BIOLOGY OF REPRODUCTION (2016) **95**(4):89, 1–10
Published online before print 31 August 2016.
DOI 10.1095/biolreprod.116.142802

Complete Human and Rat Ex Vivo Spermatogenesis from Fresh or Frozen Testicular Tissue[1]

Marie-Hélène Perrard,[3] Nicolas Sereni,[4] Caroline Schluth-Bolard,[5] Antonine Blondet,[6] Sandrine Giscard d'Estaing,[7] Ingrid Plotton,[8] Nicolas Morel-Journel,[9] Hervé Lejeune,[7] Laurent David,[4] and Philippe Durand[2,6]

[3]*INSERM U 1208, Bron, France*
[4]*Laboratoire Ingénierie des Matériaux Polymères, CNRS UMR 5223, Université Claude Bernard Lyon1, Université Lyon, Villeurbanne, France*
[5]*Hospices Civils de Lyon, Université Claude Bernard Lyon 1, CRNL, CNRS UMR5292; TIGER Team, Lyon, France Service de Génétique, Laboratoire de Cytogénétique Constitutionnelle, Bron, France*
[6]*Kallistem, Ecole Normale Supérieure de Lyon, Lyon, France*
[7]*Hospices Civils de Lyon, Université Claude Bernard Lyon 1, INSERM U 1208; Service de Médecine de la Reproduction, Hôpital Femme Mère Enfant, Bron, France*
[8]*Hospices Civils de Lyon, Université Claude Bernard Lyon 1, INSERM U 1208; Service d'Hormonologie, Endocrinologie Moléculaire et Maladies Rares, Bron, France*
[9]*Hospices Civils de Lyon. Service d'Urologie, Centre Hospitalier Lyon Sud, Chemin du Grand Revoyet, Pierre-Bénite, France*

ABSTRACT

Until now, complete ex vivo spermatogenesis has been reported only in the mouse. In this species, the duration of spermatogenesis is 35 days, whereas it is 54 days in the rat and 74 days in humans. We performed long-term (until 60 days) cultures of fresh or frozen rat or human seminiferous tubule segments in a bioreactor made of a hollow cylinder of chitosan hydrogel. Testicular tissues were obtained from 8- or 20-day-old male rats or from adult human subjects who had undergone hormone treatments leading to a nearly complete regression of their spermatogenesis before bilateral orchiectomy for gender reassignment. The progression of spermatogenesis was assessed by cytological analyses of the cultures; it was related to a dramatic increase in the levels of the mRNAs specifically expressed by round spermatids, Transition protein 1, Transition protein 2, and Protamine 3 in rat cultures. From 2% to 3.8% of cells were found to be haploid cells by fluorescence in situ hybridization analysis of human cultures. In this bioreactor, long-term cultures of seminiferous tubule segments from prepubertal rats or from adult men allowed completion of the spermatogenic process leading to morphologically mature spermatozoa. Further studies will need to address the way of optimizing the yield of every step of spermatogenesis by adjusting the composition of the culture medium, the geometry, and the material properties of the chitosan hydrogel bioreactors. Another essential requirement is to assess the quality of the gametes produced ex vivo by showing their ability to produce normal offspring (rat) or their biochemical normality (human).

bioreactor, chitosan, fertility preservation, hollow cylinder, human spermatogenesis

INTRODUCTION

Spermatogenesis is a complex process of cellular multiplication and differentiation which occurs in the testis within the seminiferous tubules. After several mitotic divisions, diploid (2C cells) spermatogonia develop into preleptotene spermatocytes which become 4C cells after premeiotic DNA replication. The first meiotic division then generates secondary spermatocytes which possess two copies of each gene (2C cells). After the second meiotic division, the secondary spermatocytes generate haploid spermatids (1C cells) which will differentiate into spermatozoa. These steps are regulated by pituitary hormones (mainly follicle-stimulating hormone [FSH] and luteinizing hormone) and by soluble and membrane bound factors produced by both the somatic cells and the germ cells of the testis [1, 2].

During the past 20 yr, we have developed two culture systems for rat germ cells together with Sertoli cells, the somatic cells of the seminiferous tubules supporting the germ cells [3, 4]. We were the first to show that the whole meiotic process could occur in vitro or ex vivo in a mammal, the rat [5]. We showed that the development of the meiotic step, in vitro or ex vivo, in the testis of prepubertal and pubertal rats was close to what happens in vivo [5, 6]. These ex vivo systems have been useful to highlight some aspect of the intratesticular regulation of spermatogenesis [2, 7–11]. However, in these culture systems, germ cell differentiation stops at early steps of spermiogenesis [5, 6].

Improved treatments with radiotherapy and chemotherapy increases survival in cancer diseases, especially in children. These treatments have side effects: they are especially responsible for testicular damages and infertility. Cryopreservation of sperm before chemotherapy and radiotherapy sterilizing treatments preserves reproductive opportunities in

[1]Supported by Cancéropole Rhône-Alpes Auvergne grant CVPDC001 and by Kallistem SAS.
[2]Correspondence: Phillipe Durand, Kallistem, Ecole Normale Supérieure de Lyon, 46 allée d'Italie 69364 Lyon Cedex 07, France.
E-mail: philippe.durand@ens-lyon.fr

Received: 17 June 2016.
First decision: 11 July 2016.
Accepted: 24 August 2016.

eISSN: 1529-7268 http://www.biolreprod.org
ISSN: 0006-3363

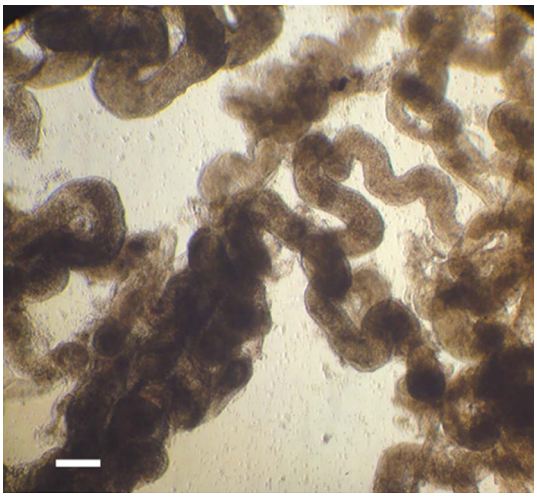

FIG. 1. Morphological aspect of isolated human seminiferous tubule segments. Seminiferous tubule segments were mechanically isolated from decapsulated testes from transsexual men by using two lancets. Tubule segment preparations appear almost completely free from interstitial tissue. Bar = 200 μm.

adult men. In the absence of mature sperm, measures to preserve the reproductive potential could not be considered in children, even though the cure rate is important at this age [12, 13]. The only currently feasible conservation measures for these children are collection and cryopreservation of testicular tissue, with no guarantee that future scientific advances will restore their fertility as part of a medically assisted parental project [12].

In the present study, we identified the conditions allowing rat or human spermatogonia to undergo the whole spermatogenesis process ex vivo, leading to morphologically mature spermatozoa from fresh or frozen testicular tissue.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats 8 or 20 days old were used (Charles River). All procedures were approved by the Scientific Research Agency (approval number 69306) and conducted in accordance with recommendations of the European Economic Community (86/609/EEC) for the care and use of laboratory animals.

Origin of Human Testicular Tissue

Testicular tissues were obtained from bilateral orchiectomy performed for gender reassignment of five male-to-female transsexual subjects between 25 and 31 years of age. The subjects received daily treatment of 50 mg of cyproterone acetate, 2 mg of 17β-estradiol, and 1 mg of finasteride orally for 2 years before orchiectomy. Written informed consent was obtained from the subjects. The protocol has been accepted by the ethical committee of the Hospices Civils de Lyon.

Freezing and Thawing of Seminiferous Tubules

Isolated human seminiferous tubule segments were cryopreserved with dimethyl sulfoxide (Sigma-Aldrich) as a cryoprotectant at a final concentration of 1.5 M and sucrose as an extracellular cryoprotectant at a final concentration of 1 M. The freezing medium contained Leibovitz L-15 medium (Eurobio) and 10% serum substitute supplement (Irvine Scientific).

The seminiferous tubule segments were resuspended in a minimum volume of Leibovitz medium after centrifugation. Segments of 5–10 mg were individually placed in cryotubes containing the cryoprotective solution. The cryotubes were incubated at 4°C for 30 min. The tubule segments were frozen in a programmable freezer (Freezal; Air Liquide Sante). A slow freezing protocol previously described was used [14]. The duration of the freezing time was 124 min. At the end of the program, the cryotubes were plunged into nitrogen liquid at −196°C. The thawing program consisted of a rapid thawing at 32°C in a water bath.

Preparation of Chitosan Hydrogel Bioreactors

Chitosan hydrogel bioreactors were prepared as described previously [15]. Such technology was derived from the structuring process of hydrogels by interrupted neutralization of chitosan aqueous solutions. A highly deacetylated chitosan with high molecular weight was produced from squid pen chitin (indexes 114, batch no. P1; Mahtani Chitosan Pvt. Ltd.). Sodium hydroxide pellets, ammonium hydroxide solution at 28%–30% (w/w), and acetic acid were purchased from Sigma-Aldrich. Physical chitosan hydrogels were prepared by gelation of an aqueous chitosan solution. The chitosan solution was first obtained by dispersing neutralized purified chitosan lyophilizates into water, and then acetic acid was added to achieve the stoichiometric protonation of $^{-}NH_2$ sites. Amounts of chitosan and water were determined to obtain the solution concentration of 2.0% (w/w). After polymer dissolution, the resulting solution was placed in syringes. The solution was then extruded (using Performus I; Nordsan EFD) through a cone extruder tip of 3 mm diameter and neutralized in sodium hydroxide solution at a concentration of 1 M in order to coagulate the external membrane of the extrudates. After a coagulation time, a tubular hydrogel was formed and placed in deionized water bath in order to stop the coagulation process. The uncoagulated inner solution was removed with air flux inside the chitosan tube, thus creating the lumen of the tube. Bioreactors with an internal diameter of 2 mm and an external membrane of 1 mm were sterilized by autoclaving and stored in deionized water at room temperature.

Ex Vivo Spermatogenesis

Isolated seminiferous tubule segments, 20–50 mm^3, prepared by mechanical dissociation (Fig. 1), were introduced into chitosan tubes which were then sealed at both ends and deposited in a conventional culture well containing 8 ml of culture medium [16].

The medium was changed every 2 days. The serum-free culture medium consisted of 15 mM Hepes-buffered F12/Dulbecco modified Eagle medium supplemented with antibiotics, 1.2 g/L $NaHCO_3$, 10 μg/ml insulin, 10 μg/ml transferrin, 10^{-4} M vitamin C, 10 μg/ml vitamin E, 3.3×10^{-7} M retinoic acid, 3.3×10^{-7} M retinol, 10^{-3} M pyruvate, 10^{-7} M testosterone (all from Sigma-Aldrich), and 1 ng/ml FSH (lot number AFP 7028 D; U.S. National Institutes of Health) obtained through National Institute of Diabetes and Digestive and Kidney Diseases. For 8-day-old rats and the transsexual men, testosterone was added to the culture medium after 5 days of culture. Incubation was carried out at 33°C in a water-saturated atmosphere of 95% air and 5% CO_2.

Histochemical Studies of Histological Sections

Pieces of rat or human testes were fixed in Bouin fluid for 12–24 h and then dehydrated and embedded in paraffin; 3-μm-thin sections of testes were deparaffinized and rehydrated and stained with Harris hematoxylin solution.

Some testis sections from 8-day-old rats or from human subjects were incubated overnight at 4°C with a mouse antivimentin monoclonal antibody (clone V9; Dako) at a 1/300 dilution in antibody diluent (Dako). The staining was performed with the EnVision+ dual link system peroxidase kit (Dako) with 3,3-diaminobenzidine (DAB) or amino-3-ethyl-9-carbazole (AEC; both from Vector Laboratories) as a chromogen; cells were counterstained with Harris or Mayer hematoxylin solution.

Cytological Studies of Cultured Cells

At selected days of culture (see *Results*), seminiferous tubule segments were extruded from the chitosan tubes and then crushed between two microscopic glass slides [17]. The nuclei were stained with Harris's hematoxylin solution, and cells were identified by microscopy examination (Axioskop; Zeiss).

Extraction of Spermatozoa from Bioreactor

Seminiferous tubule segments were extruded from the chitosan tubes with a lancet and then stretched with needles in a drop of culture medium.

Fluorescent In Situ Hybridization in Human Germ Cells

Samples obtained for fluorescent in situ hybridization were spread on a slide and then dehydrated in ethanol series (70%, 85%, and 100%) and stored at

TABLE 1. Primers used for the qRT-PCR experiments.

Primer	Sequence
Mitochondrial ribosomal protein S18B (*18S*)	
Sense	ATCATCTTCCATGCCCCATA
Antisense	AAGGTTCACCCGACAACAAC
Ribosomal protein S16 (*16S*)	
Sense	AGTCTTCGGACGCAAGAAAA
Antisense	AGCCACCAGAGCTTTTGAGA
Stathmin 1	
Sense	AAGGATCTTTCCCTGGAGGA
Antisense	TTCTCCTCTGCCATTTTGCT
Transition protein 1 *(Tp1)*	
Sense	CGACCAGCCGCAAACTAAAG
Antisense	ATCATCGCCCCGTTTCCTAC
Transition protein 2 (*Tp2*)	
Sense	GGCCTCAAAGTCACACCAAT
Antisense	CTTGCTCACTTTCCCTTCCA
Protamine 3 (*Prm3*)	
Sense	GTGGCCTGTGTGAGTCAAGA
Antisense	CCTCAGCACCATCTTGCTTT
Gap junction protein, alpha 1 (*Cx43*)	
Sense	TCCTTGGTGTCTCTCGCTTT
Antisense	GAGCAGCCATTGAAGTAGGC

−20°C before processing. Before hybridization, slides were fixed three times in Carnoy's fixative (methanol-glacial acetic acid, 3:1 dilution). Then cells were decondensed for 3 min in 1M NaOH and fixed once in Carnoy fixative for 10 min. Slides were dehydrated in ethanol series (70%, 85%, 100%).

Hybridization was performed using a FastFish kit (Cytocell) according to the manufacturer′s instructions, using probe set 1 for chromosomes 13 and 21 and probe set 2 for chromosomes X, Y, and 18. After we washed the slides, we observed them by using an AxioImager Z2 epifluorescence microscope (Zeiss), and images were processed using Isis 5.7.4 software (Metasystem).

RNA Preparation and Analysis

Total RNA was extracted from freshly prepared or cultured seminiferous tubule segments, using TRI-REAGENT (Euromedex) according to the manufacturer′s protocol. All samples were treated with DNase I (Life Technologies) before reverse transcription. First-strand cDNA was synthesized from 1 μg of RNA with Super Script III (Life Technologies) in the presence of oligo(dT) (Life Technologies) and random primers (Fermentas). Real-time PCR was performed using a Step One Plus instrument (Applied Biosystems), using SYBR-Green (Qiagen). Primers sequences are shown in Table 1. Mitochondrial ribosomal protein S18B and the ribosomal protein S16 mRNAs were used as housekeeping gene controls. The absence of contaminants was checked by RT-PCR assays of negative control samples in which the RNA samples were replaced with sterile water, or reverse transcriptase was not added.

RESULTS

Ex vivo differentiation of immature germ cells into spermatozoa was obtained in 3 of 4 experiments with 8-day-old rats; in 7 of 9 experiments with 20-day-old rats, in 4 of 5 experiments with fresh tissue from men and in 2 of 2 experiments with human frozen testicular samples. Representative examples are presented below.

Results with 20-Day-Old Rats

Similar to prepubertal human males, these animals did not display full spermatogenesis. Indeed, at the beginning of the culture, the most differentiated germ cells in their seminiferous tubules were pachytene spermatocytes (PS) from stage X [6]. Round spermatids (RS) (steps 1–4 of spermiogenesis) and elongated spermatids (ES) (step 9) as described by Russell et al. [18] were visualized on Day 9 of culture (Fig. 2, A and B). ES (step 9) and more differentiated ES (steps 10–12) were observed on Day 25 of culture (Fig. 2, C, D, and E). After 39 days of culture a cluster of ES with their flagellum was observed (Fig. 2F). Heads and flagella of ES (steps 15–17) are also shown at a higher magnification (Fig. 2G). Cell clusters were observed at Day 39 in which early meiotic cells, young spermatocytes (yS) and PS (identified by the diameter and chromatin aspects of the nucleus of each cell type and the stage of seminiferous epithelium) (Fig. 2H) were still abundant. A new wave of RS and young PS (yPS) (Fig. 2I), as associated in stages I–IV of the seminiferous epithelium [18], was also present. ES (step 9 in stage IX) were observed (Fig. 2J). A bouquet of spermatozoa, freshly extracted from the bioreactor on Day 39 of culture is shown in Figure 3.

mRNA-related qPCR products were measured in freshly isolated seminiferous tubule segments of 20-day-old rats or in seminiferous tubule segments cultured for 12, 20, or 40 days (Table 2). In cocultures, as in vivo during testis development [19], the rate of survival and/or differentiation of somatic (Sertoli cells) and germ cells may be quite different over time, making the quantitative measurement of an mRNA species at different days of the experiment rather difficult. Nevertheless, the progression of spermatogenesis was related with a dramatic increase (approximately 100-fold) in the levels of mRNAs specifically expressed by RS: Transition protein 1 (*Tp1*), Transition protein 2 (*Tp2*), and Protamine 3 (*Prm3*) up to 20 days, together with a mild decrease in stathmin mRNAs, mostly expressed by PS [20, 21]. By contrast, the expression of the gap junction protein alpha 1 (*Cx43*), which is expressed by both the Sertoli cells and some germ cells and is crucial for spermatogenesis [22, 23] was not as highly modified.

Results with 8-Day-Old Rats

Seminiferous tubule cultures from 8-day-old rats were then performed. Indeed, only (vimentin-positive) Sertoli cells and (vimentin-negative) spermatogonia were present in the testes of 8-day-old rats (Fig. 4, A and B), making them animal models for young human males. Note that vimentin is much less expressed in immature Sertoli cells than after the establishment of the blood testis barrier (see supplemental Fig. S1 associated with the article by Ryser et al. [24]). Meiotic cells yS and PS were visualized on Day 12 of culture (Fig. 4, C and D). RS and ES (step 10 of spermiogenesis) were observed on Days 20 and 32 of culture, respectively (Fig. 4, E and F). After 61 days of culture, germ cells were still observed: among them spermatozoa with their flagellum (Fig. 4G). A whole isolated spermatozoon with its head, its cytoplasmic lobe, and its flagellum is shown in Figure 4, H and I.

Despite a careful histological observation of these cultures, we could not provide an exact number of spermatozoa formed per bioreactor; it was estimated to be approximately 100 in the cultures of 20-day-old rat and 30 to 50 in cultures of 8-day-old rats.

Culture of Freshly Prepared Human Seminiferous Tubule Segments

The treatment of human patients retained in this study with both estrogens and antiandrogens led to a regression of the seminiferous tubules, which were surrounded by fibrous interstitial tissue (Fig. 5A). Only Sertoli cells, labeled with the antivimentin antibody and spermatogonia were present in the seminiferous epithelium, which possessed no differentiated germ cells (Fig. 5B).

At different days of culture of the seminiferous tubule segments of patient 1, germ cells were observed. Cultured cells had an appearance similar to those in vivo described by Clermont [25] (see Fig. 3 in reference [25]). A spermatid Sc

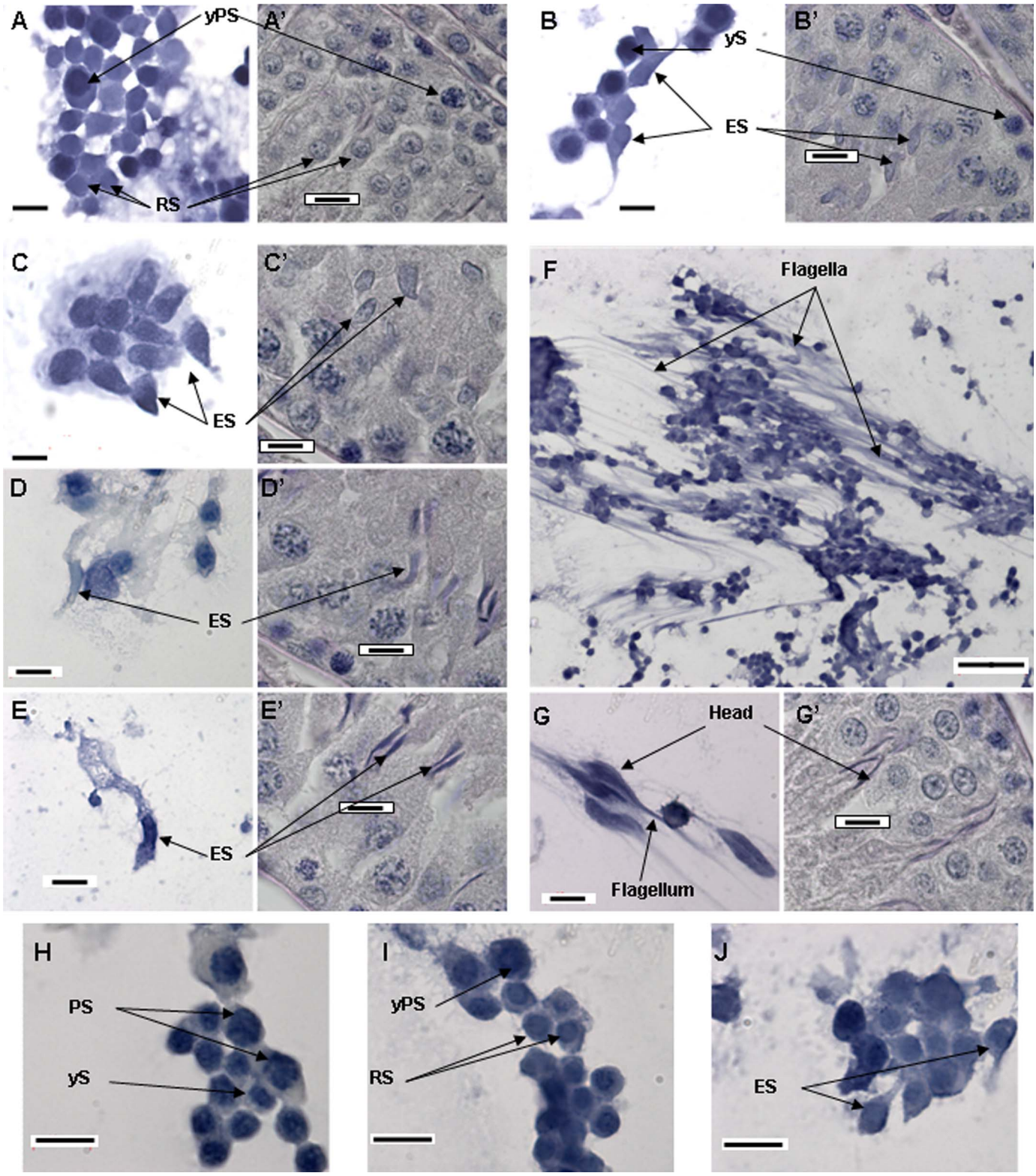

FIG. 2. Cytological analysis of cultured germ cells from 20-day-old rats. Germ cells extracted from the chitosan tubes were compared in cross-section with cells at the same stage in adult testis according to the description by Russell et al. [18]. **A**) On Day 9 of culture, young pachytene spermatocytes (yPS) and round spermatids (RS) were examined. **A**′) yPS and RS on a cross-section of stages I–IV of the seminiferous epithelium (SE). **B**) On Day 9 of culture, young spermatocytes (yS) and elongated spermatids (ES) of stage 9 of spermiogenesis were examined. **B**′) yS and ES on a cross-section of a stage IX of the SE. **C)** ES of stage 9 of spermiogenesis after 25 days of culture. **C**′) ES on a cross-section of a stage IX of the SE. **D**) On Day 25 of culture, ES of stage 11 of spermiogenesis. **D**′) ES on a cross-section of a stage XI of the SE. **E**) ES of stage 12 of spermiogenesis after 25 days of culture. **E**′) ES on a cross-section of a stage XII of the SE. On Day 39 of culture, a cluster of ES with a lot of flagella (**F**). **G**) Heads and flagella of ES of stage 15–17 of spermiogenesis at a higher magnification. **G**′) ES on a cross-section of stages I–IV of the SE. On Day 39 of culture, a cluster of early meiotic cells (**H**), yS and PS; a cluster of a new wave of RS and yPS (**I**); ES, step 9 of spermiogenesis (**J**). Note that cells on the prime-lettered pictures are from paraffin-embedded testis sections, whereas the other pictures show whole cells from cultured, crushed, seminiferous tubule segments. Bars = 10 μm (**A**–**E**, **G**–**J**) and 50 μm (**F**). Note that, ex vivo, the

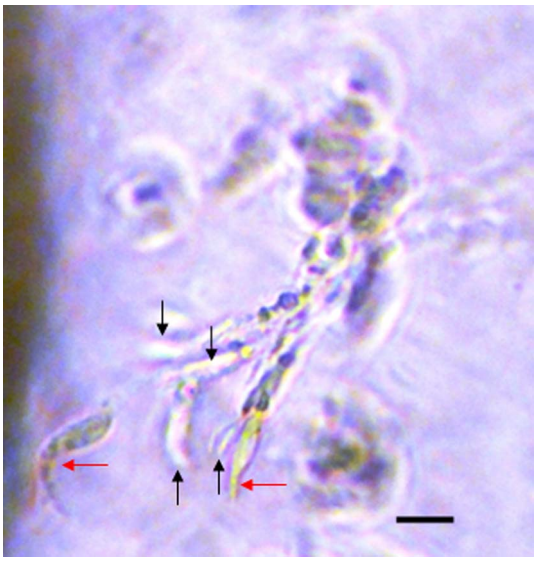

FIG. 3. Spermatozoa freshly extracted from cultured 20-day-old rat seminiferous tubule segments. On Day 39 of culture, a bouquet of spermatozoa is shown (arrows). The focus was performed using the two heads shown by the red arrows. Bar = 10 μm.

(stages V–VI of the seminiferous epithelium) was visualized on Day 25 of culture (Fig. 6A). On Day 34 of culture, spermatids at different steps of differentiation were observed: Sb (stages III–IV) (Fig. 6B), Sc (stages V–VI) (Fig. 6C). A spermatozoon on Day 55 is shown in Figure 6D. Also, a group of less differentiated cells was observed at Day 55, with yS and PS and a new wave of spermatids (Sb and Sc/Sd) (Fig. 6E).

Fluorescent in situ hybridization of cultured cells was performed on Day 60 of culture. A total of 106 nuclei were observed by using probe set 1. Four cells showed one signal for chromosome 13 and one signal for chromosome 21 and were, thus, considered haploid (3.8%). With probe set 2, 151 nuclei were observed. Two cells showed one signal for chromosome X and one signal for chromosome 18, and one cell showed one signal for chromosome Y and one signal for chromosome 18. These three cells were considered haploid (2%) (Fig. 7).

Culture of Frozen/Thawed Human Seminiferous Tubule Segments (Patient 2)

Spermatids Sa (stages I–II) of the seminiferous epithelium were observed on Day 24 of culture, one of them is shown in Figure 8A. After 34 days of culture, spermatozoa and flagella were observed (Fig. 8B). Figure 8C shows a spermatozoon at higher magnification. A cluster of yS associated with PS and a spermatid Sb (stages III–IV) observed at Day 34 is shown in Figure 8D. In both types of human cultures the number of spermatozoa ranged between 5 and 20 spermatozoa per bioreactor.

DISCUSSION

Long-term primary cultures of seminiferous tubule segments in a bioreactor made of a hollow cylinder of chitosan hydrogel in a defined, serum-free medium allowed completion of the spermatogenic process in seminiferous tubule segments from prepubertal rats which do not display full spermatogenesis or, very importantly, from adult men who had undergone hormone treatments leading to a nearly complete regression of their spermatogenesis. Until now, complete ex vivo spermatogenesis has been reported only in the mouse [26–28], in which the duration of the spermatogenic process is much shorter (35 days) [29].

The most important result presented herein is the achievement of spermiogenesis in both human and rat, leading to the ex vivo formation of morphologically normal spermatozoa. Indeed, we have shown previously that spermatogonia multiplication and the whole meiotic step occurred in primary cultures of 20-day-old rat (of 8-day-old rat and of human [Perrard et al., unpublished results]) seminiferous tubule segments in bicameral culture chambers, but that spermiogenesis stops early after the completion of the second meiotic division [3, 5]. It is important to note that, ex vivo, the germ cells most often had a morphology very similar to their appearance in vivo, despite the fact that after they were extracted from the chitosan tubes the seminiferous tubule segments had to be crushed between two microscopic glass slides to perform the microscopic observations. Actually, this allowed conserving the cellular associations characteristic of the stages of the seminiferous epithelium [17]. Careful cytological analysis of these cultures was, indeed, essential, to follow spermiogenesis, as there is no change in cell ploidy at that step, and chromatin compaction in elongating spermatids renders gene transcription virtually inactive [19].

No study using bromodeoxyuridine labeling of the germ cells was performed here, preventing the determination of the kinetics of the spermatogenic process under the present culture conditions. However, for the rat studies, the timing of the appearance of the spermatozoa was compatible with that of the in vivo data [6, 29]. Indeed, in the rat at 7–8 days of age, the only germ cells were spermatogonia (Fig. 3, A and B) and [30, 31]. In this species, the duration of spermatogenesis is 54 days [32]. After 61 days of culture under the present conditions, morphologically normal spermatozoa were observed, demonstrating that every step of the spermatogenic process occurred in culture.

For the human studies, observation of spermatozoa on Day 55 of culture of ''fresh'' seminiferous tubule segments might be expected if they originate from B spermatogonia [25]. More surprising was the observation of spermatozoa on Day 34 of culture of frozen/thawed seminiferous tubule segments. It must be underlined that some variability among subjects may occur in humans [33], leading to the ''need of revisiting the cycle of the seminiferous epithelium in humans'' [34]. It has also been reported that culture conditions may increase the speed of differentiation of immature testicular tissue [35] and references herein. Alternatively, the residual presence of a small number of early meiotic cells in the biopsies cannot be excluded [36].

Despite the fact that we could not assess precisely the number of spermatozoa produced in these cultures, the yield of human haploid cell formation in the bioreactor was similar to that obtained in rat tubule segments cultured in bicameral chambers [5]. In addition, it was close to that reported in the mouse in the organotypic culture model reported by Sato et al. [28].

germ cells had, most often, a morphology very similar to their appearance in vivo [18], despite the fact that, after they were extracted from the chitosan tubes, the seminiferous tubule segments had to be crushed between two microscopic glass slides to perform the microscopic observations. Actually, this allowed conserving the cellular associations characteristic of the stages of the seminiferous epithelium [17].

TABLE 2. Stathmin, *Tp1*, *Tp2*, *Prm3*, and *Cx43* mRNAs in rat seminiferous tubule segments.[a]

Day	*Tp1/16S, 18S*	*Tp2/16S,18S*	*Prm3/16S, 18S*	*Cx43/16S, 18S*	Stathmin/*16S, 18S*
Day 0	0.0069	0.0027	0.0005	1.5148	6.9779
Day 12	1.0390	0.4423	0.0516	1.7104	2.4694
Day 12/Day 0	(151)	(163)	(95)	(1)	(0.35)
Day 20	0.2925	0.1124	0.0270	3.2645	1.6955
Day 20/Day 0	(43)	(41)	(50)	(2)	(0.24)
Day 40	0.026	0.0029	0.0006	1.6954	1.2998
Day 40/Day 0	(4)	(1)	(1)	(1)	(0.19)

[a] The ribosomal proteins S16 and S18B were used as housekeeping gene controls. Similar results were obtained in another experiment.

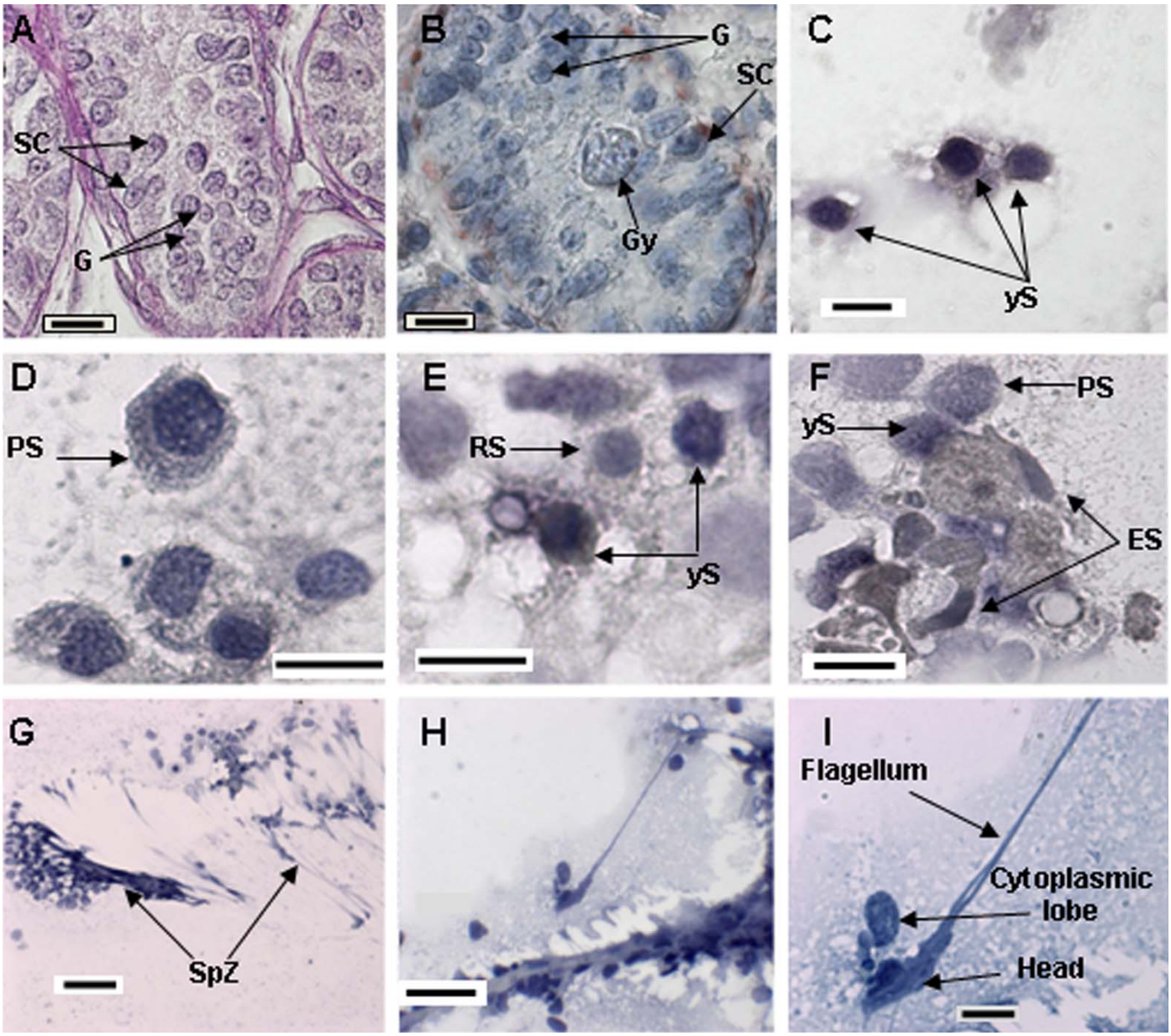

FIG. 4. Cytological analysis of cultured germ cells from 8-day-old rats. Cross sections from an 8-day-old rat testis. **A**) The nuclei and cytoplasms were stained with Harris hematoxylin and eosin, respectively. Sertoli cells (SC) and spermatogonia (G) were identified according to the morphology of their nuclei [40]. **B**) Immunocytochemical staining of vimentin (counterstaining with Mayer hematoxylin). Only (vimentin-positive) Sertoli cells (SC) with a red spot in the cytoplasm under the nucleus and (vimentin-negative) spermatogonia (G) or gonocytes (Gy) with a colorless cytoplasm are present. Note that vimentin is much less expressed in immature Sertoli cells than after the establishment of the blood-testis barrier [24]. On Day 12 of culture, three young spermatocytes (yS [**C**]) and a pachytene spermatocyte (PS [**D**]) are shown. On Day 20 of culture, a round spermatid (RS) and yS are shown (**E**). On Day 32 of culture, elongated spermatids (ES) of step 10 of spermiogenesis, a yS and a PS (**F**). After 61 days of culture, a cluster of spermatozoa (SpZ [**G**]); a whole isolated spermatozoon (**H**); and at a higher magnification (**I**), the isolated spermatozoon with its head, cytoplasmic lobe, and flagellum [18]. (**C**–**I**) Whole cells from cultured, crushed seminiferous tubule segments. Meiotic cells (yS and PS) and RS and ES were identified by the aspect of the nucleus of each cell type (diameter and chromatin aspects). Bars = 10 μm (**A**–**F** and **I**) and 50 μm (**G** and **H**).

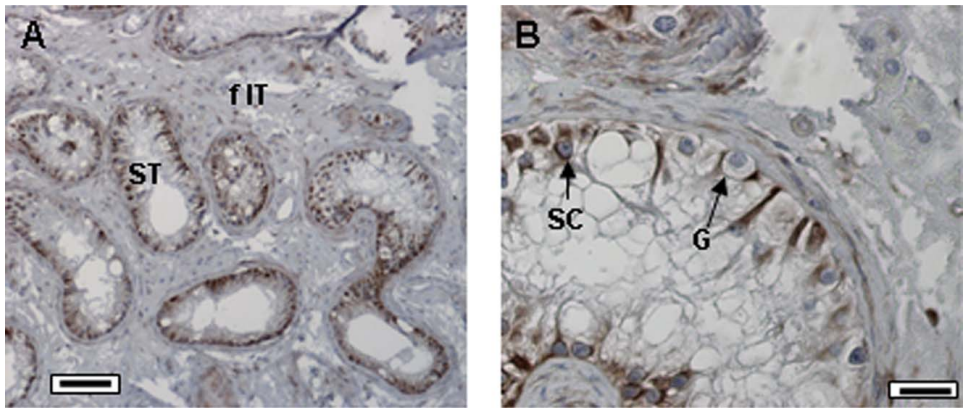

FIG. 5. Cross-section from the testis of a transsexual man. **A**) Patient treatment caused regression of the seminiferous tubules (ST) surrounded by a fibrous interstitial tissue (f IT). **B**) At a higher magnification it can be observed that patient treatment led to an absence of differentiated germ cells. Only Sertoli cells (SC) with brown cytoplasm, labeled with antivimentin antibody and spermatogonia (**G**) with a colorless cytoplasm, were present. Bars = 50 μm (**A**) and 20 μm (**B**).

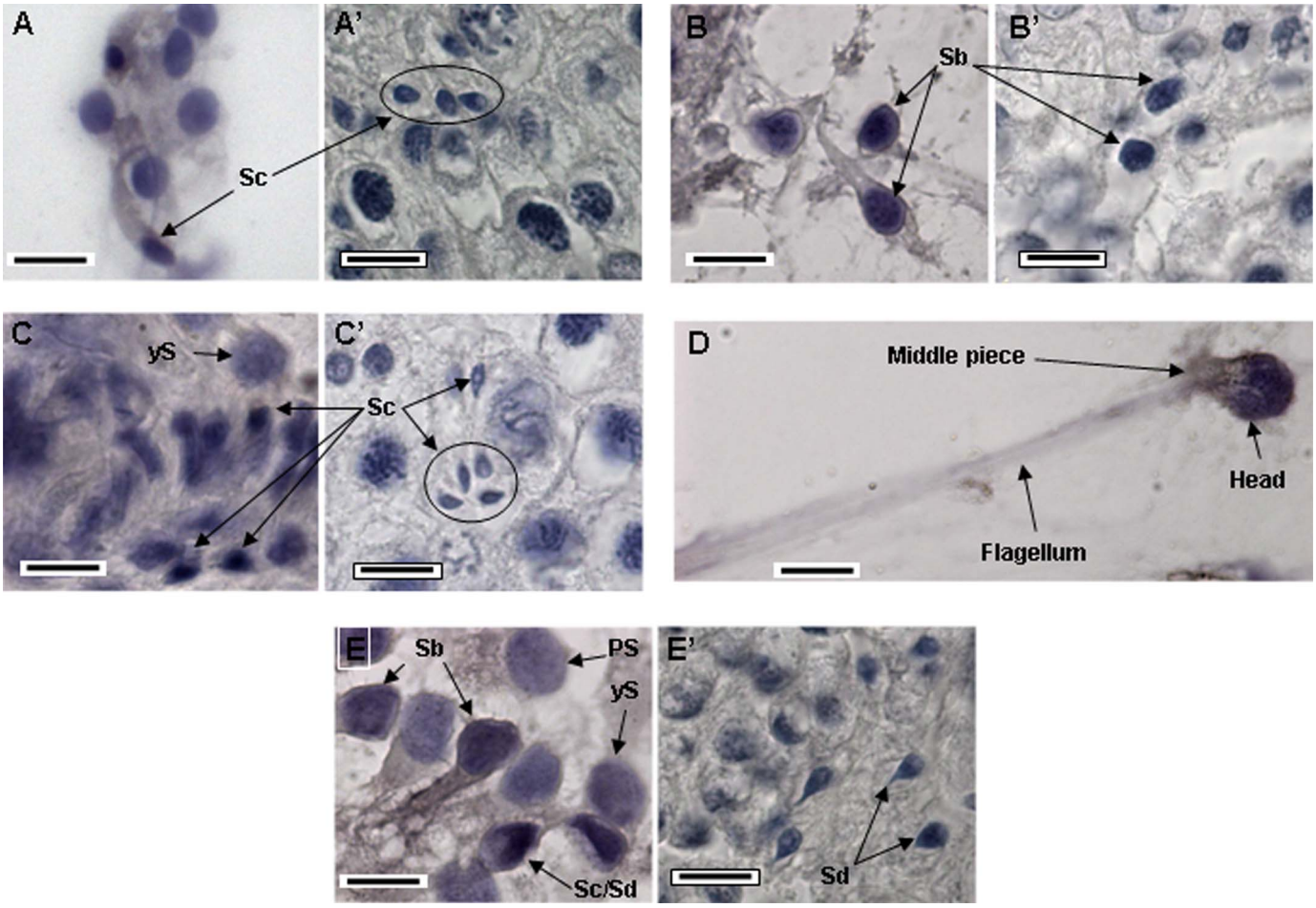

FIG. 6. Cytological analysis of a culture from human fresh seminiferous tubule segments. Germ cells extracted from the chitosan tubes are compared with cells of the same stage according to Clermont [25] on cross-sections from an obstructive azoospermic adult testis. **A**) On Day 25 of culture, a spermatid Sc. **A**′) Spermatids Sc on a cross-section of stages V–VI of the seminiferous epithelium (SE). **B**) On Day 34 of culture, spermatids Sb, Spermatids Sb on a cross-section of stages III–IV of the SE (**B**′); a young spermatocyte (yS) and spermatids Sc (**C**); spermatids Sc on a cross-section of stages V–VI of the SE (**C**′). **D**) After 55 days of culture, a spermatozoon with its head, middle piece and flagellum. **E**) A yS, a pachytene spermatocyte (PS) and spermatids Sb and Sc/Sd. **E**′) Spermatids Sd on a cross-section of stages I–II of the SE. Note that cells on the prime lettered pictures are from paraffin-embedded testis sections, whereas the other pictures show whole cells from cultured, crushed, seminiferous tubule segments. All bars = 10 μm.

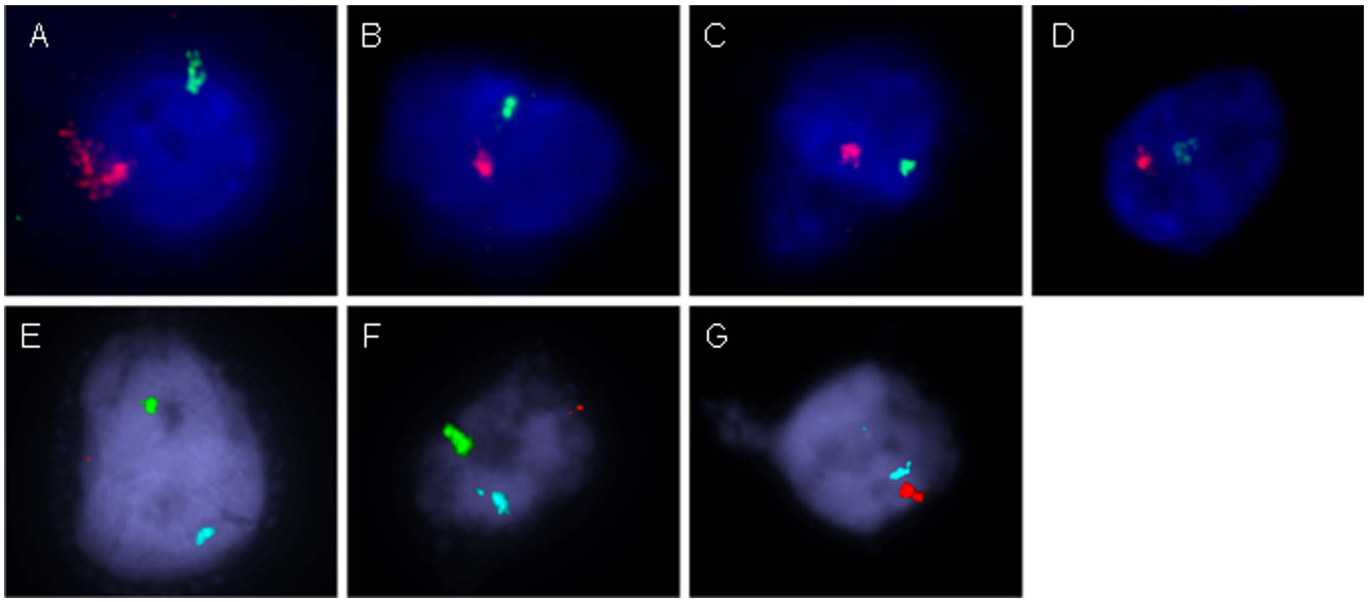

FIG. 7. Fluorescent in situ hybridization of a culture at Day 60 of human seminiferous tubule segments showing the presence of haploid cells. **A**–**D**) Hybridization with chromosome 13 probe (green) and chromosome 21 probe (red). **E**–**G**) Hybridization with chromosome X probe (green), chromosome Y probe (red) and chromosome 18 probe (aqua).

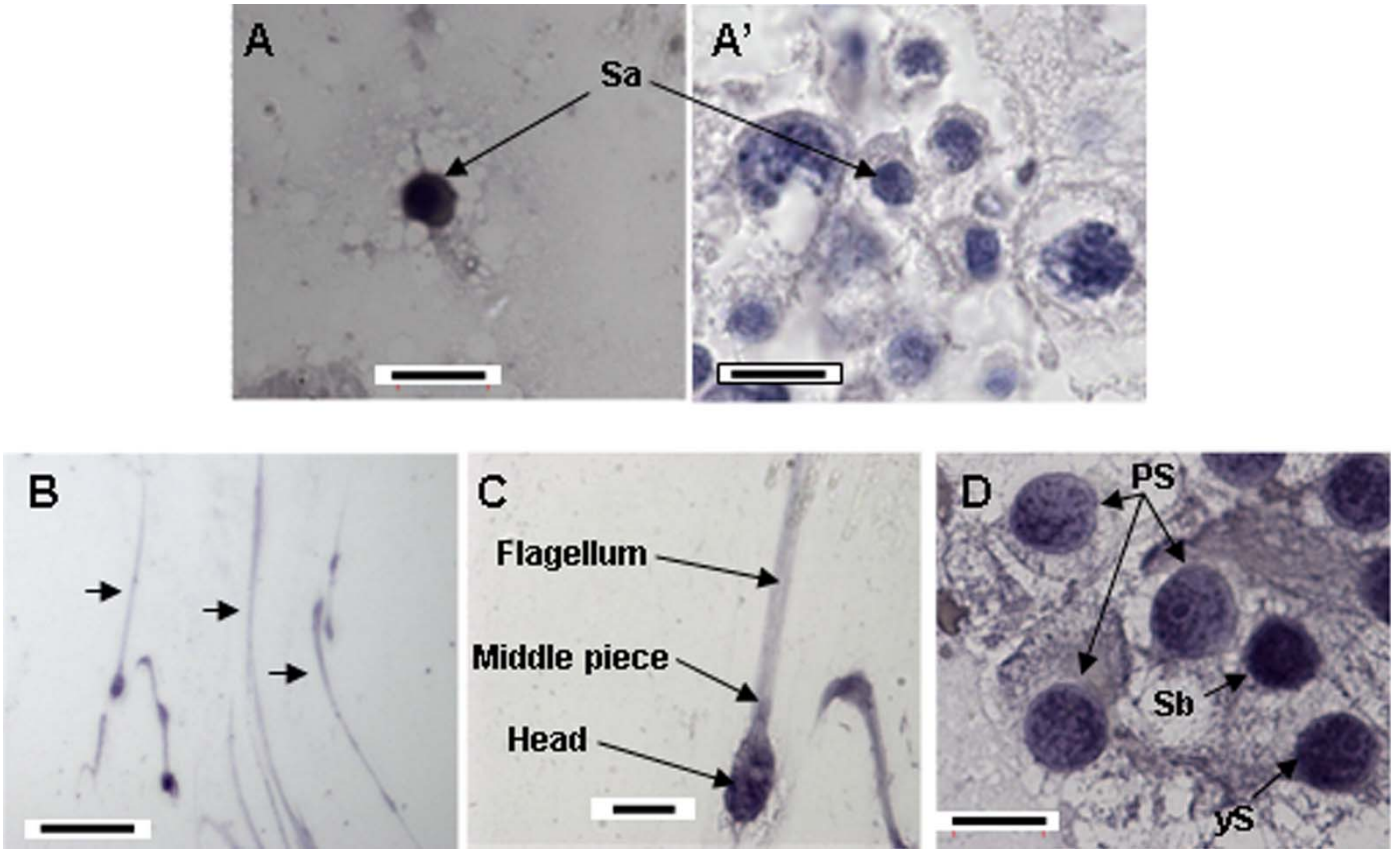

FIG. 8. Cytological analysis of a culture from human frozen /thawed seminiferous tubule segments. Germ cells extracted from the chitosan tubes are compared with cells of the same stage according to Clermont [25] on cross-sections from an obstructive azoospermic adult testis. **A**) On Day 24 of culture, a spermatid, Sa. **A'**) A spermatid, Sa, on a cross-section of stages I–II of the SE. **B**) After 34 days of culture, spermatozoa with flagella are shown by arrows. **C**) A spermatozoon with its head, middle piece, and flagellum at a higher magnification. **D**) A young spermatocyte (yS), pachytene spermatocytes (PS) and a spermatid Sb. Note that cells on the prime lettered picture are from paraffin-embedded testis sections, whereas the other pictures show whole cells from cultured, crushed, seminiferous tubule segments. Bars = 10 μm (**A**, **A'**, **C** and **D**) and 50 μm (**B**).

Under the present culture conditions, the testicular tissue was confined in the hydrogel bioreactor, thereby maintaining the 3-dimensional architecture of the seminiferous tubules close to that in vivo. This appears to be of primary importance. Indeed, numerous cellular junctions develop between Sertoli cells and germ cells during spermatogenesis [37]. Sertoli cells interact directly with developing germ cells at every step of their differentiation, mainly through gap junction communications and its primordial testicular component the *Cx43* [22, 23]. Hence, it is the cytoarchitectural arrangement, between Sertoli cells and the developing germ cells, that provides the microenvironment in which the germ cells will survive, proliferate and differentiate (see also [38]). When using the present bioreactor, the levels of *Cx43* mRNAs appeared to be maintained for the duration of the culture. By contrast, in our previous culture system in bicameral chambers, *Cx43* decreased from Day 12 of culture onward [39]. Hence it may be suggested that the microenvironment necessary to the achievement of spermatogenesis/spermiogenesis was rather well maintained in the present bioreactor. It may be noticed that in the rat cultures the levels of RS specific mRNAs were maximal on Day 12 and decreased thereafter. Nevertheless, the presence of early meiotic cells and of new RS on Day 39, together with rather high amount of Stathmin mRNAs indicates that a new wave of spermatogenesis was occurring at that time. Similarly, in the human cultures, we still observed the presence of spermatocytes and of spermatids Sb on Day 55.

In the present study, we report for the first time that whole human spermatogenesis can be achieved in vitro or ex vivo under well-defined culture conditions by using a bioreactor made of a hollow cylinder of chitosan hydrogel in which the seminiferous tubule segments are confined. Hence, the present results support the strategy that before chemotherapy treatment of young boys, a small piece of testicular pulp could be removed and cultured in our model for the production of sperm. The sperm will be frozen until the patient's parental project. However, in order to get a sufficient number of spermatozoa for intracytoplasmic sperm injection (ICSI), further studies will need to address the way of optimizing the yield of every step of spermatogenesis, by adjusting the composition of the culture medium, the geometry and material properties of the chitosan hydrogel bioreactors. Another essential requirement is to assess the quality of the gametes produced ex vivo by showing their ability to produce normal offspring (rat) or their biochemical normality (human). In the future, this technology should be helpful in clinical practice, not only to save fertility in patients subjected to radiotherapy and chemotherapy but also to correct some nonobstructive azoospermia due to Sertoli cell dysfunction.

ACKNOWLEDGMENT

We thank Dr. G. Pointis for helpful discussion of the manuscript, E. Christin for help with the cultures, and J. Bois for preparation of the manuscript.

REFERENCES

1. Parvinen M, Ventela S. Local regulation of spermatogenesis: a living cell approach. Hum Fertil 1999; 2:138–142.
2. Perrard MH, Vigier M, Damestoy A, Chapat C, Silandre D, Rudkin BB, Durand P. Beta-nerve growth factor participates in an auto/paracrine pathway of regulation of the meiotic differentiation of rat spermatocytes. J Cell Physiol 2007; 210:51–62.
3. Hue D, Staub C, Perrard-Sapori MH, Weiss M, Nicolle JC, Vigier M, Durand P. Meiotic differentiation of germinal cells in three-week cultures of whole cell population from rat seminiferous tubules. Biol Reprod 1998; 59:379–387.
4. Weiss M, Vigier M, Hue D, Perrard-Sapori MH, Marret C, Avallet O, Durand P. Pre- and postmeiotic expression of male germ cell-specific genes throughout 2-week cocultures of rat germinal and Sertoli cells. Biol Reprod 1997; 57:68–76.
5. Staub C, Hue D, Nicolle JC, Perrard-Sapori MH, Segretain D, Durand P. The whole meiotic process can occur in vitro in untransformed rat spermatogenic cells. Exp Cell Res 2000; 260:85–95.
6. Perrard MH, Hue D, Staub C, Le Vern Y, Kerboeuf D, Durand P. Development of the meiotic step in testes of pubertal rats: comparison between the in vivo situation and under in vitro conditions. Mol Reprod Dev 2003; 65:86–95.
7. Damestoy A, Perrard MH, Vigier M, Sabido O, Durand P. Transforming growth factor beta-1 decreases the yield of the second meiotic division of rat pachytene spermatocytes in vitro. Reprod Biol Endocrinol 2005; 3:22.
8. Fouchécourt S, Godet M, Sabido O, Durand P. Glial cell-line-derived neurotropic factor and its receptors are expressed by germinal and somatic cells of the rat testis. J Endocrinol 2006; 190:59–71.
9. Perrard MH, Chassaing E, Montillet G, Sabido O, Durand P. Cytostatic factor proteins are present in male meiotic cells and beta-nerve growth factor increases mos levels in rat late spermatocytes. PLoS One 2009; 4: e7237.
10. Perrard MH, Durand P. Redundancy of the effect of TGFbeta1 and beta-NGF on the second meiotic division of rat spermatocytes. Microsc Res Tech 2009; 72:596–602.
11. Vigier M, Weiss M, Perrard MH, Godet M, Durand P. The effects of FSH and of testosterone on the completion of meiosis and the very early steps of spermiogenesis of the rat: an in vitro study. J Mol Endocrinol 2004; 33: 729–742.
12. Picton HM, Wyns C, Anderson RA, Goossens E, Jahnukainen K, Kliesch S, Mitchell RT, Pennings G, Rives N, Tournaye H, van Pelt AM, Eichenlaub-Ritter U, et al. European perspective on testicular tissue cryopreservation for fertility preservation in prepubertal and adolescent boys. Hum Reprod 2015; 30:2463–2475.
13. Schwartz CL. Long-term survivors of childhood cancer: the late effects of therapy. Oncologist 1999; 4:45–54.
14. Milazzo JP, Vaudreuil L, Cauliez B, Gruel E, Masse L, Mousset-Simeon N, Mace B, Rives N. Comparison of conditions for cryopreservation of testicular tissue from immature mice. Hum Reprod 2008; 23:17–28.
15. David L, Domard A, Rivas-Araiza R, inventors. Centre National de la Recherche Scientifique Université Claude Bernard Lyon 1, assignee. Hollow, notably multi-membrane fibers, method for preparation thereof by spinning and device for applying said method. European patent WO 200944053 A2n, P.F. 5 September 2008.
16. Perrard MH, Durand P, David L, inventors. Kallistem Université Claude Bernard Lyon 1 Centre National de la Recherche Scientifique Institut National de la Recherche Agronomique Ecole Normale Supérieure, assignee. Process for implementing in vitro spermatogenesis and associated device. European patent B ET1 41330 (7B F/SC) PCT1 EP20141078887. 20 December 2013.
17. Toppari J, Parvinen M. In vitro differentiation of rat seminiferous tubular segments from defined stages of the epithelial cycle morphologic and immunolocalization analysis. J Androl 1985; 6:334–343.
18. Russell LD, Ettlin RA, Sinha Hikim AP, Clegg ED. Staging for laboratory species In: Russell LD, Ettlin RA, Sinha Hikim AP, Clegg ED (eds.), Histological and Histopathological Evaluation of the Testis. Clearwater, FL: Cache River Press; 1990; 62–118.
19. Almstrup K, Nielsen JE, Hansen MA, Tanaka M, Skakkebaek NE, Leffers H. Analysis of cell-type-specific gene expression during mouse spermatogenesis. Biol Reprod 2004; 70:1751–1761.
20. Marret C, Avallet O, Perrard-Sapori MH, Localization Durand P. and quantitative expression of mRNAs encoding the testis-specific histone TH2B, the phosphoprotein p19, the transition proteins 1 and 2 during pubertal development and throughout the spermatogenic cycle of the rat.Mol Reprod Dev 1998; 51:22–35.
21. Schluter G, Engel W. The rat Prm3 gene is an intronless member of the protamine gene cluster and is expressed in haploid male germ cells. Cytogenet Cell Genet 1995; 71:352–355.
22. Brehm R, Zeiler M, Ruttinger C, Herde K, Kibschull M, Winterhager E, Willecke K, Guillou F, Lecureuil C, Steger K, Konrad L, Biermann K, et al. Sertoli cell-specific knockout of connexin43 prevents initiation of spermatogenesis. Am. J. Pathol 2007; 171:19–31.
23. Pointis G, Segretain D. Role of connexin-based gap junction channels in testis. Trends Endocrinol Metab 2005; 16:300–306.
24. Ryser S, Glauser D, Vigier M, Zhang YQ, Tachini P, Schlegel W, Durand P, Irminger-Finger I. Gene expression profiling of rat spermatogonia and Sertoli cells reveals signaling pathways from stem cells to niche and testicular cancer cells to surrounding stroma. BMC Genomics 2011; 12:29.

25. Clermont Y. The cycle of the seminiferous epithelium in man. Am J Anat 1963; 112:35–51.
26. Arkoun B, Dumont L, Milazzo JP, Way A, Bironneau A, Wils J, Macé B, Rives N. Retinol improves in vitro differentiation of prepubertal mouse spermatogonial stem cells into sperm during the first wave of spermatogenesis. PLoS One 2015; 10:e0116660.
27. Komeya M, Kimura H, Nakamura H, Yokonishi T, Sato T, Kojima K, Hayashi K, Katagiri K, Yamanaka H, Sanjo H, Yao M, Kamimura S, et al. Long-term ex vivo maintenance of testis tissues producing fertile sperm in a microfluidic device. Sci Rep 2016;21472.
28. Sato T, Katagiri K, Gohbara A, Inoue K, Ogonuki N, Ogura A, Kubota Y, Ogawa T. In vitro production of functional sperm in cultured neonatal mouse testes. Nature 2011; 471:504–508.
29. Clermont Y. Kinetics of spermatogenesis in mammals: seminiferous epithelium cycle and spermatogonial renewal. Physiol. Rev 1972; 52: 198–236.
30. Boitani C, Politi MG, Menna T. Spermatogonial cell proliferation in organ culture of immature rat testis. Biol Reprod 1993; 48:761–767.
31. Jahnukainen K, Chrysis D, Hou M, Parvinen M, Eksborg S, Soder O. Increased apoptosis occurring during the first wave of spermatogenesis is stage-specific and primarily affects midpachytene spermatocytes in the rat testis. Biol Reprod 2004; 70:290–296.
32. Clermont Y, Harvey SC. Duration of the cycle of the seminiferous epithelium of normal, hypophysectomized and hypophysectomized-hormone treated albino rats. Endocrinology 1965; 76:80–89.
33. Misell LM, Holochwost D, Boban D, Santi N, Shefi S, Hellerstein MK, Turek PJ. A stable isotope-mass spectrometric method for measuring human spermatogenesis kinetics in vivo. J Urol 2006; 175:242–246.
34. Amann RP. The cycle of the seminiferous epithelium in humans: a need to revisit? J. Androl 2008; 29:469–487.
35. Suzuki S, Sato K. The fertilising ability of spermatogenic cells derived from cultured mouse immature testicular tissue. Zygote 2003; 11:307–316.
36. Schneider F, Neuhaus N, Wistuba J, Zitzmann M, Hess J, Mahler D, van Ahlen H, Schlatt S, Kliesch S. Testicular functions and clinical characterization of patients with gender dysphoria (GD) undergoing sex reassignment surgery (SRS). J Sex Med 2015; 12:2190–2200.
37. Cheng CY, Wong EW, Yan HH, Mruk DD. Regulation of spermatogenesis in the microenvironment of the seminiferous epithelium: new insights and advances. Mol Cell Endocrinol 2010; 315:49–56.
38. Seidl K, Holstein AF. Organ culture of human seminiferous tubules: a useful tool to study the role of nerve growth factor in the testis. Cell Tissue Res 1990; 261:539–547.
39. Carette D, Perrard MH, Prisant N, Gilleron J, Pointis G, Segretain D, Durand P. Hexavalent chromium at low concentration alters Sertoli cell barrier and connexin 43 gap junction but not claudin-11 and N-cadherin in the rat seminiferous tubule culture model. Toxicol Appl Pharmacol 2013; 268:27–36.
40. Clermont Y, Perey B. Quantitative study of the cell population of the seminiferous tubules in immature rats. Am J Anat 1957; 100:241–267.