent in-situ hybridization (FISH) analysis. **RESULTS:** Best results were achieved with both hormones, which elicited 6.9% of meiosis index and 22.7% of differentiation into normal late spermatids after 2–3 weeks of culture. In-vitro matured spermatids were microinjected into oocytes to study their developmental potential. Round spermatids elicited 37.5% of fertilization and 28.6% blastocyst rates. Abnormal elongating and elongated spermatids enabled 8.3 and 27.3% fertilization rates respectively, but none achieved the blastocyst stage. Normal elongating and elongated spermatids elicited 30.5% fertilization and 42.9% of blastocyst rates. FISH analysis showed sex chromosome anomalies in all embryos, except in the case of morulae from normal late spermatids. **CONCLUSIONS:** Results suggest that meiosis and spermiogenesis can be resumed *in vitro*, with normal differentiated spermatids showing a low fertilization potential but regular rates of blastocyst formation. However, most of the embryos did not reach the morula stage and showed major sex chromosome abnormalities.

Key words: in-vitro maturation/meiosis/non-obstructive azoospermia/spermatogenesis

Introduction

Restoration of the complete spermatogenic cycle *in vitro* is of fundamental importance for revealing the mechanisms controlling meiosis and cell differentiation, and also to provide an in-vitro system that might be helpful for the treatment of male infertility. However, this goal remains to be successfully achieved in mammals. This has been explained by the existence of complex mixtures of germ and Sertoli cells, with development appearing also to be dependent on locally secreted growth factors and interleukins (Heller and Clermont, 1964; Gnassi *et al.*, 1997; Schlatt *et al.*, 1997). More recently, co-cultures of rodent germ cells with Sertoli cells enabled meiosis reinitiation and round spermatid formation, but these then remained arrested at that step of spermiogenesis (Hue *et al.*, 1998).

Both FSH and testosterone are needed to support germ cell differentiation through direct or indirect actions on Sertoli cells (Carreau, 1994; Gnassi *et al.*, 1997; Schlatt *et al.*, 1997). They enhance Sertoli cell responses to insulin. FSH and insulin then regulate the glucose metabolism and Sertoli cell lactate production, which is needed for normal spermiogenesis and spermatocyte RNA synthesis. FSH was suggested to play a determinant role in the survival of germ cells besides increasing spermatogonia proliferation (Hikim and Swerdloff, 1995;

Foresta *et al.*, 1998; Baarends and Grootegoed, 1999), whereas testosterone, which is 50–100 times more concentrated in the testes than in serum (Gunsalus *et al.*, 1994), has been implicated in spermatogonia and spermatocyte differentiation, in the conversion of round to elongated spermatids (Huang *et al.*, 1987; McLachlan *et al.*, 1994; O'Donnell *et al.*, 1996), and as an antiapoptotic substance on spermatocytes (Erkkila *et al.*, 1997; Print and Loveland, 2000).

In the present study, human Sertoli and diploid germ cells were individually isolated and then co-cultured under different media conditions to study the role of FSH and testosterone in supporting germ cell survival in long-term in-vitro culture, resumption of meiosis and spermatid differentiation. Controls were compared with cases of meiosis arrest in order to determine if in-vitro culturing could overcome the meiotic arrest. The developmental potential of the in-vitro matured spermatids was also studied by microinjecting these cells into oocytes, with the resulting embryos being then analysed by fluorescent in-situ hybridization (FISH).

Materials and methods

Ethical aspects

In all cases, patients gave informed consent to use cells for experiments. Because this work was experimental, none of the embryos

obtained after microinjection of in-vitro matured gametes was used for transfer, having all been used for FISH analysis after long-term culture (6 days).

Male patients

Sixty-one patients with non-obstructive azoospermia and normal karyotypes participated in the present experiments that took place between November 10, 1998 and July 1, 2000. Patients referred for treatment came with a full urologist clinical evaluation, which included a diagnostic testicular biopsy. Recovery of male gametes for clinical treatment was performed by open testicular biopsy. After treatment, of the nine Sertoli cell only syndrome cases, none was confirmed as pure, as three showed one focus of spermiogenesis (presence of late spermatids/sperm) and six had one focus of meiosis arrest; of the 23 maturation arrest cases, eight had one focus of spermiogenesis (presence of late spermatids/sperm) and 15 were confirmed to be arrested at meiosis; and of the 29 cases with hypoplasia, mature sperm could be recovered in all patients.

Male germ cell isolation and culture

Each testicle biopsy was collected in sperm preparation medium (SPM; Medicult, Copenhagen, Denmark) and squeezed with surgical blades. The resultant fluid was diluted with SPM and washed by centrifuging at 500–600 *g*, twice for 5 min each. When an excessive number of erythrocytes was present, the pellet was resuspended for 5 min in 2 ml of erythrocyte-lysing buffer (Verheyen *et al.*, 1995), prepared with 155 mmol/l NH₄Cl, 10 mmol/l KHCO₃, and 2 mmol/l EDTA in water, pH 7.2 with KOH (all from Sigma, Barcelona, Spain, cell culture tested), and filtered by 0.2 µm (TPP, Switzerland). After washing, samples were digested (Crabbé *et al.*, 1997) for 1 h at 37°C, in a solution of SPM containing 25 µg/ml of crude DNase and 1000 U/ml of collagenase-IV (Sigma). After washing, the pellet was resuspended in IVF medium (Medicult) and incubated at 30–32°C, 5% CO₂ in air until use. A sample was then diluted in SPM, spread on a tissue culture plate and covered with light mineral oil (Medicult). Sertoli cells, elongated type-A spermatogonia, round spermatogonia and primary spermatocytes, secondary spermatocytes, and round spermatids were individually selected in an inverted Nikon microscope, equipped with Hoffman optics and a heated stage (32°C), using Narishige micromanipulators (Nikon, Tokyo, Japan) and micropipettes of 15–20 µm in diameter (SweMed, Frolunda, Sweden). Selected cells were mixed in a culture drop containing 40 µl of Vero cell conditioned medium, with or without 25 U/l of recombinant (r)FSH (Serono, Geneva, Switzerland) and 1 µmol/l of testosterone (Sigma, water-soluble) prepared as described by Tesarik *et al.* (1998a), and cultured at 32°C with 5% CO₂ in air, for up to 21 days. Vero cells (Vircell SL, Santa Fe, Granada, Spain) were prepared in IVF medium (BM1; Lab. Elliós, Paris, France) with 10% synthetic serum substitute (Irvine Scientific, CA, USA). The overlaying solution was taken for germ cell culture as conditioned medium, 2 days after monolayer formation (Cremades *et al.*, 1999).

Biochemical assays

In Vero cell conditioned medium, FSH concentrations were assayed by enzyme-linked immunosorbent assay (BioMérieux kit; Vidas Systems), and testosterone by radioimmunoassay (Tecam). Assays were repeated at three different experiments.

Microinjection experiments

Oocytes used for testing the developmental potential of in-vitro matured spermatids came from patients that had donated spare oocytes for research. Female patients with normal karyotypes elected for ICSI clinical treatment cycles were treated with a long gonadotrophin-

releasing hormone analogue suppression protocol combining busserelin acetate (Suprefact; Hoechst, Frankfurt, Germany) with pure (p) FSH (Metrodin HP; Serono) or rFSH (Gonal F; Serono; or Puregon; Organon, Oss, The Netherlands). Ovulation was induced with human chorionic gonadotrophin (HCG, Pregnyl; Organon; or Profasi; Serono). Oocytes were recovered from large ovarian follicles by ultrasound-guided follicular aspiration, 36 h after HCG administration, using flush medium (Medicult) (Sousa *et al.*, 1999). Oocytes were microinjected in SPM using the strong dislocation of the ooplasm (Tesarik and Sousa, 1995), and then cultured for up to 6 days over Vero cell monolayers. Normal fertilization was assessed 10–18 h after injection, and embryo cleavage and quality were evaluated 42 h later (Staessen *et al.*, 1995).

Fluorescent in situ hybridization

For FISH, a published technique (Coonen *et al.*, 1994) was followed. Briefly, embryos were washed in Tyrode's salt solution (TSS; Sigma), and partially depellucidated in TSS containing 0.1 mg/ml of pronase (Merck, Darmstadt, Germany). After washings, they were transferred to 1–2 µl of lysis buffer (0.01 N HCl/0.1% Tween 20; Sigma) on poly-L-lysine (Sigma) coated slides. Isolated germ cells were prepared by direct transfer to lysis buffer. After nuclei isolation, slides were air dried, rinsed in water and phosphate-buffered saline (PBS; Sigma) for 5 min each, rapidly dehydrated in an ethanol series, and stored in dark conditions at –20°C until use. Slides were then incubated with pepsin (100 µg/ml; Sigma) for 20 min at 37°C, rinsed in water and PBS, fixed in 4% paraformaldehyde (Bio-Rad, Watford, Herts, UK) at 4°C for 10 min, rinsed in PBS and water, and dehydrated. The probe mixture, made of 6 µl of hybridization buffer (Vysis Inc., Downers Grove, IL, USA), 1 µl of each directly labelled α -satellite centromeric DNA probes and 1 µl of water, was applied to each slide under a coverslip. Probes used (Vysis) were CEP 18 (region 18p11.1–q11.1, locus D18Z1; Spectrum Orange, 0.5 µl, and Spectrum Green, 0.5 µl), and CEP X (bands Xp11.1–q11.1, locus DXZ1; Spectrum Green)/CEP Y (bands Yp11.1–q11.1, locus DYZ3; Spectrum Orange).

Probes and nuclear DNA were denatured simultaneously at 75°C for 3 min, and then left to hybridize for 2–4 h at 37°C in a moist chamber. Post-hybridization washes consisted of 60% formamide (Fluka Chemika, Switzerland)/2× standard saline citrate (SSC)/0.05% Tween 20, at 42°C for 5 min, 2×SSC, for 5 min at 42°C, and 4×SSC/0.05% Tween 20, for 5 min at room temperature. After rinsing in water and PBS, slides were dehydrated, air-dried and mounted in 10 µl Vectashield antifade medium containing 1.5 µg/ml 4',6-diamidino-2-phenylindole (DAPI) to counterstain the nuclei (Vector Laboratories, Burlingame, USA). The efficiency of the FISH procedure was controlled using metaphase chromosomes and interphase nuclei from cultured lymphocytes. For this, peripheral blood cells from healthy male patients were stimulated with phytohaemagglutinin (Difco Laboratories, Detroit, USA) and cultured for 72 h at 37°C. A total of 100 lymphocytes were examined, and 95% of them gave positive signals for the expected number of copies. FISH images were recorded in a Nikon (Eclipse, E-400) epifluorescence microscope fitted with a CCD camera and appropriate software (Cytovision Ultra, Applied Imaging International, Sunderland, UK).

Statistical analysis

Where appropriate, the χ^2 -test was used to evaluate the significance of difference between the percentages of two groups.

Results

Culture conditions

Early germ cells and Sertoli cells were isolated from 61 patients with non-obstructive azoospermia. Of these, 19 cases

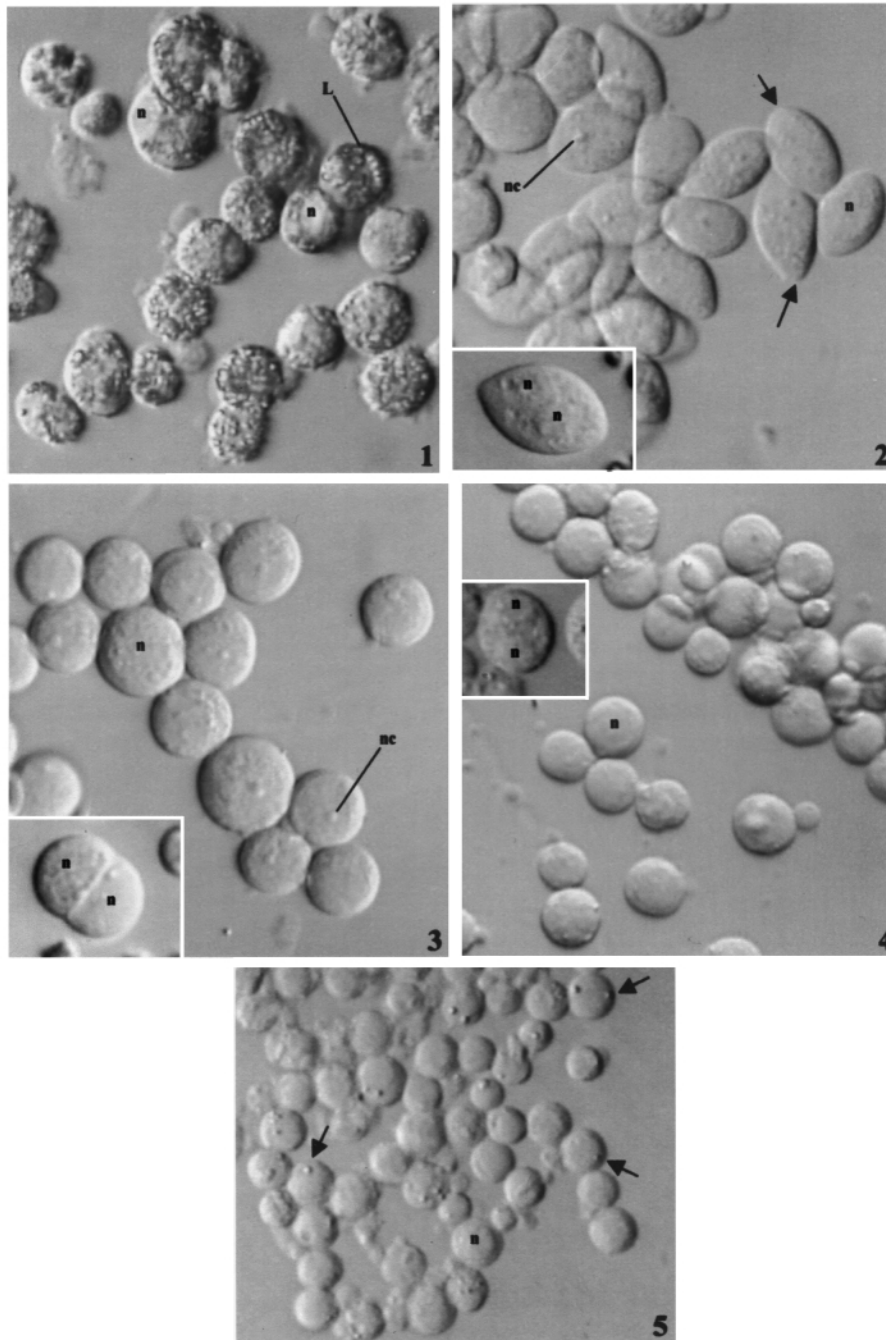


Figure 1. Sertoli cells. Note the cytoplasm rich in lipid droplets (L) and the pale nuclear region (n).

Figures 2–5. Round early germ cells. For size comparison all images have the same magnification ($\times 700$). **Figure 2.** Elongated spermatogonia. Note the ellipsoidal contour with fusiform extremes (arrows). Inset: mitotic division ($\times 700$). **Figure 3.** Pool of round spermatogonia and primary spermatocytes. Inset: meiosis I division ($\times 900$). **Figure 4.** Secondary spermatocytes. Inset: meiosis II division ($\times 900$). **Figure 5.** Round spermatids. Cells are at the Golgi phase (1–2 round acrosomal vesicles: arrows) or at the cap phase (no visible acrosome). N = nucleus; NC = nucleolus.

were used for culture in conditioned medium, 17 for conditioned medium supplemented with rFSH, and 25 for conditioned medium supplemented with rFSH and testosterone. Biochemical assays of conditioned medium have not shown any significant levels of both hormones. Controls were those in which late spermatids/sperm were found: all cases with hypoplasia and cases with incomplete maturation arrest (MA) and incomplete Sertoli cell-only syndrome (SCOS). The case

study group comprised those in whom no germ cells more mature than round spermatids were found: all cases of complete MA, and cases of SCOS in which a focus of meiosis arrest was found at treatment.

About 30–80 Sertoli cells, 10–30 elongated spermatogonia, and 200 primary spermatocytes and round spermatogonia were always used in each culture test (Figures 1–3). When available, ~10–20 secondary spermatocytes (Figure 4) and a variable

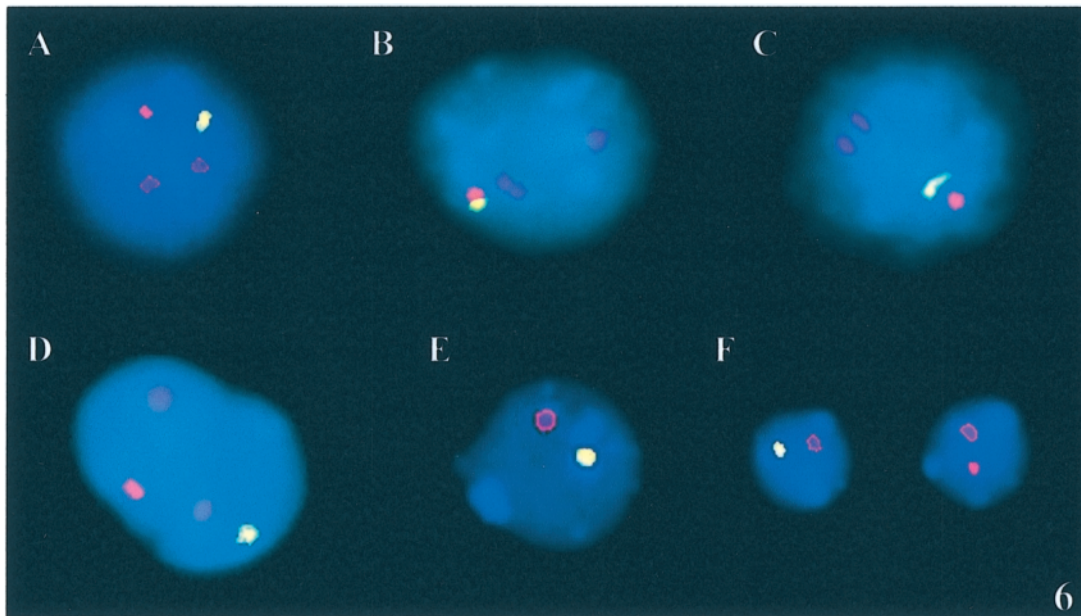


Figure 6. Fluorescent in-situ hybridization analysis of isolated early germ cells using probes to chromosomes X (yellow), Y (red) and 18 (violet). (A) Elongated spermatogonium; (B–D) primary spermatocytes. Note the presence of chromatids by a larger fluorescence signal (B–D) or by a double contiguous signal (B, C), and the pairing of the sexual (B, C) and somatic (C) chromosomes. In (D), the primary spermatocyte is dividing, with segregation of homologues; (E) secondary spermatocyte; (F) round spermatids. Original magnification $\times 1000$.

number of round spermatids (Figure 5) were also added to the culture medium. In two control cases, round spermatids could not be found to be added to culture.

Early germ cells were initially differentiated accordingly to morphological criteria (Holstein and Roosen-Runge, 1981; Sofikitis *et al.*, 1998b). Briefly, elongated spermatogonia (A pale, A cloudy and A pale long) were selected by their ellipsoidal shape, with fusiform extremes; the pool of primary spermatocytes could not be distinguished from round spermatogonia (A pale, A dark and B), and thus includes both types of diploid germ cells; primary (19–24 μm in diameter) and secondary (14 \pm 1 μm in diameter) spermatocytes were distinguished based on the relative cell and nuclear sizes; round spermatids (8–10 μm in diameter) were distinguished from spermatocytes based on the relative cell and nuclear sizes and also on the presence of the acrosomal vesicle. Cell purity was then analysed by in-situ hybridization, which confirmed cell stage diagnosis in $\sim 95\%$ of the cells (Figure 6).

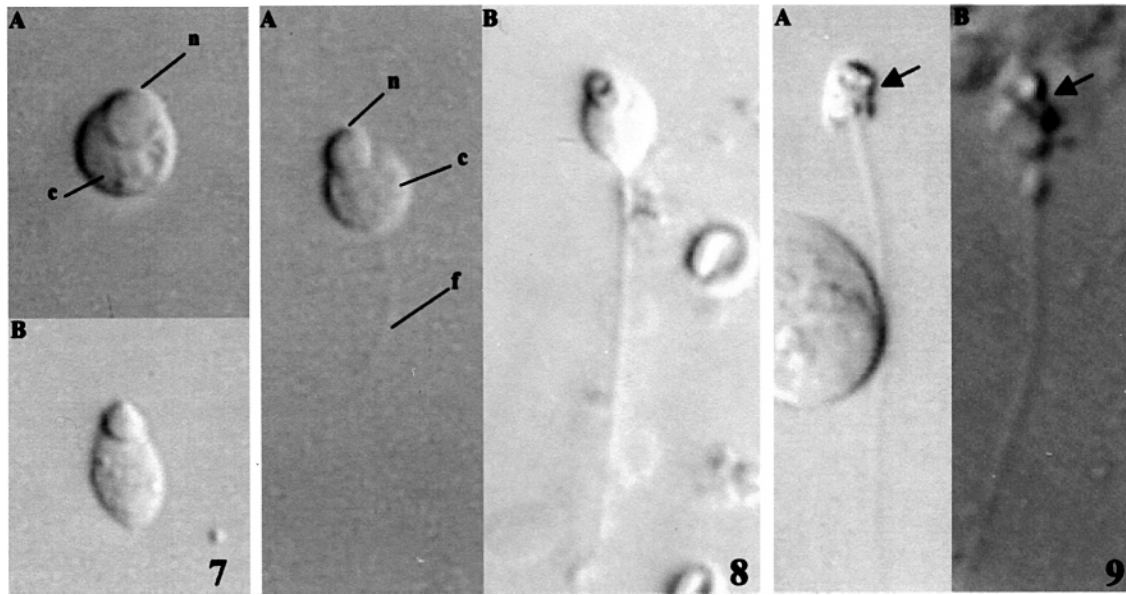
Co-cultures of germ cells with Sertoli cells were followed daily and spermatids counted individually. In most of the cultures, ~ 24 h after mixing the cell subtypes, many of the cells became aggregated into cell clusters that detached from the tissue culture plate, thus suggesting that Sertoli and germ cell contacts were re-acquired very shortly after culturing. Resumption of meiosis and spermiogenesis differentiation were not restricted to those cell clusters and were similarly observed on isolated cells, although this occurred in monolayers of side connected cells. Elongated spermatogonia were found in division, but this occurred at a very low rate (Figure 2). A few of the primary and secondary spermatocytes were observed to enter meiotic divisions, which took ~ 2 –4 days per stage (Figures 3 and 4). Most of the round spermatids in culture did not further differentiate, some matured into abnormal (without

flagellum) elongating and elongated spermatids (Figure 7), and only a few developed a flagellum after 1–2 days of culture. Some of the round spermatids that developed a flagellum then further developed into normal elongating spermatids after 2–3 further days of culture, and, among the latter, a few became elongated spermatids after more 3–4 days of culture (Figure 8). Of the early elongated spermatids formed *in vitro* some differentiated into late elongated spermatids after more 2–3 days of culture, and of these some matured into sperm in the next 2–3 days of culture. However, most of these late spermatids/sperm displayed abnormal nuclei (Figure 9). In total, the *in-vitro* spermiogenic process was thus obtained after a mean time of 10–15 days of culture. Cell degeneration was found persistently throughout culturing and increased with time, being especially marked on Sertoli and early germ cells. This could be partially inhibited by FSH, and especially with FSH and testosterone.

In-vitro maturation in conditioned medium

In controls (Table I), newly formed round spermatids were observed in only one case (9.1%). Of all round spermatids, 62% remained arrested, 22.2% matured into abnormal (without flagellum) elongating spermatids, and 15.8% grew a flagellum (91% of cases). About 40.6% of the latter remained arrested, whereas 59.4% developed into normal elongating spermatids (90.9% of cases). Of the normal elongating spermatids, 42.1% arrested in culture, and 57.9% further progressed to early elongated spermatids (72.7% of cases). Of these, 81.8% remained arrested, and 18.2% matured into late elongated spermatids (18.2% of cases), with all maturing into sperm with highly abnormal heads.

In case studies (Table I), round spermatids could be isolated for culturing in 75% of the patients and new round spermatids



Figures 7–9. **Figure 7.** Abnormal (without flagellum) spermatids. The elongating spermatid (**A**) has a round nucleus slightly bulging from a round cytoplasm (magnification $\times 1200$), whereas the elongated spermatid (**B**) shows an elongated and more condensed nucleus protruding from an elongated cytoplasm (magnification $\times 1200$). **Figure 8.** Normal elongating (**A**, magnification $\times 1500$) and early elongated (**B**, magnification $\times 1400$) spermatids. **Figure 9.** Late elongated spermatids with abnormal heads (arrows). N = nucleus; c = cytoplasm; f = flagellum (magnification $\times 1400$).

were observed in only two cases (25%). Of all round spermatids, 77.6% remained arrested, 14.9% developed into abnormal elongating spermatids, and 7.5% extruded a flagellum (87.5% of cases). Of the latter, 26.7% remained arrested and 73.3% developed into normal elongating spermatids (75% of cases). About 72.7% of the normal elongating spermatids arrested, whereas 27.3% matured into early elongated spermatids (25% of cases), which then remained arrested.

In-vitro maturation in conditioned medium supplemented with rFSH

In controls (Table II), newly formed round spermatids were observed in only three cases (23.1%), and of all round spermatids, 46.2% remained arrested, 27.8% developed into abnormal elongating spermatids, and 26% grew a flagellum (100% of cases). Of the latter, 23.9% remained arrested, whereas 76.1% developed into normal elongating spermatids (84.6% of cases). About 88.9% of the normal elongating spermatids arrested in culture, and only 11.1% progressed to early elongated spermatids (15.4% of cases), of which all have remained arrested.

In case studies (Table II), round spermatids could be isolated for culturing in only one case (25%), and new round spermatids were observed in two cases (50%). Of all round spermatids, 66.1% remained arrested, 18.6% matured into abnormal elongating spermatids, and 15.3% developed a flagellum (50% of cases). Of these, 33.3% remained arrested and 66.7% developed into normal elongating spermatids (50% of cases). About 83.3% of the normal elongating spermatids arrested in culture, whereas 16.7% progressed to early elongated spermatids (25% of cases), which then remained arrested.

In-vitro maturation in conditioned medium supplemented with rFSH and testosterone

In controls (Table III), round spermatids could be retrieved for culture in 87.5% of the cases, and newly formed round spermatids were observed in all cases. Of all round spermatids, 22.7% remained arrested, 38.8% developed into abnormal elongating spermatids, and 38.5% extruded a flagellum (75% of cases). Of the latter, 17.3% remained arrested and 82.7% developed into normal elongating spermatids (68.8% of cases). About 63.2% of the normal elongating spermatids then arrested in culture, whereas 36.8% progressed to early elongated spermatids (56.3% of cases). Of these, 37.1% arrested and 62.9% developed into late elongated spermatids (31.3% of cases), of which >80% matured into sperm, with most exhibiting an abnormal nuclear morphology.

In case studies (Table III), round spermatids could be isolated for culturing in 66.7% of the patients, and new round spermatids were observed in 77.8% of the cases. Of all round spermatids, 29.4% remained arrested, 38.7% developed into abnormal elongating spermatids, and 32% formed a flagellum (66.7% of cases). Of the latter, 29% remained arrested, whereas 71% developed into normal elongating spermatids (66.7% of cases). About 43.2% of the normal elongating spermatids arrested in culture, and 56.8% matured into early elongated spermatids (44.4% of cases). Of these, 36% remained arrested, whereas 64% developed into late elongated spermatids (44.4% of cases), of which the majority matured into sperm, all with abnormal nuclear morphology.

Comparison between the three different media

Comparisons within and between groups (Table IV) suggest that although meiosis induction is increased by FSH, significant

Table I. Results of culture in conditioned medium

| Study | Diagnosis | No. of cases | Days of culture | DGC | Previous | | New | | | | |
|----------|-----------|--------------|-----------------|-----|----------|-----|-----|-----|-----|-----|-----|
| | | | | | Sa1 | Sa1 | Sa2 | Sb1 | Sb2 | Sd1 | Sd2 |
| Controls | SCOS | 1 | 11 | 200 | 10 | 0 | 2 | 0 | 2 | 2 | 0 |
| | HP | 1 | 5 | 200 | 16 | 0 | 3 | 5 | 3 | 1 | 0 |
| | | 2 | 9 | 200 | 37 | 0 | 3 | 14 | 3 | 0 | 0 |
| | | 3 | 11 | 200 | 10 | 10 | 2 | 3 | 2 | 2 | 0 |
| | | 4 | 10 | 200 | 10 | 0 | 3 | 3 | 2 | 1 | 1 |
| | | 5 | 8 | 200 | 10 | 0 | 2 | 1 | 1 | 1 | 0 |
| | | 6 | 20 | 200 | 10 | 0 | 2 | 0 | 2 | 1 | 1 |
| | | 7 | 14 | 200 | 20 | 0 | 1 | 5 | 1 | 0 | 0 |
| | | 8 | 7 | 200 | 10 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 9 | 16 | 200 | 10 | 0 | 2 | 2 | 1 | 1 | 0 |
| 10 | 13 | 200 | 50 | 0 | 12 | 12 | 2 | 2 | 0 | | |
| Cases | MA | 1 | 2 | 200 | 35 | 0 | 2 | 0 | 2 | 0 | 0 |
| | | 2 | 8 | 200 | 29 | 0 | 2 | 7 | 2 | 1 | 0 |
| | | 3 | 10 | 200 | 36 | 0 | 4 | 7 | 2 | 0 | 0 |
| | | 4 | 4 | 200 | 53 | 0 | 2 | 14 | 2 | 0 | 0 |
| | | 5 | 9 | 200 | 10 | 0 | 0 | 1 | 0 | 0 | 0 |
| | SCOS | 1 | 7 | 200 | 30 | 0 | 1 | 0 | 1 | 0 | 0 |
| | | 2 | 10 | 200 | 0 | 4 | 2 | 1 | 0 | 0 | 0 |
| | | 3 | 12 | 200 | 0 | 4 | 2 | 0 | 2 | 2 | 0 |

SCOS = Sertoli cell-only syndrome; MA = maturation arrest; HP = hypoplasia. DGC = diploid germ cells; Sa1 = early round spermatids; Sa2 = late round spermatids (with flagellum); Sb1 = abnormal elongating spermatids (without flagellum); Sb2 = normal elongating spermatids (with flagellum); Sd1 = early elongated spermatids; Sd2 = late elongated spermatids.

Table II. Results of culture in conditioned medium + rFSH

| Study | Diagnosis | No. of cases | Days of culture | DGC | Previous | | New | | | | |
|----------|-----------|--------------|-----------------|-----|----------|-----|-----|-----|-----|-----|-----|
| | | | | | Sa1 | Sa1 | Sa2 | Sb1 | Sb2 | Sd1 | Sd2 |
| Controls | MA | 1 | 7 | 200 | 10 | 3 | 9 | 4 | 9 | 0 | 0 |
| | SCOS | 1 | 10 | 200 | 10 | 0 | 3 | 5 | 3 | 0 | 0 |
| | HP | 1 | 10 | 200 | 40 | 10 | 16 | 8 | 16 | 0 | 0 |
| | | 2 | 9 | 200 | 50 | 0 | 2 | 32 | 2 | 0 | 0 |
| | | 3 | 8 | 200 | 10 | 0 | 3 | 5 | 3 | 0 | 0 |
| | | 4 | 7 | 200 | 10 | 0 | 11 | 1 | 9 | 0 | 0 |
| | | 5 | 3 | 200 | 10 | 0 | 7 | 4 | 5 | 2 | 0 |
| | | 6 | 7 | 200 | 10 | 0 | 2 | 0 | 1 | 0 | 0 |
| | | 7 | 5 | 200 | 10 | 0 | 1 | 0 | 0 | 0 | 0 |
| | | 8 | 7 | 200 | 10 | 0 | 2 | 0 | 1 | 0 | 0 |
| | | 9 | 8 | 200 | 50 | 0 | 6 | 0 | 0 | 0 | 0 |
| 10 | 6 | 200 | 10 | 0 | 1 | 5 | 1 | 0 | 0 | | |
| 11 | 9 | 200 | 10 | 20 | 8 | 12 | 4 | 4 | 0 | | |
| Cases | MA | 1 | 11 | 200 | 0 | 4 | 4 | 1 | 1 | 1 | 0 |
| | | 2 | 9 | 200 | 50 | 0 | 0 | 5 | 0 | 0 | 0 |
| | SCOS | 1 | 7 | 200 | 0 | 5 | 5 | 5 | 5 | 0 | 0 |
| | | 2 | 7 | 200 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

For abbreviations, see Table I.

differences only appear in the presence of FSH and testosterone. No major differences were found within each tested medium regarding the in-vitro maturation rate of round spermatids. On the contrary, significant differences were found between groups of different culture media, suggesting that FSH plays a role in the maturation to elongating spermatids (in controls), and that FSH plus testosterone induces significant increases of maturation in early and late spermiogenesis (in controls and case studies).

Microinjection experiments

Although these experiments were highly dependent on the availability of donated spare oocytes, we could nevertheless test 30 cultures. No significant differences were observed in the fertilization and blastocyst rates between controls and case studies. The fertilization rate with in-vitro matured round spermatids (26 oocytes) was 37.5% (1PN), all cleaved, and two reached the morula stage (28.6%). With abnormal elongating spermatids (27 oocytes), the fertilization rate was 8.3% (2PN),

Table III. Results of culture in conditioned medium + rFSH + testosterone

| Study | Diagnosis | No. of cases | Days of culture | DGC | Previous | | New | | | | | |
|----------|-----------|--------------|-----------------|-----|----------|-----|-----|-----|-----|-----|-----|---|
| | | | | | Sa1 | Sa1 | Sa2 | Sb1 | Sb2 | Sd1 | Sd2 | |
| Controls | SCOS | 1 | 7 | 200 | 6 | 1 | 4 | 3 | 4 | 2 | 0 | |
| | | MA | 1 | 11 | 200 | 10 | 10 | 10 | 10 | 10 | 0 | 0 |
| | | | 2 | 6 | 200 | 5 | 10 | 2 | 0 | 2 | 2 | 2 |
| | | | 3 | 5 | 200 | 5 | 10 | 0 | 0 | 0 | 0 | 0 |
| | | | 4 | 6 | 200 | 0 | 10 | 0 | 3 | 0 | 0 | 0 |
| | | | 5 | 7 | 200 | 5 | 5 | 0 | 0 | 0 | 0 | 0 |
| | | | 6 | 9 | 200 | 0 | 5 | 0 | 2 | 0 | 0 | 0 |
| | 7 | 7 | 200 | 20 | 10 | 4 | 13 | 4 | 4 | 3 | | |
| | HP | 1 | 4 | 200 | 5 | 7 | 10 | 4 | 7 | 3 | 0 | |
| | | 2 | 6 | 200 | 10 | 10 | 14 | 2 | 13 | 2 | 0 | |
| | | 3 | 3 | 200 | 20 | 12 | 4 | 20 | 3 | 0 | 0 | |
| | | 4 | 8 | 200 | 10 | 11 | 9 | 12 | 7 | 1 | 0 | |
| | | 5 | 11 | 200 | 10 | 15 | 10 | 15 | 10 | 10 | 10 | |
| | | 6 | 6 | 200 | 10 | 26 | 36 | 15 | 28 | 8 | 5 | |
| | | 7 | 10 | 200 | 5 | 15 | 5 | 6 | 3 | 3 | 2 | |
| 8 | | 8 | 200 | 5 | 3 | 2 | 6 | 0 | 0 | 0 | | |
| Cases | MA | 1 | 11 | 200 | 0 | 10 | 0 | 0 | 0 | 0 | 0 | |
| | | 2 | 7 | 200 | 0 | 10 | 2 | 2 | 1 | 1 | 1 | |
| | | 3 | 7 | 200 | 20 | 23 | 33 | 10 | 31 | 14 | 12 | |
| | | 4 | 12 | 200 | 10 | 17 | 17 | 10 | 2 | 2 | 1 | |
| | | 5 | 13 | 200 | 10 | 15 | 1 | 4 | 1 | 0 | 0 | |
| | | 6 | 6 | 200 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | |
| | | 7 | 12 | 200 | 6 | 20 | 1 | 20 | 1 | 0 | 0 | |
| | | 8 | 6 | 200 | 3 | 30 | 8 | 19 | 8 | 8 | 2 | |
| | SCOS | 1 | 5 | 200 | 20 | 0 | 0 | 10 | 0 | 0 | 0 | |

For abbreviations, see Table I.

Table IV. Results of culture: comparison between the three different culture media

| | Conditioned medium (CM) | | CM + rFSH | | CM + rFSH + testosterone | |
|---------------------------------|-------------------------|------------|---------------------|------------|--------------------------|-------------------------|
| | Controls | Cases | Controls | Cases | Controls | Cases |
| No. of patients | 11 | 8 | 13 | 4 | 16 | 9 |
| Mean days of culture (range) | 11.3 (5–20) | 7.8 (2–12) | 7.4 (3–10) | 8.5 (7–11) | 7.1 (3–11) | 8.8 (5–13) ^d |
| Meiotic index (new Sa1/DGC) (%) | 0.45 | 0.5 | 1.3 | 1.1 | 5 ^c | 6.9 |
| Total Sa1 | 203 | 201 | 273 | 59 | 286 | 194 |
| Sa2 (%) | 15.8 ^a | 7.5 | 26 ^b | 15.3 | 38.5 ^c | 32 ^d |
| Sb2 (%) | 9.4 | 5.5 | 19.8 ^{a,b} | 10.2 | 31.8 ^{a,c} | 22.7 ^d |
| Sd1 (%) | 5.4 ^a | 1.5 | 2.2 | 1.7 | 12.2 ^c | 12.9 ^d |
| Sd2 (%) | 1 | 0 | 0 | 0 | 7.7 ^c | 8.2 ^d |

^a $P < 0.05$ between control and case groups within each culture medium.

^b $P < 0.05$ between control groups of CM and CM + rFSH.

^c $P < 0.05$ between control groups of the three different culture media.

^d $P < 0.05$ between case groups of the three different culture media.

For abbreviations, see Table I.

all cleaved with good morphological grade, but no blastocyst was obtained (Table V). Normal elongating and elongated spermatids (50 oocytes), elicited 31.3% of fertilization rate (6.3% with 2PN), 94.4% cleaved (82.4% with good morphological grade), and the morula/blastocyst rate was 46.2% per total high quality embryos. Late elongated spermatids and sperm with abnormal nuclear morphology (14 oocytes) enabled 27.3% of fertilization rate (9.1% with 2PN), all embryos cleaved (33% with good morphological grade), but no blastocyst formed (Table VI).

FISH analysis

FISH analysis was performed in 15 embryos (Table VII, Figure 10). Of the nine embryos resulting from round spermatid injection, seven were analysed by FISH, including those attaining the morula stage. All exhibited an abnormal constitution of the sex chromosomes (mosaicism), except one morula that had a clear prevalence of disomy (63.6% of the nuclei). Of the two embryos generated by using abnormal elongating spermatids, all were submitted to FISH analysis, which revealed one mosaic and one haploid embryo

Table V. Results of microinjection using round spermatids, and elongating spermatids without flagellum

| Type of spermatid injected | No. of cases | MII oocytes | Degenerated | Intact | Non-fertilized 0PN, 1PB | Activated 0PN, 2PB | Abnormal fertilization 1PN, 2PB | Normal fertilization 2PN, 2PB | Cleaved | Embryo grade | | Morula/ blastocyst |
|----------------------------|--------------|-------------|-------------|--------|-------------------------|--------------------|---------------------------------|-------------------------------|---------|----------------|----------------|--------------------|
| | | | | | | | | | | AB | CD | |
| Sa1 | 1 | 4 | 0 | 4 | 2 | 0 | 2 | 0 | 2 | 1 ^a | 1 | 0 |
| | 2 | 6 | 0 | 6 | 5 | 0 | 1 | 0 | 1 | 1 ^a | 0 | 0 |
| | 3 | 4 | 0 | 4 | 1 | 0 | 3 | 0 | 3 | 2 ^a | 1 ^a | 1 ^a |
| | 4 | 2 | 0 | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 5 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 6 | 6 | 2 | 4 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Sa2 | 1 | 2 | 0 | 2 | 0 | 0 | 2 | 0 | 2 | 2 ^a | 0 | 1 ^a |
| | 2 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 0 |
| Sb1 | 1 | 4 | 0 | 4 | 3 | 0 | 0 | 1 | 1 | 1 ^a | 0 | 0 |
| | 2 | 4 | 0 | 4 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 3 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 4 | 4 | 0 | 4 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 5 | 8 | 2 | 6 | 6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 6 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 7 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 8 | 2 | 0 | 2 | 1 | 0 | 0 | 1 | 1 | 1 ^a | 0 | 0 |
| | 9 | 2 | 0 | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

^aProcessed for fluorescent in-situ hybridization analysis.
 PN = pronucleus; PB = polar body. For other abbreviations, see Table I.

Table VI. Results of microinjection using normal elongating and early elongated spermatids, and late elongated spermatids with abnormal nuclear morphology

| Type of spermatid injected | No. of cases | MII oocytes | Degenerated | Intact | Non-fertilized 0PN, 1PB | Activated 0PN, 2PB | Abnormal fertilization 1PN, 2PB | Normal fertilization 2PN, 2PB | Cleaved | Embryo grade | | Morula/ blastocyst |
|----------------------------|--------------|-------------|-------------|--------|-------------------------|--------------------|---------------------------------|-------------------------------|---------|----------------|----|--------------------|
| | | | | | | | | | | AB | CD | |
| Sb2/Sd1 | 1 | 16 | 0 | 16 | 16 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 2 | 9 | 0 | 9 | 3 | 0 | 4 | 2 | 6 | 6 | 0 | 4 ^b |
| | 3 | 9 | 0 | 9 | 2 | 1 | 6 | 0 | 6 | 5 ^a | 1 | 2 ^a |
| | 4 | 5 | 0 | 5 | 4 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| | 5 | 3 | 0 | 3 | 2 | 0 | 0 | 1 | 1 | 1 ^a | 0 | 0 |
| | 6 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 7 | 2 | 0 | 2 | 1 | 0 | 1 | 0 | 1 | 1 ^a | 0 | 0 |
| | 8 | 5 | 2 | 3 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Sd2-abn | 1 | 2 | 0 | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 2 | 5 | 0 | 5 | 2 | 0 | 2 | 1 | 3 | 1 ^a | 2 | 0 |
| | 3 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 4 | 2 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 5 | 4 | 2 | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

^aProcessed for fluorescent in-situ hybridization analysis.
^bTwo cases from 1PN zygotes and two cases from 2PN zygotes.
 PN = pronucleus; PB = polar body. For other abbreviations, see Table I.

(both from 2PN zygotes). Of the 14 embryos obtained after injection of normal elongating and elongated spermatids, nine were not analysed, five because they had degenerated during long-term culture, and four because they had attained the blastocyst stage. Of the five embryos analysed by FISH, including two morulae, embryos were mosaic ($n = 3$), haploid ($n = 1$, from a 2PN zygote) or chaotic ($n = 1$), but both morulae exhibited a marked disomic prevalence (78.6% of the nuclei). Only one out of the three embryos generated by using abnormal late elongated spermatids survived after long-term culture, whose FISH analysis revealed them to be chaotic (from a 2PN zygote).

Discussion

In several patients with non-obstructive azoospermia, mature sperm are not found at treatment. As an alternative, late or round spermatids have been used as gametes (Hannay, 1995; Fishel *et al.*, 1995, 1996; Tesarik *et al.*, 1995; Vanderzwalmen *et al.*, 1995, 1997; Amer *et al.*, 1997; Antinori *et al.*, 1997a,b; Araki *et al.*, 1997; Barak *et al.*, 1998; Bernabeu *et al.*, 1998; Kahraman *et al.*, 1998; Sofikitis *et al.*, 1998a; Sousa *et al.*, 1999). On the contrary, patients with complete Sertoli cell-only syndrome and maturation arrest had to be treated with donor sperm.

Because round spermatid injection proved to be of seldom

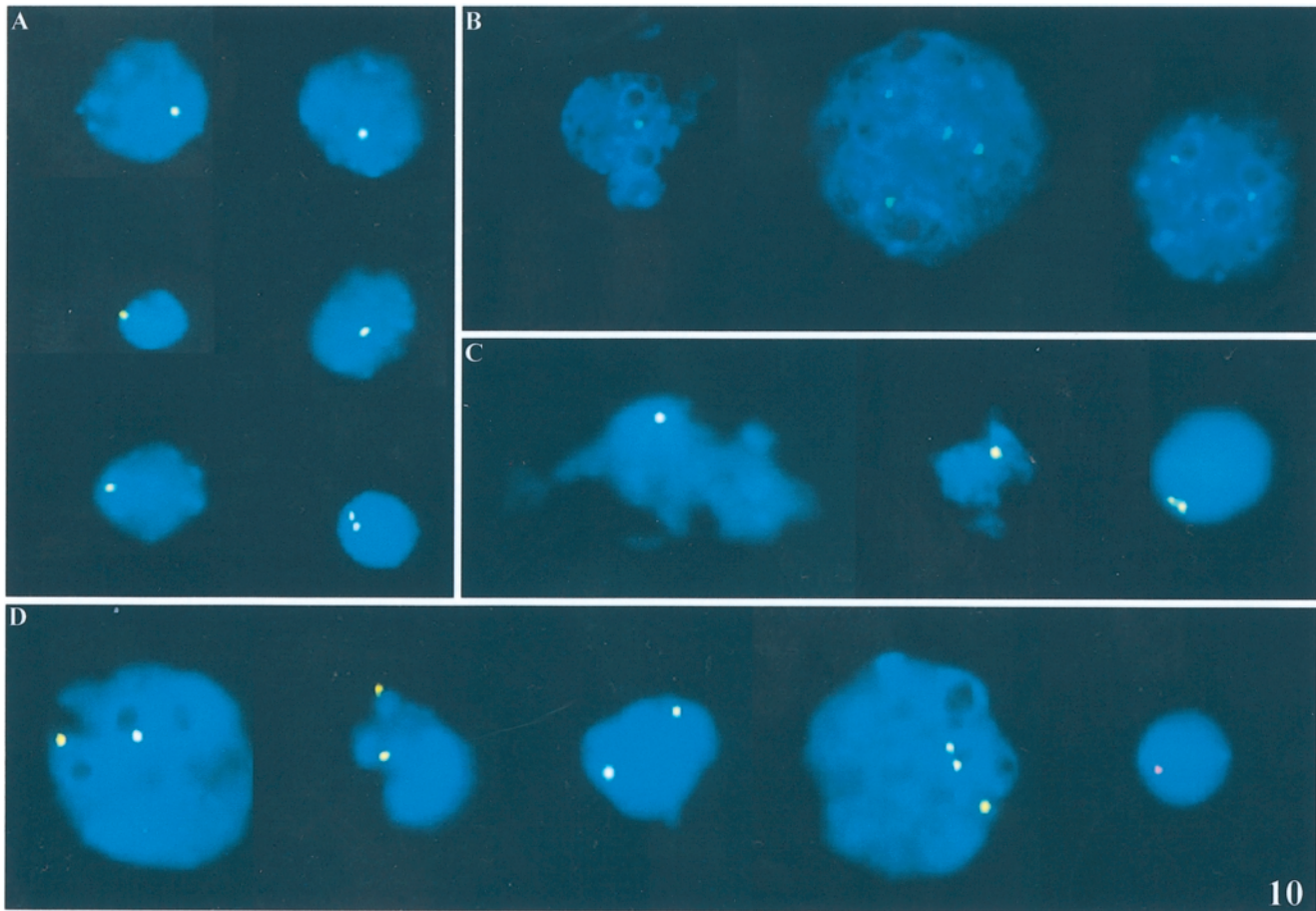


Figure 10. Fluorescent in-situ hybridization analysis of embryos using probes to chromosomes X (yellow/green) and Y (red). **(A)** Two-cell arrested embryo from injection of an early round spermatid. There were three nuclei per blastomere, five with a single X chromosome signal, and one with a diploid X constitution. **(B)** Morula from injection of a normal elongating spermatid. Two blastomeres had one single X chromosome, one blastomere showed four X signals, and eleven blastomeres had a normal diploid X constitution. **(C)** Three-cell arrested embryo from injection of a normal elongating spermatid, each with one nucleus and a single X chromosome signal. **(D)** Four-cell arrested embryo from injection of a late elongated spermatid with abnormal nucleus. One blastomere had one nucleus with two X chromosome signals, the second blastomere had two nuclei each with two X signals, the third blastomere had one nucleus with three X signals, and the fourth blastomere had a single Y chromosome signal. Original magnification $\times 1000$.

Table VII. Results of embryo analysis by fluorescent in-situ hybridization (FISH)

| Spermatid injected | No. of cases | Case ^a | Embryo grade | Zygote origin | FISH signals | Diagnosis |
|--------------------|--------------|-------------------|---------------------|---------------|--|--------------------------------|
| Sa1 | 1 | 1 | 6B | 1PN | X(3)/XXX(fragment)/XXXX(2)/0(2) | Mosaic |
| | 2 | 2 | 2B ^b | 1PN | X(5)/XX(1)/0(1 + 1 fragment) | Mosaic: prevalence of monosomy |
| | 3 | 3 | Morula | 1PN | X(4)/XX(14)/XXX (4) | Mosaic: prevalence of disomy |
| | 4 | 3 | 5B | 1PN | X(2)/XX(3)/0(6) | Mosaic |
| | 5 | 3 | 6C | 1PN | X(3)/XX (3) | Mosaic |
| Sa2 | 1 | 1 | Morula | 1PN | X(2)/XX(4)/XXX(6)/XXXX(1)/XXXXXX(5)/XXXXXX(1)/XXXXXX(1)/0(2) | Mosaic |
| | 2 | 1 | 5B | 1PN | X(2)/XX(1) | Mosaic |
| Sb1 | 1 | 1 | 5B | 2PN | X(2)/0(7) | Haploid |
| | 2 | 8 | 5B | 2PN | X(2)/XX(3) | Mosaic |
| Sb2/Sd1 | 1 | 3 | 5B | 1PN | X(1)/XXXXXXXXXXY(1)/XXXXXXXXYY(1) | Caotic |
| | 2 | 3 | Morula ^b | 1PN | X(2)/XX(11)/XXXX(1)/0(2) | Mosaic: prevalence of disomy |
| | 3 | 3 | Morula | 1PN | X(3)/XX(11) | Mosaic: prevalence of disomy |
| | 4 | 5 | 3B ^b | 2PN | X(3)/0 (2) | Haploid |
| | 5 | 7 | 3B | 1PN | X(1)/XX(2) | Mosaic |
| Sd2-abn | 1 | 2 | 4A ^b | 2PN | XX(3)/XXX(1)/Y(1)/0(1) | Caotic |

^aRefer to Tables V–VI.

^bRefer to Figure 10.

PN: pronucleus. For other abbreviations, see Table I.

beneficial interest, in-vitro culture of these cells has then been experimentally initiated. Using IVF medium, it was shown that ~22% of round spermatids can grow flagella in ~1–2 days, but these cells then became arrested and were incapable of inducing normal embryo development (Fishel *et al.*, 1997; Aslam and Fishel, 1998; Balaban *et al.*, 2000). On the contrary, by using Vero cell monolayers, isolated round spermatids were shown to be able to mature into late spermatids and sperm in ~7–12 days (Cremades *et al.*, 1999). More recently, co-cultures of Sertoli and diploid germ cells have shown that FSH is needed for stimulating spermatogenesis and testosterone for inhibiting Sertoli cell apoptosis (Tesarik *et al.*, 1998a,b, 2000a,b). With this method, differentiation of elongated spermatids from primary spermatocytes was reported to occur within 1–2 days, and was proven to be able to generate viable normal pregnancies (Tesarik *et al.*, 1999). However, this pace was high compared with that of the normal testicular cycle which needs more than 1 month to proceed through meiosis and spermiogenesis, or at least 16 days to develop from the late pachytene or secondary spermatocyte stages to elongated spermatids (Steele *et al.*, 1999).

To ascertain if the human spermatogenic cycle could be restored *in vitro*, we have developed an alternative protocol where, to avoid any possibility of contamination by a hidden focus of elongating or elongated spermatids, round germ cells and Sertoli cells were first individually isolated, and then mixed and co-cultured. Because in-vivo spermatogenesis needs a complex set of growth factors and interleukins (Kierszenbaum, 1994; Gnessi *et al.*, 1997; Schlatt *et al.*, 1997), we have used Vero cell conditioned medium, once it is known that these cells secrete several of those factors (Huang *et al.*, 1997; Desai and Goldfarb, 1998). Comparison between different tested media suggests that FSH increases cell viability, besides stimulating meiosis and the early differentiation of spermatids up to the elongating stage, and that the association between FSH and testosterone further inhibits apoptosis, increases the meiotic index, and improves the maturation rate of all spermiogenic stages. These results confirm previous findings in rodents which have suggested that FSH plays a determinant role in the survival of the seminiferous epithelium and in spermatogonia proliferation (Huang *et al.*, 1987; Hikim and Swerdloff, 1995; Foresta *et al.*, 1998; Baarends and Grootegoed, 1999), but suggest primarily that it plays a role in the conversion of round to elongating spermatids. In relation to testosterone, our results confirm that it inhibits human Sertoli cell apoptosis (Tesarik *et al.*, 1998a) and increases the viability of human diploid germ cells (Erkkila *et al.*, 1997; Print and Loveland, 2000). They also confirm previous data from rodents showing that testosterone induces spermatogonia and spermatocyte proliferation and acts as a crucial element in the conversion of round to elongated spermatids (Huang *et al.*, 1987; McLachlan *et al.*, 1994; O'Donnell *et al.*, 1996).

In-vivo spermatogenesis in the human has been studied by Heller and Clermont (1964). The complete cycle from dark spermatogonia to sperm took ~90 days, and ~74 days from pale spermatogonia (the mitotic product of dark spermatogonia) to sperm. Conversion of pale spermatogonia to primary spermatocytes through the intermediate production of spermatogonia

B lasted for ~26 days. Preleptotene spermatocytes then needed 16 days to develop to the late pachytene stage, and these entered meiosis and developed into round spermatids during the next 16 days. Finally, spermiogenesis took ~16 days to attain the elongated spermatid stage and 2–3 more days to mature into sperm. Our experiments reproduced these physiological time delays, with round spermatids evolving into elongating spermatids in ~3–5 days, into early elongated spermatids in ~6–11 days, into late elongated spermatids in ~8–12 days, and into sperm in ~10–16 days. Similarly, we observed that some primary spermatocytes (probably at pachytene) finished meiosis I in 2–3 days, and that secondary spermatocytes also took 2–3 days to finish meiosis II and give rise to early round spermatids.

Although cultures could remain viable for 2–3 weeks, most of the newly formed round spermatids must have originated from late pachytene spermatocytes and secondary spermatocytes, since most of them appeared in the first 3–4 days of culture. This is in accordance with previous rodent in-vitro studies, which have shown that the information needed for meiosis completion and to begin spermiogenesis is only translated at the late pachytene stage (Nakamura *et al.*, 1978; Toppari and Parvinen, 1985; Kierszenbaum, 1994; Hue *et al.*, 1998). Nevertheless, the present data show that meiosis can be reinitiated *in vitro* at a rate of 5–7%, at least from samples containing late pachytene and secondary spermatocytes, and that normal late spermatids can differentiate at a rate of 12–32% from round haploid cells. On the contrary, cells from patients with complete meiotic arrest showed a lower rate of meiosis reinitiation (3.3%), with only 5% of the newly formed round spermatids differentiating into late spermatids.

It remains to be explained how meiosis and spermatid differentiation could be resumed in cases of complete meiotic arrest. Two possible mechanisms were envisaged. First, secondary spermatocytes could have been misdiagnosed, and then those cases with a positive progression were in reality wrong diagnoses of meiosis arrest. Second, the mutation causing meiosis arrest could have happened *de novo* at an age when pachytene spermatocytes had already developed, or alternatively the mutation did not attain all foci of germ cells (Vogt, 1996; Page *et al.*, 1999).

The present experiments also revealed that the optimized conditions were not met, as some control cases were unable to progress through spermiogenesis. Possible causes could be the need for specific factors secreted by connective tissue cells that surround the seminiferous epithelium, the rupture of cell connections during cell dissociation, which are essential for sharing gene products such as mRNA encoded by sex chromosomes (Morales *et al.*, 1998), the loss of the compartmentalization into apical and basal systems as determined by Sertoli cells *in vivo*, and the absence of renewal of the culture medium. However, the present investigation was conducted with isolated cells in microdrops in order to assure that no mature spermatids could be hidden in the tissue. Now that culture conditions and the pace of cell differentiation *in vitro* are better defined, culturing can be performed in tubes, avoiding cell dissociation and enabling the change of the upper part of the culture medium.

The developmental potential of the in-vitro matured

spermatids, from either controls or case studies, was assessed by microinjection on spare mature oocytes donated for research. In-vitro matured round spermatids and normal elongating and elongated spermatids elicited a low fertilization rate (31–38%), regular rates of blastocyst formation (28.6% with round cells and 46.2% with late spermatids), but most of the embryos displayed an abnormal sex chromosome constitution, except in the case of morulae from late spermatids. The low fertilization rate and the high abnormal genetic constitution of the embryos may not result from a deficient meiotic process *in vitro* but rather to the immaturity of the spermatid. Immaturity of the cytoplasm could cause improper oocyte activation, or the presence of two centrioles could induce abnormal chromosomal segregation such as those found in haploid embryos derived from 2PN zygotes (Sousa *et al.*, 1996, 1998; Tesarik *et al.*, 1998c). The oocyte activating competence was not found at the round spermatid stage in mice (Ogura *et al.*, 1994), but it was demonstrated in hamsters (Ogura *et al.*, 1993), rabbits (Sofikitis *et al.*, 1996a,b) and bovines (Goto *et al.*, 1996). In humans, a normal activating competence was demonstrated at the round spermatid stage (Sousa *et al.*, 1996) and even at the secondary spermatocyte stage (Sofikitis *et al.*, 1998b). Similarly, nuclear immaturity could retard X chromosome decondensation, thus inducing aneuploidy through mitotic errors (Luetjens *et al.*, 1999). Although the culture system needs improvements, such problems may also be an inevitable consequence when using germ cells from non-obstructive azoospermic patients, which are supposed to have an intrinsic genetic problem, such as mutations affecting specific genes in the AZF region that are responsible for loss of spermatid differentiation (Vogt, 1996), as also shown in rabbits with primary testicular damage (Sofikitis *et al.*, 1996b).

We have also tested abnormal (without flagellum) elongating spermatids, as they represented the majority of the in-vitro differentiated haploid cells, and late elongated spermatids with abnormal nuclear morphology, which represented the majority of the terminal in-vitro differentiated cells. As expected, due to the evident centriole abnormalities and deficient nuclear maturation, the fertilization rate was low (8.3 and 27.3% respectively), no blastocysts formed, and all embryos showed sex chromosome abnormalities.

In conclusion, the present experiments ensured long-term (2–3 weeks) in-vitro co-cultures of human Sertoli and diploid germ cells, enabling, at a physiological pace, some degree of spermatogonia proliferation, resume of meiosis of late primary and of secondary spermatocytes, and differentiation of round spermatids to elongated spermatids. However, if in-vitro differentiated normal late spermatids could originate blastocysts at a regular rate and with a normal chromosome constitution, they nevertheless displayed a low fertilization potential with most of the embryos showing developmental arrest and sex chromosome anomalies.

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