Human testis in organotypic culture: application for basic or clinical research

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BACKGROUND: Over recent decades, recurring efforts have been devoted to developing testicular cell or tissue cultures for basic and clinical research. However, there remains much confusion, particularly concerning the fate of human germ cells in culture. OBJECTIVE: To reassess the status of human testicular cell types as well as the ability of germ cells to divide and differentiate in organotypic culture. METHODS: Human testicular fragments were maintained for 2 weeks in culture. The viability and functionality of testicular cells were assessed using light and electronic microscopy, apoptotic cell labelling, 5-bromo-2’-deoxyuridine (BrdU) incorporation, immunohistochemistry and quantitative PCR against specific cell markers. RESULTS: A gradual loss of meiotic and post-meiotic germ cells occurred throughout the culture period, irrespective of the presence of gonadotrophins. However, all germ cell types remained traceable for up to 16 days, some still dividing and differentiating at a rate compatible with the in vivo situation. Good maintenance of the general architecture of the explants associated with clearly quantifiable levels of several somatic cell markers was observed. CONCLUSION: Although this culture model is clearly unsuitable for preparing germ cells for therapeutic purposes, it does represent a most valuable tool for testing the effects of biological and chemical agents on testicular tissue.

Key words: human/organotypic culture/testis

Introduction

The first published attempt to develop a culture system for testicular tissues was that of Champy in 1920 who maintained testis fragments in a culture environment constituted by a plasma clot (Champy, 1920). The development of mammalian testis organotypic culture peaked in the 1960s, when several systems using adult mammalian gonads, including human, were designed. Initially, reproductive biologists placed their hopes in such models to improve the understanding of the complex process of gametogenesis (Steinberger, 1975; Kierszenbaum, 1994; Staub, 2001; Jégou et al., 2002; Sofikitis et al., 2005). Subsequently, the need for basic research but also, and principally, for various possible clinical applications led to major efforts to develop culture systems which would allow testicular germ cells to survive and achieve differentiation (Jégou et al., 2002; Sofikitis et al., 2005). However, after >80 years of research on in vitro mammalian spermatogenesis which has mobilized different technical approaches, a great degree of confusion still exists on the actual ability of human spermatogenetic cells to divide and differentiate in culture and on the influence that hormones display on these cells. This confusion arises from the wide diversity of methodologies developed by different laboratories working in this domain, but also because rigorous cellular and molecular assessments of the evolution of the cultures were often not applied to the analysis of the different culture systems (Jégou et al., 2002), partly because of the paucity of human materials available.

The aim of the present study was to totally reassess the survival of human testis in organotypic culture through a culture period of >2 weeks using an unprecedented battery of techniques, including light and transmission electronic microscopy (TEM) together with molecular, immunological and biochemical approaches.
Organotypic culture of human testis explants

The protocol was approved by the local ethics committee of Rennes, and informed consent was obtained from the donors. Normal testes, obtained from prostate cancer patients (77.94 ± 6.84 years, not subjected to any hormone therapy), were transported in fresh medium on ice, immediately following orchiectomy. The clinical status of the patients revealed no reproductive abnormalities or testicular infections. The materials were assessed by transillumination for the occurrence of full spermatogenesis before their subsequent use.

Testicular tissues were carefully dissected with scissors into 3 mm³ fragments. In each well of a six-well plate, two fragments were placed onto a PET insert (Falcon Labware; Becton Dickinson, Lincoln Park, NJ, USA) and cultured at the interface between air and 2 ml of medium (DMEM supplemented with antibiotics, 10% FCS, 1 mM sodium pyruvate, 4 mM glutamate, 100 ng/ml of vitamin A, 200 ng/ml of vitamin E, 50 ng/ml of vitamin C, 10 μg/ml of insulin and 5 μg/ml of transferrin) supplemented or not with 200 ng/ml of FSH and 1 IU/ml of HCG, as specified in the text. For each condition tested, a minimum of two wells (i.e. four fragments) was analysed. The culture was established for up to 16 days, as preliminary experiments indicated important germ cell loss and seminiferous tubules disorganization beyond that stage. The culture was performed in a humidified atmosphere containing 5% CO₂ at 34°C, and media were changed every 2 days before their storage at –20°C for testosterone assay. On days 4, 8, 12 and 16, testis explants were either fixed in neutral buffered 4% formaldehyde for immunohistochemical analysis or fixed in 4% glutaraldehyde, 0.1 M cacodylate buffer (pH 7.4) for microscopic analysis (light microscopy and TEM) or frozen and stored at –80°C until RNA extraction or cyclic adenosine monophosphate (cAMP) assay. Only testis explants that presented a normal histology before culture were used for subsequent studies.

Light microscopy

For histological analysis, testicular explants (3 mm³) were fixed in neutral buffered 4% paraformaldehyde for 2 h at 4°C, dehydrated by immersion in a series of graduated alcohol concentrations, embedded in paraffin, sectioned at 5.0 μm and stained with Masson’s hemalum for examination.

Transmission electron microscopy

Cultured explants were fixed in, successively, 2% glutaraldehyde in 0.1 M buffered sodium cacodylate (pH 7.4) for 1 h at room temperature and then in 4% glutaraldehyde in 0.1 M buffered sodium cacodylate (pH 7.4) for at least 24 h at 4°C. Post-fixation was performed with 2% OsO₄ and 1.5% potassium ferricyanide in 0.1 M sodium cacodylate buffer at room temperature for 1.5 h. Fixed explants were then dehydrated by three successive 10-min immersions in gradual ethanol concentrations (70, 95 and 100%, respectively) and, subsequently, submitted to progressive impregnations in epon resin by three successive 45-min incubations in epoxypar (1:2, 1:1, 2:1 v:v, respectively) and pure epox, overnight at 4°C. Polymerization was carried out at 60°C for 48 h. Observations of ultrathin sections were performed with a Philips CM10 electron microscope at 80 kV.

Immunostaining

To estimate the lifespan of interstitial cells in cultured testis explants, immunohistochemistry using avidin–biotin–peroxidase complex (ABC) technique was performed on formaldehyde-fixed, paraffin-embedded tissues. Tissue sections (5 μm) were deparaffinized, rehydrated and incubated in an antigen-retrieval solution (10 mM citrate, pH 6) for 20 min, as previously described (Cuevas et al., 1994) and then washed in 0.05 mol/l phosphate-buffered saline (PBS) (pH 7.6). Endogenous peroxidase was inactivated in deparaffinized sections with a 5-min treatment in PBS 3% H₂O₂. Slides were saturated with PBS supplemented with 2% bovine serum albumin (BSA) to block the non-specific sites, before overnight binding at 4°C with the appropriate antibody diluted in PBS 2% BSA. The following antibodies were used: rat anti-human Ins13 (1/2000), a previously described specific Leydig cell marker (Ivell et al., 1997); mouse anti-human CD68 (1.2 μg/ml), a marker for monocytes, macrophages and dendritic cells (Pulford et al., 1990); mouse anti-human myeloid/histiocyte MAC387 (8.6 μg/ml), a marker for a subset of reactive/infiltrating monocytes/macrophages (Flavell et al., 1987); and mouse anti-human CD3 (7.3 μg/ml), a marker for T lymphocytes (Alibaud et al., 2000). The primary antibody was replaced by appropriate isotype control (rat serum or mouse IgG1) in control sections at the same concentration. All other steps were performed at room temperature. After two washes with PBS, biotinylated secondary antibodies were applied to the sections, rinsed again with PBS and then incubated with peroxidase-conjugated streptavidin (1:500, Dako) for 30 min. After a final wash with PBS, the sections were incubated with either aminomethylcarba- zole substrate for 2–30 min or 3,3′-diaminobenzidine substrate for 1–10 min to reveal the specific staining. The nuclei were counterstained with Masson’s hemalum. The sections were photographed through an Olympus AX60TF microscope with monochromatic objectives (Olympus, Paris, France), coupled to a digital macro camera (Kigamo, Metis, France).

Quantification of cell number

The counting of the cells specifically stained by the marker was undertaken in 1000 interstitial cells in three independent cultures using the Cast™ software (Olympus, Lille, France) and their number expressed as a percentage of total interstitial cells. The number of primary spermatocytes and early spermatids per 1000 Sertoli cells was also counted in three independent cultures. Germ cell types and Sertoli cells were identified on morphological criteria. All cells present in a field were counted.

RT–PCR and real-time quantitative RT–PCR

Total RNA was extracted from the testis explants at days 0, 4, 8, 12 and 16 of culture using the RNasy isolation kit (Quiagen, Courtaboeuf, France) and depleted of contaminating DNA by DNase treatment (Promega, Charbonnières, France). cDNA was generated from 4 μg of total RNA by using the first strand step of the SuperScriptIII kit (Invitrogen, Cergy-Pontoise, France). Gene expression along the culture of the germinal-specific gene deleted in azoospermia (DAZ), expressed by spermatogonia (Menke et al., 1997), PGK2, expressed by late spermatocytes from pachytene stage to round early spermatids (McCarrey et al., 1996), and protamine-2, expressed by early and
elongated spermatids (Lee et al., 1998), was first confirmed with general RT–PCR with previously described primer pairs (Song et al., 2000). Glyceraldehyde-3-phosphatedehydrogenase (GAPDH) was used as an internal control in the RT–PCR. We then used quantitative RT–PCR to independently determine the level of gene expression for eight genes specific to different cell types in at least three independent organ cultures: PGK2, protamine-2, CHK2 for spermatogonia (Bartkova et al., 2001); LH receptor and laminin 5α for Sertoli cells (Grissold et al., 1995; Sharpe et al., 2003); class B scavenger receptor type I for both Leydig and Sertoli cells (Nakagawa et al., 2004); and CD68 for monocytes, macrophages and dendritic cells (Pulford et al., 1990). PCR was carried out on 4 ng and 40 ng of equivalent RNA with the ABI7500 (Applied Biosystems, Foster City, CA, USA) using commercially available target probes and mastermix (Applied Biosystems): Hs00200485_m1 (CHK2), Hs00371072_s1 (PGK2), Hs00172518_m1 (protamine-2), Hs00174885_m1 (LH receptor), Hs00174865_m1 (FSH receptor), Hs00245699_m1 (laminin 5α), Hs00605123_m1 (3β-HSD II) and Hs00194092_m1 (scavenger receptor). The relative gene expression in three independent cultures at the various time points (days 0, 4, 8, 12 and 16) was normalized to 18S expression by use of the standard curve method, as described by Applied Biosystems. The Ct value of each gene was calculated with the ABI sequence detection system 1.9 program (Applied Biosystems) and normalized to the level of ribosomal 18S (Hs99999901_s1).

Measurement of testosterone production
Testosterone secreted into the medium was assayed in duplicate using a commercial radioimmunoassay based on competitive binding with 1,25-dihydroxyvitamin D (Sigma), labelled testosterone (Beckman Coulter, Villepinte, France), according to the manufacturer’s recommendations. Unknown values were obtained from the standard curve interpolation and were expressed in ng/ml.

Measurement of cAMP
Intracellular cAMP content was measured using a commercially available immunoassay (cAMP Immunoassay, R&D System Europe, Abingdon, UK).

Analysis of DNA fragmentation
Apoptotic cells were detected in situ in explant sections using the TUNEL (terminal deoxynucleotidyl transferase [TdT]-mediated dUTP nick end labelling) method that was performed using an in situ Apoptosis Detection kit (Boehringer Mannheim, Mannheim, Germany), according to the manufacturer’s instructions. Briefly, paraffin-embedded sections were dewaxed and rehydrated. Tissue sections were incubated successively in hydrogen peroxide 0.3% to block endogenous peroxidase, permeabilizing solution (0.1% TritonX-100 in sodium citrate 3.5 mM) and buffer containing TdT at 0.6 U/ml and fluorescein-dUTP. The sections were incubated with an anti-fluorescein antibody conjugated to peroxidase, stained with dianimobenzidine tetrahydrochloride (DAB) (Sigma) and counterstained by brief immersion in Masson’s hemalum, following the appearance of positive cells with brown nuclei under a light microscope. Positive controls were incubated with deoxyribonuclease I (100 µg/ml) (Boehringer Mannheim) for 10 min at 37°C to induce DNA strand breaks. Negative controls were incubated without TdT. Sections were rinsed in PBS between each step.

Analysis of cell replication
Experiments were incubated with 5-bromo-2′-deoxyuridine (BrdU) (labeling reagent diluted 1:1000 in culture medium), according to the manufacturer’s protocol (Sigma), for either 1 h pulse or a 5 h incubation period following dissection (day 0 of culture). The explants incubated for 1 h were then fixed for analysis of BrdU incorporation of germ cells in S phase at the beginning of the culture. The explants incubated for 5 h were kept in culture for 1 week before being fixed and analysed to follow germ cell survival and differentiation. In another series of experiments, explants were incubated for 5 h after the 7-day culture period and were cultured for a further period of 1 week before being processed and analysed for germ cell survival and differentiation. All the BrdU-labelled tissues were fixed for 2 h in Bouin’s fluid, processed, embedded in paraffin and sectioned. Paraffin-embedded sections were dewaxed and rehydrated. The sections were incubated successively in permeabilizing solution (PBS TritonX-100 0.03%), in 3% hydrogen peroxide to block endogenous peroxidase and in denaturation solution (alcohol 50% NaOH 0.07 N). BrdU incorporation was revealed using a mouse anti-BrdU monoclonal antibody (1:100, Dako) in Tris-buffered saline (TBS) with 0.5% TritonX-100, in the dark, at room temperature for 1 h 30 min. The antibody bound to the nuclei was detected by incubation for 1 h with an anti-mouse biotinylated antibody (1:500, Dako), followed by peroxidase-conjugated streptavidin (1:500, Dako) for 30 min. DAB or 3-amino, 9 ethyl-carbazole (AEC) substrates (Sigma) were used for colour development. Sections were counterstained with Masson’s hemalum. Identification of the stained cell types was on the basis of both cellular morphological criteria and nuclei sizes. To evaluate the differentiation of the replicating germ cells during the culture, the diameter of 200 BrdU-stained positive nuclei diameter was measured using the Cast1 software (Olympus, Lille, France), and data were plotted according to the box plot methodology.

Statistics
All values (excepting box plot) are the mean ± SEM. The significance of the differences between values was evaluated using paired t-test. A value of P < 0.05 was considered statistically significant.

Results
Morphological analysis
As observed using light microscopy, the overall morphology of the seminiferous tubules and the integrity of the interstitial tissue were maintained in the absence of gonadotrophins for up to 12 days in our organotypic culture system (Figure 1). By day 8, in some tubules, the number of spermatocytes and spermatids appeared to have decreased. This phenomenon was associated with the occurrence of vacuoles within seminiferous tubules (Figure 1B). By day 12, the seminiferous epithelium was clearly disorganized in all tubules because of a marked intensification of germ cell loss. However, in most tubules, all testicular cell types were still present, including spermatocytes and early spermatids (Figure 1C). When primary spermatocytes and early spermatids were numbered (Figure 1D), it appeared that indeed both cell types tended to decrease by day 8 of culture, this decrease being statistically significant by day 12. These results were reproducible between tissues from three independent patients. The addition of LH and FSH in the culture medium had no influence on germ cell losses (data not shown).

At the TEM level, before culture, Sertoli cells presented large and invaginated nuclei comprising a pale nuclear content and lipid droplets in the cytoplasm (Figure 2A). These cells were associated with spermatogonia resting on the lamina propria, primary spermatocytes and elongated spermatids near the lumen (Figure 2A). By day 4, spermatogonia, and primary...
spermatocytes containing numerous mitochondria displaying widened spaces in the cristae, were seen in close association with the Sertoli cell cytoplasm (Figure 2B). By day 12, clusters of primary spermatocytes at various degrees of differentiation, and with large nucleus and patches of heterochromatin, some of them degenerating, were still observed (Figure 2C). Early spermatids displaying acrosome formation (Figure 2D) were also observed by day 12 of culture, as well as adherent junctions between early spermatids and Sertoli cells (Figure 2D). After 16 days of culture, Sertoli cells generally maintained their ultrastructural characteristics and their phagocytic activity, as represented by numerous cytoplasmic lysosome-like structures and clear vacuoles (Figure 2E). At this time of culture, spermatagonia surrounded by Sertoli cell cytoplasm also kept their morphological features, including large nuclei and nucleolus, cytoplasm with few round mitochondria and rare endoplasmic reticulum cisternae. Occasionally, newly formed early spermatids were observed, with endoplasmic reticulum cisternae that appeared more tubular in shape (Figure 2F). These early spermatids could have survived throughout the culture or may have originated from germ cells, which would have undergone the whole meiotic process in vitro. At this step of the culture, degenerating spermatids and giant multinucleated spermatids were found (Figure 2F) together with increased Sertoli cell lipid stores and loss of immature germ cells.

**Analysis of cell division and differentiation**

BrdU pulse–chase experiments were conducted at day 0 and day 7 of culture to check for the presence of cells in S phase and for survival/differentiation of replicating germ cells (day 7 and day 14). As expected, only BrdU-labelled spermatogonia and pre-leptotene spermatocytes were observed at the beginning of the culture (Figure 3A). At this stage, the diameter of BrdU-labelled nuclei ranged from 6.2 to 10.15 μm (median size of 7.7 μm) (Figure 3D). After 1 week of culture, labelled germ cell nuclei were larger (Figure 3B), as they ranged from 6.94 to 12.69 μm (median size of 9.5 μm) (Figure 3D). Data comparable to the latter were obtained when the BrdU incubation was performed at day 7 (instead of day 0) and processed a week later (range from 6.98 to 13.91 μm; median size of ~9.00) (Figure 3C and D). Despite several attempts, we did not observe any effect of a combination of HCG and FSH on germ cell differentiation (data not shown).
DNA fragmentation analysis

To identify the testicular cell types most prone to apoptosis in our organotypic system, DNA fragmentation was evaluated using the TUNEL method. Negative control sections (incubated without TdT) were always negative (Figure 4A), whereas cells in DNaseI-treated section were almost all positive for TUNEL staining (Figure 4B), with the exception of a very few spermatogonia and spermatids. At the beginning of the culture, a few apoptotic germ cells were already present, essentially spermatocytes (Figure 4C). By day 4, a few spermatids appeared labelled, in addition to some spermatocytes (Figure 4D). This phenomenon increased by day 12, most notably for spermatocytes, whereas spermatogonia and somatic cells were rarely labelled (Figure 4E). A significant proportion of the remaining spermatids appeared unlabelled, although we cannot completely rule out that this results from restricted access of reagents because of condensed chromatin.

Maintenance of interstitial cells during the culture

Antibodies directed against Insl3, CD68, MAC387 and CD3 were used to analyse the nature and the fate of the different interstitial cell types during the culture. No stained cells were ever detected in the seminiferous tubule cells nor in peritubular cells, whereas specific staining for Insl3, CD68, MAC and CD3 was observed within the interstitium (data not shown).
CD68+ cells represented an average of 11% of total interstitial cells at the start of the culture, among which about 6% were MAC+, whereas CD3+ T lymphocytes represented 8% of interstitial cells and Leydig cells 55% (Figure 5). For each of these antigens, the percentage of positive cells was maintained throughout the culture period, indicating stability of the interstitial cell pool in our culture system (Figure 5).

Testosterone secretion
Basal and HCG-stimulated testosterone secretions were measured in culture supernatants every 2 days throughout the culture period. Basal testosterone levels decreased by an average of 50% between day 2 and day 4 of culture, but remained in the same range thereafter until the end of the culture (Figure 6). These levels appeared unchanged by the addition of 1 IU/ml of HCG into the culture medium throughout the culture period (Figure 6). A lower (0.1 IU/ml) and a higher (10 IU/ml) concentration of HCG was also tested and, similarly, did not modify the testosterone secretion observed in basal conditions (data not shown). The duration of HCG exposure was then modified from continuous to alternate (stimulation with 1 IU/ml for 48 h followed by 48 h retrieval and so on throughout the culture), but no difference was observed (data not shown).

Testicular explant cAMP content
cAMP acts as a ubiquitous and ‘central’ second messenger in all cell types. Basal intracellular cAMP levels were measured to evaluate the maintenance of metabolic activity of testicular cells in the cultures, whereas FSH-stimulated cAMP levels were assayed to assess Sertoli cell activity.

In the absence of FSH, cAMP production decreased by an average of 60% between the beginning of culture and day 4 and remained stable thereafter (Figure 7). FSH stimulation, whether continuous or iterative (3 h of exposure every 4 days), failed to induce an increase in overall cAMP levels (Figure 7).

RT–PCR quantitative analysis of cell-specific mRNAs
Using standard RT–PCR, we first analysed the presence throughout the culture duration of three germ cell-specific mRNAs, namely DAZ [encoded by spermatogonia (Menke et al., 1997)]; PGK2 [late spermatocytes from pachytene stage to early spermatids (McCarrey et al., 1996)] and protamine-2 [Prot2; early and late spermatids (Lee et al., 1998)]. All three genes were expressed in the starting material up to day 16 with, however, an apparent progressive decrease for PGK2 and protamine-2 cDNAs expression (Figure 8A–C). Real-time quantitative RT–PCR was then performed on cDNAs obtained from three independent cultures of distinct testicular tissues.
Expression of CHK2 [a spermatogonia marker (Bartkova et al., 2001)] relative to the level of the ubiquitous 18S housekeeping gene was reduced by about 20–30% in testis explants cultured for 4 days when compared with the starting material but remained stable thereafter (Figure 8E). This confirmed the profile of expression obtained using semiquantitative RT–PCR (Figure 8A). In contrast, consistent with our previous RT–PCR, both PGK2 and Prot2 relative gene expression decreased...
by about 50% after 4 days in culture. This decrease continued as the duration of the culture increased to reach, by 16 days, 5 and 10% of the initial levels, respectively (Figure 8F and G).

Many somatic cell markers were then quantified: laminin 5α and FSH receptor for mature Sertoli cells (Sharpe et al., 2003), 3β-HSD II and LH receptor for Leydig cells (Labrie et al., 1994), class B scavenger receptor type I for both Leydig and Sertoli cells (Nakagawa et al., 2004) and CD68 for cells of myeloid lineage such as macrophages (Pulford et al., 1990). The relative copy number of laminin 5α, 3β-HSD II and CD68 mRNAs standardized to the copy number of the ubiquitous 18S mRNA increased between the beginning and the end of the culture (Figure 9A, B and F). In contrast, a dramatic decrease was observed for both LH and FSH receptor mRNAs between the beginning and the end of the culture (Figure 9A, B and F). In contrast, a dramatic decrease was observed for both LH and FSH receptor mRNAs between the beginning and the end of the culture (Figure 9A, B and F). In contrast, a dramatic decrease was observed for both LH and FSH receptor mRNAs between the beginning and the end of the culture (Figure 9A, B and F). In contrast, a dramatic decrease was observed for both LH and FSH receptor mRNAs between the beginning and the end of the culture (Figure 9A, B and F). In contrast, a dramatic decrease was observed for both LH and FSH receptor mRNAs between the beginning and the end of the culture (Figure 9A, B and F). In contrast, a dramatic decrease was observed for both LH and FSH receptor mRNAs between the beginning and the end of the culture (Figure 9A, B and F). In contrast, a dramatic decrease was observed for both LH and FSH receptor mRNAs between the beginning and the end of the culture (Figure 9A, B and F). In contrast, a dramatic decrease was observed for both LH and FSH receptor mRNAs between the beginning and the end of the culture (Figure 9A, B and F).

Figure 6. Testosterone secretion by testicular explants in culture, in the absence (■) or in the presence (□) of HCG (1 IU/ml) in the culture medium. Culture supernatants were changed and analysed every 48 h. Each bar represents the mean ± SEM of eight independent experiments performed on tissues from different donors. For basal testosterone secretion, *P < 0.05 in the paired statistical comparison with day 2 value by the t-test. There was no statistically significant difference between basal and HCG testosterone production on the same day of culture.

Figure 7. Intracellular cyclic adenosine monophosphate (cAMP) accumulation within testicular explants during the culture, in the absence (■) or the presence (□) of FSH (200 ng/ml). Each bar represents the mean ± SEM of three independent cultures from different donors. *P < 0.05 in the paired statistical comparison with day 0 value by the t-test.

Discussion
There are several important reasons that justify the need for a thorough characterization of a culture system for human testis. These include the development of basic research, various possible clinical applications and the need to study the influence of environmental factors on human testicular function, whether they be physical (e.g. radiation and heat), chemical (e.g. endocrine disruptors) or biological (e.g. viruses such as mumps or human immunodeficiency virus (HIV)).

In their pioneering work, Steinberger’s group developed an approach combining thorough morphological observations with the incorporation of tritiated thymidine in small fragments of human testis cultured on a filter at the interface between air and medium (Steinberger, 1967). This group reported survival of spermatogonia and Sertoli cells for several weeks (partial spermatogenesis, i.e. differentiation of primary spermatocytes from pre-leptotene to pachytene stage) and degeneration of early spermatids during the first days of culture, whatever the culture medium and supplements used (Steinberger, 1967). Inspired by the same culture technique but with a rotation of the culture plate and a different supplementing medium including gonadotrophins, Ghatnekar et al. (1974) identified human germ cells that were supposed to have almost passed the entire meiosis in vitro in one man of the 16 different testis biopsies used, most with testicular anomalies. However, whether trans-meiosis differentiation had occurred in this culture system remains uncertain due to the lack of reproducibility and the rapidity of the in vitro process described (14 days), which did not match the normal duration of spermatogenesis (Clermont, 1963).

More recently, prompted by the development of assisted reproductive technologies (ARTs), a marked renewal in interest for the development of culture of human testis fragments or cells has arisen, in a context of controversy about the potential dangers of microinjections of immature or cultured germ cells for the treatment of male infertility (Jégou, 1998; Jégou et al. 2002; Cremades et al., 1999, 2001; Sousa et al., 2002; Tesarik et al., 2002; Sofikitis et al., 2005). According to several laboratories using different culture systems, some human germ cells have the ability to differentiate trans-meiotically and to complete spermiogenesis in vitro. However, there are still important discrepancies between such studies, notably concerning the speed at which human germ cells differentiate in vitro (Ghatnekar et al., 1974; Tesarik et al., 1998a,b; Cremades et al., 2001; Sousa et al., 2002; Tanaka et al., 2003) and the necessity or not of gonadotrophins for their differentiation (Tesarik et al., 1998b; Cremades et al., 2001; Sousa et al., 2002; Tanaka et al., 2003). Furthermore, to date, the most advanced culture systems favourable for germ cell differentiation use Vero cells or Vero cell-conditioned medium (Cremades et al., 2001; Sousa et al., 2002; Tanaka et al., 2003) and, therefore, cannot be envisaged for preparing germ cells used for microinjections because of the inherent risks of transferring infectious materials.
The recurrent conflicting results between studies have led us to undertake a thorough re-evaluation of the behaviour of whole human testicular fragments in vitro. We postulated that the conserved morphological interactions between testicular cells within the seminiferous tubules and between the latter and interstitial compartment are much more likely to allow the viability of the system than isolated cells or disintegrated testicular biopsies; this explains our choice of using cultured human testicular fragments.

The culture principle herein presented combines a unique mix of morphological, biochemical and molecular parameters and is based on both ancient but reliable published literature and our own long-standing experience in the culture and co-culture of animal testicular cells (Le Magueresse et al., 1986; Le Magueresse and Jégou, 1988; Pineau et al., 1990; Le Magueresse-Battistoni et al., 1991; Gerard et al., 1992; Syed et al., 1995). To avoid anoxic culture conditions, small testis fragments of 3 mm3 (a small size being required to avoid necrosis) were placed onto a permeable PET membrane insert. The temperature of the human testis in vivo has been found to range from 30.5 to 34.5°C (Newman and Wilhelm, 1950; Zorgniotti et al., 1979). Thus, an incubating temperature of about 31–34°C is generally used for testis explants culture (Steinberger and Dixon, 1959). We also added transferrin, as we observed a dramatic drop in the secretion of this factor in the culture supernatant after 4 days (data not shown). The effect of gonadotrophin addition was also tested.

On the basis of both morphological criteria and the previously described sizes of the nuclei of germ cells (Johnson et al., 1999), we observed the differentiation of BrdU-labelled pre-leptotene spermatocytes into pachytene spermatocytes during the first week of culture. These results are compatible with the timing of human spermatogenesis in vivo, with pre-leptotene spermatocytes differentiating into pachytene spermatocytes in 7–14 days (Clermont, 1963). This contrasts with the previous work that showed a dramatic acceleration of germ cell differentiation in vitro (Ghatnekar et al., 1974; Tesarik et al., 2002; Tanaka et al., 2003). As in the latter studies, germ cells were cultured in isolation or in conditions where testicular fragments were disintegrated, the so-called accelerated differentiation of...
germ cells observed is most likely to be artefactual, raising very real concerns about their use for artificial fertilization by microinjection. It is noteworthy that there was no change in the speed at which germ cell differentiation was observed when animal germ cells were cultured (Perrard et al., 2003). In our model, the presence of BrdU-labelled pachytene spermatocytes at day 14, following BrdU incorporation at day 7, demonstrates that germ cells keep replicating and differentiating between 1 and 2 weeks of culture. The presence of apoptotic germ cells was noted in testis fragments before culture. Indeed, selective germ cell death is a classical component of normal spermatogenesis (Pentikäinen et al., 2003) and appears to be accentuated in testes of elderly men (Johnson, 1989) as well as in culture of human seminiferous tubules in the presence of testosterone (Erkkila et al., 1997). Moreover, the presence of degenerating and multinucleated spermatids as well as the increased lipid accumulation in the Sertoli cells observed hereafter more than 2 weeks of culture has also been previously discussed in the testes of aged men (Holstein and Eckmann, 1986; Holstein et al., 1988). However, in our research, the number of apoptotic germ cells was found to increase throughout the culture period, mainly affecting the most differentiated germ cells and confirming that the in vitro environment is largely deleterious for meiosis and spermiogenesis. This germ cell loss was also clearly established at the molecular level, as the relative abundance of germ cell-specific mRNAs decreased progressively throughout the culture. In contrast, we observed an increase throughout the culture period of the relative abundance of three somatic cell markers (laminin 5α, 3β-HSD and scavenger receptor). By using immunohistochemistry, we showed that the number of Insl3+ cells (i.e. Leydig cells) and CD68+ cells (i.e. macrophages) remained stable during the culture and none of the analysed somatic cell types divided in culture, as indicated by our BrdU experiments. Thus, although an enhanced expression of these mRNAs linked to culture conditions cannot be ruled out and may explain at least part of this effect, their continuous increase throughout the culture period most probably reflects a selective enrichment in somatic cells as germ cell numbers gradually decrease. Indeed, this increase correlates with the decrease in germ cell markers, and the ratio of laminin 5α mRNA copy number to either 3β-HSD II mRNA or CD68 mRNA or scavenger receptor mRNA indicates a similar increase in rate of these four independent and cell-specific transcripts. In contrast to the other somatic cell markers, the relative expression of two important markers of Leydig and Sertoli cells, namely LH and FSH receptors, respectively, dramatically decreased as early as 4 days into the culture period. In other in vitro experiments, also using the organ culture technique, an exposure to HCG for 24 h induced a dose-related significant loss of the specific 125I-HCG bindings for 5 days in
human testes due to down-regulation of the binding sites (Namiki et al., 1988). Thereafter, the loss was gradually recovered. The authors reported similar findings concerning FSH receptors expression following FSH exposure (Namiki et al., 1987a). However, in our experience, the down-regulation of LH and FSH receptors observed did not appear ligand induced because similar levels of mRNAs expression were observed whether in the presence or absence of LH and FSH in the culture medium.

The presence of gonadotrophins in tissue cultures has been reported to have no effect on in vitro spermatogenesis (Steinberger and Steinberger, 1967), which is consistent with the data reported here. Furthermore, no effect of HCG alone on tritiated thymidine incorporation in spermatogonia of cultured human testis was seen by Okuyama et al. (1989). In contrast, the same author described an increase in thymidine incorporation following exposure to human menopausal gonadotrophin (a mix of LH, HCG and FSH), whilst according to Tesarik et al. (2002), pharmacological concentrations of FSH and testosterone are beneficial for in vitro maturation of germ cells from some men with in vivo maturation arrest. In our experience, there was no significant effect of either HCG or FSH or a combination of the two gonadotrophins on germ cell differentiation nor on testosterone or global cAMP production. To assess whether the lack of testosterone secretion stimulation could be due to a shortage in factors involved into the steroidogenic process in our culture conditions, medium supplementation with the testosterone precursor hydroxycholesterol (30 μM) and steroidogenic enzymatic co-factors NAD/NADP (0.4 μmol/ml each) was tested but failed to enhance basal testosterone secretion either in the presence or in the absence of 1 IU/ml of HCG (data not shown). To test whether a poor penetration of HCG within the explants could be responsible for this absence of stimulation, we then incubated the explants directly with medium containing 5 IU/ml HCG into a test tube agitated overnight. Under these conditions, testosterone secretion remained independent of the presence of HCG, even for uncultured human testis explants at day 0, whilst testis explants from 90-day-old rats under the same conditions produced 3-fold more testosterone in the presence of HCG (data not shown). One of the possible causes of the observed lack of response to LH of human Leydig cells may be the age of the Leydig cells present in the human tissue used in this study. Clearly, in our experiments, basal testosterone level and 3β-HSD mRNA levels remained stable throughout the culture, indicating that the human Leydig cell steroidogenic activity in organ culture is not affected per se, but rather that the cells are unable to respond to LH stimulation. Indeed, age-related decline in testosterone production is established in men (Zirkin and Chen, 2000), the topical question being whether this decline results from a diminished LH signalling, as serum LH level do not decline significantly in older males. In support to this hypothesis, it has recently been shown that LH-stimulated adenylate cyclase activity declines with ageing and that administration of the diffusable cAMP analogue dibutyryl cAMP restored levels of testosterone production in aged rat Leydig cell cultured in vitro for 3 days, whereas LH treatment was ineffective (Chen et al., 2004). Although LH receptor numbers decrease in old Leydig cells by about 50% (Chen et al., 2002), the authors postulated that the reduced cAMP production is more likely to result from defects in the LH receptor itself or in coupling of the LH receptor to adenyl cyclase, through affected membrane fluidity.

In conclusion, our results clearly indicate that despite many different and new trials undertaken to improve the culture systems of human adult testicular fragments, we were unable to prevent the gradual loss of the most differentiated germ cells (i.e. spermatocytes and spermatids) and reverse the unresponsiveness of the explants to gonadotrophins. However, the in-depth characterization of this culture system herein presented also univocally establishes that adult human testis explants in culture for up to 2 weeks retain critical features such as the overall structural integrity of both the interstitial and the seminiferous tubules compartments, the presence of all germ cell types (some of them differentiating) associated with Sertoli cells presenting classical ultra-structural characteristics and the expression of many somatic cell markers. Therefore, if this model is clearly unsuitable for preparing germ cells usable for therapeutic purposes due to the risk for oxidative stress for germ cells or for other modification of their intrinsic integrity as recently reviewed (Sofikitis et al., 2005), it does represent a most valuable tool for toxicological or infection studies. It is currently successfully being used in our laboratory to study HIV replication in the human testis and could benefit other applications.

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References
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