TOXICOLOGICAL SCIENCES **116(1)**, 286–296 (2010)
doi:10.1093/toxsci/kfq099
Advance Access publication April 1, 2010

Validation of a Rat Seminiferous Tubule Culture Model as a Suitable System for Studying Toxicant Impact on Meiosis Effect of Hexavalent Chromium

Cendrine Geoffroy-Siraudin,*,† Marie-Hélène Perrard,‡ Florence Chaspoul,* André Lanteaume,* Philippe Gallice,* Philippe Durand,‡ and Marie-Roberte Guichaoua*,†,[1]

*Laboratoire de Biogénotoxicologie et Mutagenèse Environnementale (EA1784), Université de la Méditerranée, Faculté de Médecine et de Pharmacie, 27 Boulevard Jean Moulin, 13385 Marseille Cedex 5, France. FR CNRS 3098-Ecosystemes Continentaux et Risques Environnementaux ECCOREV, Europôle de l'Arbois, 13150 Aix-en Provence, France; †Laboratoire de Biologie de la Reproduction, AP-HM, Hôpital de la Conception, 147 Boulevard Baille, 13385 Marseille Cedex 5, France; and ‡Institut de Génomique Fonctionnelle de Lyon, UMR 5242 CNRS INRA Ecole Normale Supérieure de Lyon, Université de Lyon 1, 46 allée d'Italie, F-69364 Lyon Cedex 07, France

[1]To whom correspondence should be addressed. Fax: 04-91-38-38-97. E-mail: mguichaoua@ap-hm.fr.

Received January 6, 2010; accepted March 23, 2010

There is evidence that exposure to environmental factors is at least partly responsible for changes in semen quality observed over the past decades. The detection of reproductive toxicants under Registration, Evaluation and Authorisation of Chemicals (REACH) will impact animal use for regulatory safety testing. We first validated a model of culture of rat seminiferous tubules for toxicological studies on spermatogenesis. Then, using this model of culture, we assessed the deleterious effects of 1, 10, and 100 μg/l hexavalent chromium [Cr(VI)] on meiotic cells. The prophase I of meiosis was studied *in vivo* and *ex vivo*. Bromo-2′-deoxyuridine (BrdU) was used to describe the kinetics of germ cell differentiation. SCP3 labeling allowed to establish the distribution of the stages of the meiotic prophase I and to perform a qualitative study of the pachytene stage in the absence or presence of Cr(VI). The development of the meiotic step of pubertal rats was similar *in vivo* and *ex vivo*. The number of total cells appeared not affected by the presence of Cr(VI) irrespective of its concentration. However, the numbers of late spermatocytes and of round spermatids were decreased by Cr(VI) even at the lower concentration. The percentage of synaptonemal complex abnormalities increased slightly with the time of culture and dramatically with Cr(VI) concentrations. This model of culture appears suitable for toxicological studies. This study shows that Cr(VI) is toxic for meiotic cells even at low concentrations, and its toxicity increases in a dose-dependent manner.

Key Words: **meiosis; spermatogenesis; rat; culture; synaptonemal complex; hexavalent chromium.**

Since the meta-analysis of Carlsen *et al.* (1992), numerous studies have dealt with the sharp decline in sperm quality in both fertile and infertile men (Irvine *et al.*, 1996; Lackner *et al.*, 2005). There is now evidence that exposure to environmental factors is at least partly responsible for such remarkable changes in semen quality. Recently, Jensen *et al.* (2008) hypothesized that the decreased conception rate among young cohorts of Danish women may partly be explained by the poor semen quality of their partners. Many chemicals present in our environment may be involved in reproduction disruption, but their cellular and molecular mechanisms are still obscure. Indeed, most studies did not precisely evaluate the germ cell damages and the stages of spermatogenesis that were altered. Yet, to appreciate the risks of a toxicant for men and their progeny, it is essential to know when and how the substance can act on germ cell differentiation. Today, Registration, Evaluation and Authorisation of Chemicals (REACH), a European community regulation on chemicals and their safe use (1907/2006), imposes to industrials toxic and reprotoxic studies for products sold with more than 100 T/year. The Joint Research Centre of the European Commission (Pedersen *et al.*, 2003) estimated necessary to test 2893 chemicals for reproductive toxicity and 2135 chemicals for developmental toxicity. By following the guidelines for regulatory safety testing, millions of animals would be required only for the detection of reproductive/developmental toxicants (Hartung and Rovida, 2009). In this context, new experimental models need to be developed to study the sites and the mechanisms of action of potential male reproductive toxicants.

In this study, we aimed to validate the system of culture of seminiferous tubules settled by Durand and coworkers for toxicological studies on spermatogenesis. Indeed, this system that allows studying the mitotic phase of spermatogenesis, the entire meiotic phase, and the first steps of spermiogenesis over a 4-week culture period in the rat (Fouchécourt *et al.*, 2006; Hue *et al.*, 1998; Staub *et al.*, 2000) has been extensively validated from the physiological point of view over the past 10 years (see review in Perrard *et al.*, 2009). In addition, it

must be underlined that this model of culture in bicameral chamber allows studying the effects of a toxic substance added to the basal compartment of the chamber and thus mimics what could happen *in vivo* in the testis. Indeed, the intercellular junctions are maintained in this system of culture (Perrard *et al*., 2009; Staub *et al*., 2000), and therefore, before reaching the germ cells, the toxic substances must cross the barrier of Sertoli cells (main component of the blood-testis barrier). Conversely, in "conventional" culture chambers, an agent may be toxic to differentiating germ cells because it is placed directly in contact with these cells, whereas *in vivo*, it may not have access to the compartment of the seminiferous tubules where these populations of germ cells are located. Another interest of this culture system is the reduction of the number of animals used and of the testing costs. Indeed, testicular germ cells from one rat allow performing 10–20 different assays. In the present work, we chose to study more specifically the prophase I of meiosis, during which the mechanisms of pairing and recombination could be strategic targets for toxicants (Martin, 2006). These two meiotic phenomena greatly influence the quality of spermatogenesis.

Using this culture model, we assessed the deleterious effects of the hexavalent chromium [Cr(VI)] on meiotic cells and its mechanisms. Cr(VI) compounds find extensive application in diverse industries and induce dose-dependent acute and chronic toxicity (Barceloux, 1999; Von Burg and Liu, 1993). A growing body of studies are available regarding the reproductive effects of chromium in men and animals (Aruldhas *et al*., 2005; Danadevi *et al*., 2003; Pereira *et al*., 2004), but a detailed analysis of spermatogenesis is not available. Kawanishi *et al*. (1986) have demonstrated that Cr(VI) produce noxious ROS including superoxide anion, singlet oxygen, and hydroxyl radicals through the formation of chromium (V) [Cr(V)] intermediates. In male mice exposed to Cr(V), the major finding reported was the alteration of permeability of the blood-testis barrier (Pereira *et al*., 2004).

MATERIALS AND METHODS

Animals

Wistar rats, 100 days old (adult rat), 42 days old, or 23 days old, having undergone no treatment were used. For meiotic *in vivo* studies, three rats of each age were used. Both testes were quickly removed and frozen in Ham F10/10% glycerol in liquid nitrogen. For cultures (*ex vivo* studies) in order to counterbalance inter-animal variations, testes from six to ten 23-day-old rats were pooled in every experiment and immediately used as described below.

Preparation and Culture of Seminiferous Tubule Segments

This technique has been published previously (Hue *et al*., 1998; Staub *et al*., 2000). Incubation was carried out at 32°C in the culture medium, supplemented as above, in a water-saturated atmosphere of 95% air and 5% CO_2.

Three cultures were performed in the absence or presence of Cr(VI). When required, Cr(VI) was added from day 2 of the cultures as follows: An aqueous solution stock (20 mg/l) of potassium dichromate (RP Normapur Prolabo, France) was diluted in the culture medium in order to obtain a final concentration of Cr(VI) of 1 or 10 or 100 μg/l. The Cr(VI) solutions were then added in the basal compartment of the bicameral chambers of culture. These concentrations of chromium were selected on the basis of the mean plasma concentrations found in nonexposed healthy men (0.3–0.5 μg/l) and in workers exposed to hexavalent chromium (73.4 μg/l) (Li *et al*., 2001; Morris *et al*., 1988). Only basal media (with or without Cr(VI)) were renewed every 2 days. Two additional cultures were performed in the presence of bromo-2′-deoxyuridine (BrdU) to study the sequential appearance of the various categories of germ cells during the prophase I of meiosis and to follow the fate of the labeled cells during the cultures. Immediately after seeding the cells, BrdU (1μM) was added in both compartments of the bicameral chamber and left during 20 h, which corresponds roughly to the duration of the pre-meiotic S phase in the rat (Perrard *et al*., 2003).

At selected days of culture, cells were detached from the permeable membrane of the bicameral chambers by trypsinization. An aliquot of the cell suspension was used to determine the number of cells and to assess cell viability by trypan blue exclusion. Aliquots of cells were frozen at −80°C in culture medium supplemented with 10% glycerol. Remaining cells were fixed with ice-cold 70% ethanol for Fluorescence Activated Cell Sorter (FACS) analysis.

TABLE 1
Operational Parameters of ICP-MS

Parameter	Value
Argon gas flow rates (l/min)	
Plasma	15.0
Auxiliary	0.9
Carrier	1.0
Collision gas (He 97%, H_2 3%) (l/min)	1.2
Sampling depth (mm)	6.8
Nebulizer	Meinhard
Spray chamber	Double pass, quartz
Spray chamber temperature (K)	275
Sample uptake (ml/min)	1.0

Measurement of the Chromium Concentration in the Cultured Cells

Chromium analysis was performed using inductively coupled plasma mass spectrometry (ICP-MS) as previously described (Chardin *et al*., 2002) on days 5, 9, and 16 of the cultures performed in the absence or presence of Cr(VI) as follows. Cell suspensions (200–800 μL) were diluted by adding nitric acid 63% ultrapure (40 μL) and ultrapure water 18 MΩ (2 ml). The solutions obtained were then analyzed in triplicate by ICP-MS using a Thermo Series II ICP-MS apparatus equipped with a collision cell (Thermo-Electron, Les Ulis, France). Operational parameters are given in Table 1. The calibration curve was obtained by dilution of a multi-element solution in 1% nitric acid to yield 0.5, 1.0, 10.0, and 100.0 μg/l of chromium. Metal concentrations were finally determined using Plasmalab software (Thermo-Electron), and results were expressed as cellular chromium concentrations (nanograms per 10^6 cells).

Cytological Methods

Spreading and fixation of germ cells. Cell suspensions were pre-fixed before spreading in 0.1% paraformaldehyde (PAF) (Merck, Germany) in medium culture for 5 min. Stock solution of PAF at 2% was prepared in distilled water. To perform immunocytochemical analysis, meiotic cells were spread by cytocentrifugation at 30 × g according to Metzler-Guillemain and Guichaoua (2000).

Immunocytological localization of axial elements and lateral elements of synaptonemal complexes in spermatocytes. A rabbit polyclonal anti-SCP3 antibody (Abcam, UK) was used to reveal axial elements and lateral elements of synaptonemal complexes in freshly prepared adult rat spermatocytes or in cultured spermatocytes. Immunocytochemistry was performed according to Metzler-Guillemain and Guichaoua (2000).

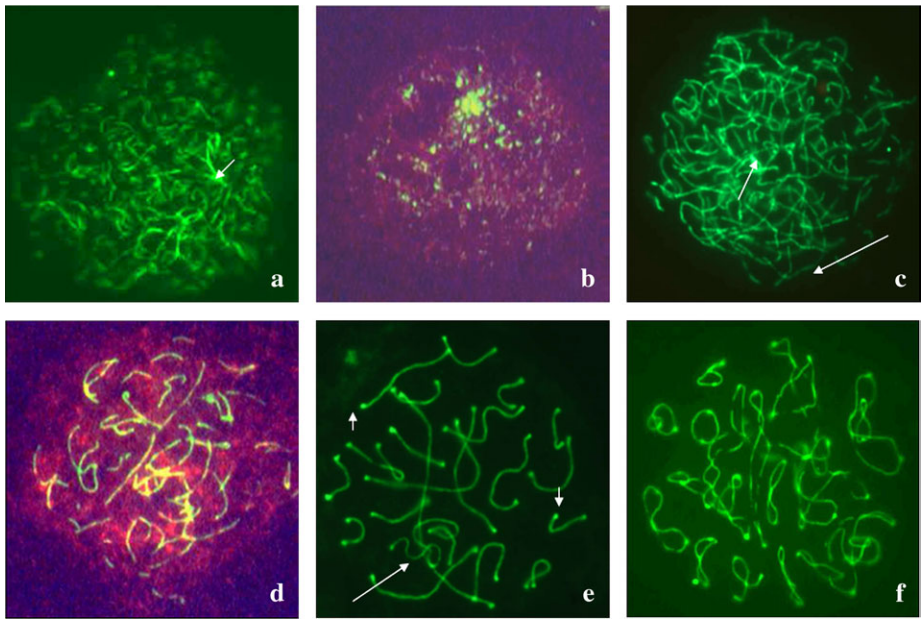

FIG. 1. Different substages of meiosis in the rat. Nuclei of spermatocytes I were stained by an anti-SCP3 antibody (green). A monoclonal anti-BrdU antibody revealed preleptotene spermatocytes that underwent DNA synthesis at the beginning of the culture (red). (a) Leptotene nuclei showing discontinuous axial elements; SC are initiated in two homologues (arrow). (b) Polarized leptotene spermatocyte double stained for SCP3 and BrdU. (c) Zygotene nuclei showing paired (short arrow) and unpaired regions (long arrow). (d) Zygotene nuclei with double staining for SCP3 and BrdU in which most homologous chromosomes are paired. (e) Pachytene nuclei in which all homologous chromosomes are paired and showing SC large ends (long arrow = sex chromosomes, short arrows = two large ends). (f) Diplotene showing separated lateral elements of SC, except at chiasma sites.

Immunocytological localization of axial elements and lateral elements of synaptonemal complexes in BrdU-labeled spermatocytes. Dual-color immunocytochemistry was performed with a rabbit polyclonal anti-SCP3 antibody and a mouse monoclonal anti-BrdU antibody (Dako cytomation, Denmark) to reveal cells that underwent DNA synthesis at the beginning of the culture (spermatogonia and preleptotene spermatocytes). Cells were stained with the anti-SCP3 antibody as described in Metzler-Guillemain and Guichaoua (2000). The cells were then rinsed in PBS 1× and mounted in Vectashield. On the following day, the cells were fixed again in 2% PAF/0.03% SDS, then in 2% PAF for 3 min before denaturing in formamide at 74°C for 3 min, and immersed in alcohol at −20°C. Cells were incubated with the anti-BrdU antibody at 1:25 dilution for 2 h at room temperature. After washing in 0.4% photoflo/PBS and then in 0.01% Triton/PBS and blocking buffer, detection was performed with a tetramethyl rhodamine isothiocyanate (TRITC)–conjugated rabbit anti-mouse immunoglobulin G (IgG) (Abcam) at 1:100 dilution for 1 h at 37°C. Cells were then rinsed in PBS and mounted in Vectashield.

Microscope Analysis

Spermatocyte nuclei were observed using a Zeiss Axioplan 2 fluorescent photomicroscope (Zeiss, Germany). For *in vivo* differentiated spermatogenic cells, 1000 nuclei were analyzed at every age studied. For cells differentiated in culture, 300 nuclei were analyzed at each day studied.

Immunolabeling of Cultured Cells for Flow Cytometric Analysis

To distinguish germ cells from Sertoli cells, fixed cultured cells were immunolabeled with a monoclonal antibody against vimentin (clone V9; DAKO SA, Trappes, France), followed by a rabbit anti-mouse IgG phycoerythrin (PE)–conjugated antibody as previously described in detail (Godet *et al.*, 2000, 2004, 2008). Hoechst 33342 at a final concentration of 0.12 μg/ml was added to the immunolabeled cells from cultured tubules to assess their DNA content.

After immunolabeling, cells were analyzed using a FACSVantage SE cell sorter (BD Biosciences, CA) equipped with an Enterprise II argon ion laser emitting simultaneously a 50 mw line tuned to UV (351 nm) and a second line tuned to 448 nm at 130 mw to respectively excite Hoechst and PE. Emission of PE fluorescence (λ.reem = 585 nm) was acquired after logarithmic amplification through a 585/40 nm filter. Emission of Hoechst 33342 fluorescence (λ.reem = 424 nm) was acquired after linear amplification through a 424/44 nm filter. Acquisition and analysis were performed using CellQuest ProTM 4.0.2 software. Analyses were performed as previously described in detail (Godet *et al.*, 2000). The vimentin-positive somatic cells and the vimentin-negative 4C, 2C, and 1C germ cells were separated with the bivariate analysis: DNA content/vimentin. Then, the bivariate linear forward light scatter and linear side angle light scatter analysis allowed the identification of young spermatocytes, middle-to-late pachytene spermatocytes, secondary spermatocytes, and round spermatids (Godet *et al.*, 2000).

Statistical Analysis

Statistical analysis was performed using the SPSS program (statistical package for the social sciences). Regression analysis with linear and quadratic correlation and the chi-square test were used. Differences were considered significant at $p < 0.05$.

RESULTS

Description of the First Meiotic Prophase In Vivo

In the adult rat. Studying the meiosis of adult rats (100 days old) by using the anti-SCP3 antibody provided a comprehensive description of the different substages of prophase I (Fig. 1). At leptotene stage, thin and discontinuous axial cores hold all the nucleus area (Fig. 1a). By the end of leptotene, some axial cores are grouped within a limited area (Fig. 1b). During zygotene, homologues have begun to come close

TABLE 2
Quantitative Aspects of the First Meiotic Prophase *In Vivo*

Age of rats (days)	Leptotene (%)	Zygotene (%)	Pachytene (%)	Diplotene (%)
23	18	15.7	65.1	1.2
42	3.7	12.5	67.8	16
100	2.3	8.1	83.1	6.5

Note. The proportions of leptotene, zygotene, pachytene, and diplotene spermatocytes were obtained in 23-, 42-, and 100-day-old rats after immunostaining by an anti-SCP3 antibody. Three rats of each age were sacrificed, and 1000 nuclei were analyzed for each age. The percentage of leptotene spermatocytes was significantly higher in 23-day-old rats than in 42- or 100-day-old rats ($p < 0.001$); no significant difference was observed for zygotene and pachytene nuclei for the three ages; the percentage of diplotene spermatocytes was significantly higher in the 42-day-old rats than in the other ages ($p < 0.01$). Results are the mean of 1000 nuclei from three rats of each age.

together and thin discontinuous axial cores coexist with short more intense stained synaptonemal complexes (SCs) (Fig. 1c). An asynchrony was observed in pairing progression between homologous chromosomes, some being partially paired, whereas others are still totally unsynapsed (Fig. 1d). At pachytene stage, all homologues are paired and 21 intensely stained SCs are clearly visible in each nuclei, one of which, the sexual bivalent, is easily identifiable by its peculiar configuration (Fig. 1e). Whereas a certain degree of condensation occurs during the pachytene stage, no condensed sex chromosomes are seen, as observed in the human species (Guichaoua *et al*., 2005). At diplotene stage, lateral elements of SCs are separated, except at chiasma sites (Fig. 1f); the latter are preferentially located in the telomeric regions of the bivalents. At pachytene and diplotene stages, telomeres exhibit large attachment sites to the nuclear envelope. Quantitative analysis showed that leptotene spermatocytes were rare on the preparations (2.3%), the percentage of zygotene spermatocytes was 8.1%, pachytene was the most frequent stage (83.1%), and diplotene spermatocytes represented 6.5% (Table 2).

The three main types of SC abnormalities in pachytene nuclei were fragmented SC (Fig. 2a), asynapsed SC (Fig. 2b), and dotted SC, defined as regular discontinuities of SC and interpreted as defective condensation of SC (Fig. 2c). Each of them could be alone or associated with one or the two others. The most frequent abnormality was the fragmentation of the SC (9.3%), short asynapsis limited to few bivalents were less frequent (4.2%), and dotted SCs were rare on the preparations (0.4%) (Table 3).

In the 23- and 42-day-old rat. The same morphological stages than those in adult rat were observed but with different frequencies (Table 2). For the 23-day-old rats, spermatocytes at the leptotene stage were significantly more frequent than those in the adult rat (18 vs. 2.3%, $p < 0.001$). Zygotene stages seemed more frequent in young rats (15.7%) than those in the adult rat, but the difference was not significant. Similarly, the frequency of pachytene stages (65.1%) was lower but not significantly different from that of the adult rat. Diplotene stages were significantly less frequent (1.2%) than those in adult rat (6.5%, $p < 0.001$).

For the 42-day-old rats, the percentages of leptotene, zygotene, and pachytene spermatocytes (3.7, 12.5, and 67.8%, respectively) were not significantly different from those of the adult rat. By contrast, diplotene spermatocytes were significantly more numerous (16%) than those in the rats at the two other ages ($p < 0.01$).

As in the adult rat, the most frequent abnormality in the 23- and 42-day-old rats was fragmentation of the SC (10.5 and 8.5%, respectively). The frequencies of asynapsis were 3.7 and 3.9% for the 23- and 42-old-day rats, respectively, similar to the adult rat (Table 3). There was no significant difference between the percentages of these two abnormalities at the three ages studied. The percentage of dotted SC in 23-day-old rats was significantly higher than that in the 42- and 100-day-old rats (3.5 vs. 0.6% and 0.4%, respectively, $p < 0.001$).

Study of the Meiotic Prophase in Culture of Seminiferous Tubules

Time course of differentiation of BrdU-labeled germ cells. Culture of seminiferous tubules of 23-day-old rats with BrdU served to establish the kinetics of differentiation of

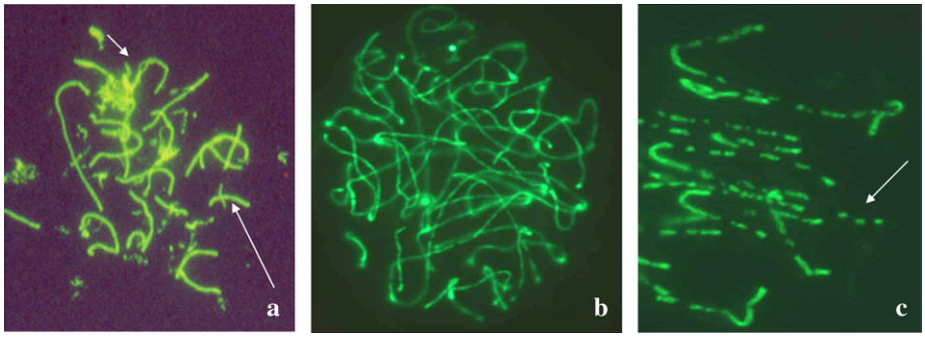

FIG. 2. The different types of SC abnormalities in rat pachytene nuclei. (a) Fragmentation (long arrow) of SC with asynapsed regions (short arrow). (b) Extended asynapsed regions. (c) Dotted SC (regular discontinuities of SC corresponding to defective condensation of SC).

TABLE 3
SC Abnormalities in Meiotic Prophase *In Vivo*

	23 days	42 days	100 days
% Nuclei with asynapsis	3.7	3.9	4.2
% Nuclei with fragmented SC	10.5	8.5	9.3
% Nuclei with dotted SC	3.5	0.6	0.4
Total % of abnormal nuclei	12	11	11

Note. For the three ages of rats, the percentage of nuclei with SC fragmentation was significantly higher than that with asynapsis and dotted SC ($p < 0.001$). The total percentage of abnormal nuclei did not significantly differ between the three ages studied. Results are the mean of 1000 nuclei from three rats of each age. SCs were immunostained by an anti-SCP3 antibody.

cultured germ cells (Fig. 3). Spread cells from two independent cultures were treated by dual-color immunocytochemistry. SCP3 labeling allowed the precise identification of each substage of prophase I, from leptotene to diplotene, and BrdU allowed to highlight the sequential appearance of the various substages. Spermatogonia and preleptotene spermatocytes were only labeled by the anti-BrdU antibody; no element of the SC was present at these two stages, which were therefore pooled. The same differentiation pattern was obtained in the two cultures. A small percentage of cells were BrdU positive in the two cultures (7.5%), but the total number of BrdU-labeled germ cells was sufficient to interpret the results.

The greatest number of BrdU-labeled spermatogonia/preleptotene spermatocytes was obtained on the first day of analysis, day 2 of culture (62%) (Fig. 3); then, it decreased until the end of the cultures as expected. BrdU-labeled leptotene spermatocytes were present from day 2 onward. Their proportion increased until day 7 (59%) and then decreased to disappear on day 14. Labeled zygotene nuclei were first observed on day 5 (13.3%); their percentage increased until day 9 (27%) and then decreased until day 14 (14.3%). BrdU-labeled pachytene nuclei appeared on day 9 and were maximal on day 14 (53.3%). The first BrdU-labeled diplotene nuclei appeared at the end of the cultures: 14.3% on day 14.

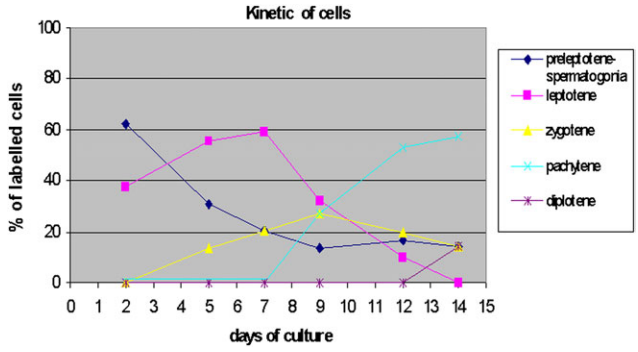

FIG. 3. Kinetics of spermatocyte differentiation *ex vivo*. Distribution profile of the five stages of the first meiotic prophase after double staining with anti-SCP3 and anti-BrdU antibodies. Spermatogonia and preleptotene spermatocytes are only stained by BrdU. Results are the mean of three to five pooled replicate culture wells in two different cultures. Three hundred nuclei were analyzed on each day in each culture. Each value is the mean of the results of the two cultures.

Quantitative aspects of the first meiotic prophase. The greatest percentage of leptotene spermatocytes was obtained on day 5 of culture (15.5% ± 3.1) (Table 4). This percentage decreased significantly with time until the end of the cultures ($R = 0.90$, $p < 0.01$). Zygotene spermatocytes showed a parabolic evolution with a maximum on day 7 (25.2% ± 4.7, $R = 0.88$, $p < 0.05$). On day 7 of culture, a few zygotene nuclei with a polarized aspect in "bouquet" form were observed (Fig. 4a). This very transient stage in rats, corresponding to early zygotene (Zickler and Kleckner, 1998), was never observed on the meiotic preparations from the 100-day-old rats. The percentage of pachytene spermatocytes did not vary with time ($R = 0.32$, $p = 0.47$). The long duration of this stage, 11 days in rats, explains the stability of its percentage (from 54.3% ± 3.6 to 65.2%) throughout the culture. The mean percentage of diplotene spermatocytes showed a linear increase throughout the culture ($R = 0.98$, $p < 0.001$). A transient diplotene stage corresponding to early diplotene was observed in these *ex vivo* preparations displaying partial separation of homologues and long regions of bivalents still paired (Fig. 4b). These early diplotene represented roughly 20% of diplotene spermatocytes observed. Note that the percentages of the four stages were similar on the last day (D16) of culture of seminiferous tubules (Table 4) and in the 42-day-old rats (Table 2).

SC abnormalities in pachytene spermatocytes. The same abnormalities than *in vivo* were found but with different frequencies (Table 5). The percentage of abnormal nuclei showed a significant linear increase from D2 to D16 of culture ($R = 0.95$, $p < 0.01$). On day 2 of culture, the mean percentage of nuclei with fragmented SC (4.6%) was significantly lower than that observed *in vivo* in 23-day-old rats (10.5%, $p < 0.001$) (Table 3), whereas the mean percentage of nuclei with asynapsis was similar. These two abnormalities showed a significantly linear increase from day 5 to day 16 ($R = 0.97$, $p < 0.001$, for SC fragmentation; $R = 0.93$, $p = 0.02$, for asynapsis). From day 12 onward, the percentage of nuclei with fragmented SC became significantly higher in culture (14.5% ± 1.6) than *in vivo* ($p < 0.001$); nuclei with asynapsis became significantly higher in culture than *in vivo* from day 9 (12.2% ± 0.2) ($p < 0.001$). The percentage of nuclei with dotted SC was as low *ex vivo* as *in vivo* (Table 3); no linear increase was observed from D5 to D16.

Effect of Cr(VI) on the Meiotic Step Ex Vivo

Determination of chromium concentrations in the cultured cells. Our model of culture in bicameral chambers allows studying the effects of a toxic substance added to the basal compartment of the culture chamber in order to mimic what

TABLE 4
Quantitative Aspects of the First Meiotic Prophase *Ex Vivo* With and Without Cr(VI)

		D2	D5	D7	D9	D12	D14	D16
Control	Leptotene	15%	15.5% ± 2.2	7.7% ± 0.3	8.1% ± 1.3	4.4% ± 1.2	5.5%	4.3% ± 0.5
	Zygotene	16.2%	19.3% ± 2.8	25.2% ± 4.7	24.8% ± 5.7	20.2% ± 2.9	14%	14% ± 2.6
	Pachytene	65.2%	58% ± 5.7	60% ± 1.7	54.3% ± 3.6	57.6% ± 2.9	60%	60% ± 0
	Diplotene	3.6%	7.2% ± 1.2	7.1% ± 0.6	12.8% ± 2.1	17.8% ± 5.3	20.5%	21.7% ± 2.1
Cr(VI) 1 µg/l	Leptotene		11.4% ± 2.8	6.2% ± 2.9	4.7% ± 0.5	6.7% ± 1	3.5%	4% ± 0
	Zygotene		22.9% ± 6.4	22.4% ± 1.2	20% ± 2	16.3% ± 0.6	11%	15.3% ± 0.7
	Pachytene		59.6% ± 6.4	64.7% ± 4.7	63.8% ± 5	61.7% ± 1.7	66.5%	64% ± 0.7
	Diplotene		6.1% ± 2.8	6.7% ± 1.2	11.5% ± 2.5	15.3% ± 1.2	19%	16.7% ± 2.7
Cr(VI) 10 µg/l	Leptotene		14.5% ± 0.8	10.5% ± 1.7	7.2% ± 0.1	6.2% ± 0.6	6%	2.3% ± 0.5
	Zygotene		16.7% ± 6.4	20.3 ± 3	19.8% ± 1.1	15.2% ± 1.9	14%	14% ± 3
	Pachytene		63.3 ± 3.3	63% ± 2.7	61.5% ± 2.3	59.4% ± 3.3	58%	64.7% ± 1
	Diplotene		5.5% ± 0.5	6.2% ± 0.2	11.5% ± 1.3	19.2% ± 1.5	22%	19% ± 1.5
Cr(VI) 100 µg/l	Leptotene		12.2% ± 0.6	8% ± 0.7	5% ± 2	5% ± 0.9	3%	2.5% ± 0.5
	Zygotene		21.8% ± 3.1	22% ± 1.2	20.5% ± 1.5	15.2% ± 0.6	12%	16.5% ± 4.5
	Pachytene		59.3% ± 3.5	60.7% ± 0.6	59% ± 0	63.6% ± 0.3	62%	64% ± 0
	Diplotene		6.7% ± 0.2	9.3% ± 0.3	15.5% ± 1.5	16.2% ± 1.6	23%	17% ±4

Note. Analysis was performed in control cultures and in cultures with different concentrations of Cr(VI) in the culture medium. Spermatocytes were identified according to their staining with an anti-SCP3 antibody. Each value is the mean (±SEM) of three cultures except on days 2 and 14. In the control cultures, the percentage of leptotene stages decreased significantly from D5 to D16 ($R = 0.90$, $p < 0.01$). No linear correlation with time was observed for zygotene and pachytene stages. The percentage of diplotene spermatocytes increased significantly from D5 to D16 ($R = 0.98$, $p < 0.001$). The percentages of the four stages did not vary between control cultures and cultures with Cr(VI) whatever its concentration.

could happen *in vivo* in the testis. Indeed, the cellular junctions, between Sertoli cells and between germ cells and Sertoli cells, which are essential for spermatogenesis, are maintained in our cultures (Perrard *et al*., 2009; Staub *et al*., 2000). Therefore, before reaching the germ cells in our system, the toxic substance must cross the barrier of Sertoli cells (main component of the blood-testis barrier). Therefore, we assessed first whether Cr(VI) added to the basal compartment of the bicameral chamber could be detected in the cultured cells.

Cellular concentrations of chromium at selected days of cultures exposed to 1, 10, or 100 µg/l of the metal are presented in Table 6. In control cultures, no metal was detected as expected. By contrast, in the cell suspensions from cultures performed in the presence of Cr(VI), the cellular chromium concentration increased with the concentration of Cr(VI) in the basal culture medium.

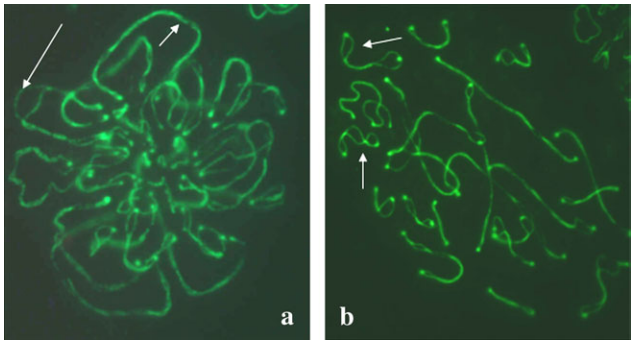

FIG. 4. Transient zygotene and diplotene stages observed only in *ex vivo* preparations. (a) ''Bouquet form'' zygotene nuclei labeled with the anti-SCP3 antibody showing SCs (short arrow) and continuous unpaired axial element (long arrow). (b) Early diplotene displaying partial separation of homologues (short arrow), whereas long regions of bivalents are still paired.

Effect of chromium on the number of Sertoli and meiotic cells in culture. The number of total cells decreased during the culture period as expected (Hue *et al*., 1998; Staub *et al*., 2000). Nevertheless, the number of cells per well was not affected by the presence of Cr(VI) irrespective of its concentration (Fig. 5). Flow cytometric analysis allowed refining these results (Fig. 6). The small decrease in the number of Sertoli cells, observed during the culture period, appeared not affected by any concentration of Cr(VI), whatever the day of sampling (107 ± 7% of the control values). Likewise, the number of young spermatocytes was roughly similar under control conditions and in cultures performed in the presence of Cr(VI) (88 ± 3%). Conversely, the numbers of middle-to-late pachytene spermatocytes (79 ± 3%), secondary spermatocytes (81 ± 2%), and round spermatids were somewhat lower in the presence of either concentration of Cr(VI) from day 4 onward; the amplitude of the decrease was more marked for *ex vivo*–formed round spermatids (65 ± 3% of control values).

Quantitative aspects of the first meiotic prophase in the presence of CR(VI). The repartition of the stages of the first meiotic prophase in cultures performed in the presence of the three concentrations of Cr(VI) is shown in Table 4. As in control cultures, the highest percentages of leptotene spermatocytes were obtained on day 5 of culture and then they decreased with time. These decreases were statistically

TABLE 5
SC Abnormalities During the First Meiotic Prophase *Ex Vivo* With and Without Cr(VI)

		D2	D5[a,b,c]	D7[a,b,c]	D9	D12[a,b,c]	D14[a,b,c]	D16[a]
Control	% Asynapsed	4.3%	4.9% ± 1.3	6.1% ± 1.6	12.2% ± 0.2	11% ± 1.5	11.7%	16.2% ± 0.4
	% Fragmented	4.6%	4.9% ± 0.2	6.8% ± 1.2	9.5% ± 0.3	14.5% ± 1.6	17.6%	17.7% ± 1.5
	% Dotted	0.6%	0	0.9% ± 0.2	0.5% ± 0.3	0.9% ± 0.5	0	3.8% ± 2.5
	% Of abnormal nuclei[d]	8%	9% ± 1.9	11% ± 0.8	15% ± 1.5	21% ± 3	19%	22% ± 2.5
Cr(VI) 1 µg/l	% Asynapsed		16.9% ± 2.4	21.9% ± 1.1	28% ± 0.1	28.6% ± 1.5	27.8%	26.2% ± 2.1
	% Fragmented		14.9% ± 1.6	14.1% ± 3.4	16.6% ± 4.4	25.7% ± 1.1	23.3%	21.2% ± 4.6
	% Dotted		1.5% ± 1	1.5% ± 0.7	1.8% ± 0.4	2.8% ± 0.2	3%	2.3% ± 0.1
	% Of abnormal nuclei[d]		30% ± 1.2	31% ± 3.2	38% ± 2.1	42% ± 3.4	39%	40% ± 1
Cr(VI) 10 µg/l	% Asynapsed		23.3% ± 1.6	26.9% ± 0.9	29.3% ± 5.1	38% ± 4.2	51.7%	29.4% ± 7.9
	% Fragmented		16.9% ± 2.8	19.6% ± 3.4	15.5% ± 5.7	31.3% ± 1.7	37.9%	25.8% ± 3.9
	% Dotted		0.4% ± 0.3	0.5% ± 0.2	0	1.7% ± 0.5	5.1%	0.5% ± 0.1
	% Of abnormal nuclei[d]		32% ± 2	41% ± 2	40% ± 4.6	58% ± 3.2	66%	57% ± 2.5
Cr(VI) 100 µg/l	% Asynapsed[d]		27.2% ± 2.3	31.9% ± 1.5	35.2% ± 2.1	40.9% ± 1.3	58.1%	57% ± 1.9
	% Fragmented[d]		20.5% ± 1.3	21.9% ± 2.9	23.7% ± 3.3	38.9% ± 4.6	56.5%	50.8% ± 1.1
	% Dotted		1.4% ± 0.7	0.8% ± 0.5	1.3% ± 0.5	0.6% ± 0.4	4.8%	3.1% ± 1.4
	% Of abnormal nuclei[d]		44% ± 2.2	52% ± 3.6	55% ± 3.7	64% ± 3.5	84%	76% ± 1.5

Note. SC abnormalities were analyzed in control cultures and in cultures performed in the presence of different concentrations of Cr(VI). Each value is the mean (± SEM) of three cultures except on days 2 and 14. The percentages of abnormal nuclei showed a significant linear increase from D5 to D16 for control cultures and for all concentrations of Cr(VI) ($p < 0.05$). This increase was significant for asynapsis and SC fragmentation for Cr(VI) 100 µg/l ($p < 0.05$). The percentage of abnormal nuclei also showed a linear increase with the concentration of Cr(VI) (from 1 to 100 µg/l) at each day of culture ($p \leq 0.05$) except on D9. This increase was significant for asynapsis and SC fragmentation ($p \leq 0.05$) from D5 to D14 except on D9. The percentage of dotted nuclei was not affected by chromium, whatever its concentration.

[a]Significant with Cr(VI) for abnormal nuclei.
[b]Significant for fragmented SC.
[c]Significant for asynapsis.
[d]Significant with days of culture.

significant for Cr 10 µg/l and Cr 100 µg/l ($R = 0.95$, $p < 0.01$ and $R = 0.93$, $p < 0.01$, respectively) and at the limit of significativity for Cr 1 µg/l ($R = 0.79$, $p = 0.057$). The percentages of zygotene and pachytene spermatocytes were stable throughout the culture for the three concentrations of Cr(VI). For diplotene spermatocytes, the same linear increase as in control was found with Cr 1 µg/l ($R = 0.93$, $p < 0.01$), Cr 10 µg/l ($R = 0.92$, $p < 0.01$), and Cr 100 µg/l ($R = 0.85$, $p < 0.05$). At each day of culture, the percentages of the four stages were similar irrespective of the concentrations of Cr(VI) (Table 4).

SC abnormalities in pachytene spermatocytes in the presence of Cr(VI). The results are shown on Table 5. The percentages of abnormal nuclei showed a significantly linear increase from D5 to D16 for the three concentrations of chromium ($R = 0.84$, $p < 0.05$; $R = 0.90$, $p < 0.05$; $R = 0.93$, $p < 0.01$; for 1, 10, and 100 µg/l, respectively). Asynapsis and

TABLE 6
Cellular Chromium Concentrations (nanograms per 10^6 cells) in Germ Cell Suspensions

Cr(VI) in culture medium (µg/l)	Cellular chromium concentration (ng/10^6 cells)		
	Days of culture		
	D5	D9	D16
0	< 0.1	< 0.1	< 0.1
1	0.35 ± 0.02	0.63 ± 0.03	1.18 ± 0.06
10	3.78 ± 0.19	1.95 ± 0.1	2.20 ± 0.11
100	3.00 ± 0.15	10.15 ± 0.51	12.25 ± 0.61

Note. Each value is the mean (±SEM) of triplicate determinations in two cultures.

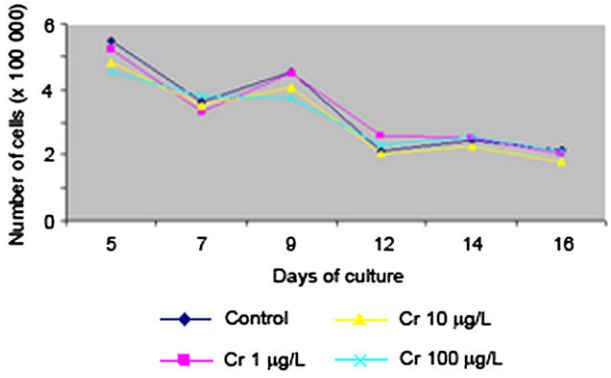

FIG. 5. Changes in the mean number of total cells by well in control cultures and in cultures with Cr(VI) at the three concentrations studied. Each value is the mean value of three cultures; for clarity, SEM has been omitted.

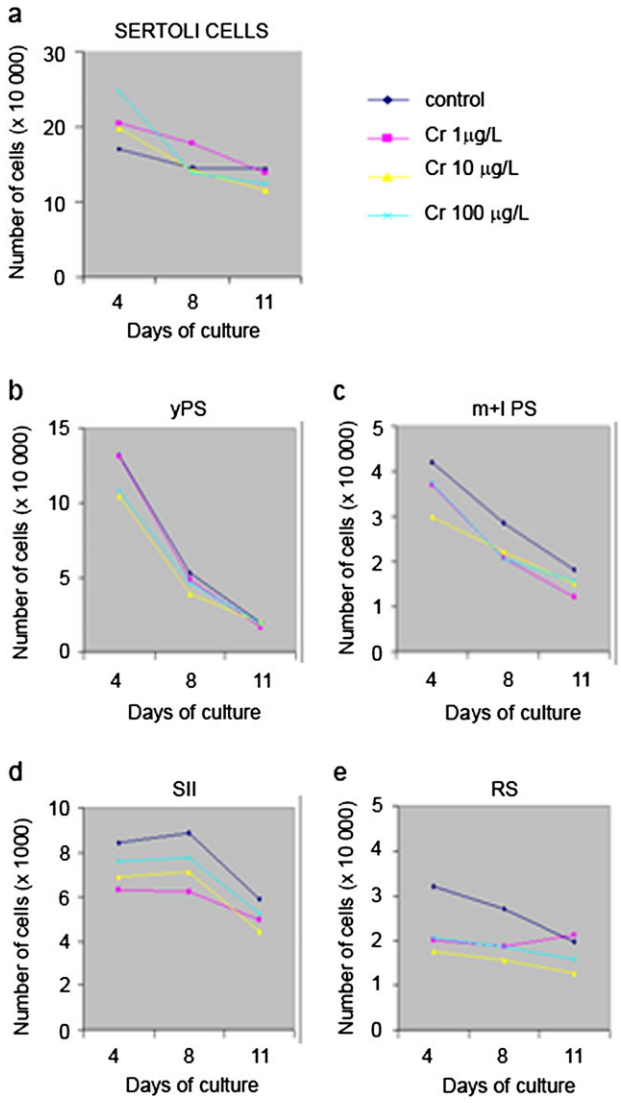

FIG. 6. Changes in the number of Sertoli cells and germ cells during a culture of testicular tubule segments from 23-day-old rats in the absence (control) or presence of Cr(VI) (1 or 10 or 100 µg/l). Sertoli cells (a). Young pachytene spermatocytes (yPS) (b). Middle-to-late pachytene spermatocytes (m + l PS) (c). Secondary spermatocytes (SII) (d). Round spermatids (RS) (e). Results are the mean of three wells per day for every condition. Similar results were obtained in another experiment.

fragmented SC were the most frequent abnormalities for the three concentrations of chromium, the frequencies of nuclei showing these two abnormalities increased with time, but this increase was significantly linear only for Cr(VI) 100 µg/l ($R = 0.95$, $p < 0.01$; $R = 0.92$, $p < 0.01$; respectively).

The percentage of abnormal nuclei increased from controls to Cr(VI) 100 µg/l for every day. The increase was linear and significant for D7, D12, D14, and D16 ($R = 0.98$, $p = 0.01$; $R = 0.97$, $p = 0.02$; $R = 0.99$, $p = 0.003$; and $R = 1$, $p < 0.001$; respectively) at the limit of significativity for D5 ($R = 0.95$, $p = 0.050$) and no significant for D9. The frequency of nuclei with asynapsis increased in the presence of chromium, and the increase was linear with the concentration and significant for D5, D7, D12, and D14 ($R = 0.97$, $p = 0.03$; $R = 0.95$, $p = 0.04$; $R = 0.94$, $p = 0.05$; and $R = 0.98$, $p = 0.02$; respectively) and no significant for D9 and D16. The frequency of nuclei with fragmented SC showed a linear increase with the concentration of chromium. This increase was significant for D7, D12, and D14 ($R = 0.97$, $p = 0.02$; $R = 0.99$, $p = 0.01$; and $R = 0.97$, $p = 0.02$; respectively) at the limit of significativity for D5 ($R = 0.94$, $p = 0.05$) and no significant for D9 and D16. The percentage of nuclei with dotted SC was not affected by chromium, whatever its concentration.

DISCUSSION

We have described a reliable *ex vivo* model for toxicological studies, allowing the study of the mechanisms by which environmental toxicants may interfere with the first prophase of meiosis. Our results demonstrate that the meiotic prophase is severely impaired by Cr(VI); the higher is the concentration, the more frequent are the abnormalities.

Validation of the System of Culture of Seminiferous Tubules for Meiotic Studies

We identified the four stages of the meiotic prophase I revealed by SCP3 labeling in adult rats. Our results on the chromosomal behavior from the leptotene to the diplotene stages are coherent with those obtained by Clermont (1972), Clermont and Perey (1957), and Scherthan and Schönborn (2001). The chronology of the *ex vivo* meiotic prophase, based on the double staining BrdU/SCP3 (Fig. 2), was in general accordance with the results of Hue *et al.* (1998), Staub *et al.* (2000), and Perrard *et al.* (2003). However, in our study, the percentage of BrdU-labeled spermatocytes was lower than that in the work of Staub *et al.* (2000) (7.5 vs. 53%). Two differences in the technical conditions should explain this discrepancy: (1) in Staub *et al.* (2000), germ cells were labeled *in vivo* and (2) the use of formamide in the present study, instead of NAOH, for denaturing chromatin before revelation of BrdU. The choice of a gentle denaturing procedure in this model was imposed by the necessity of protecting the SCP3 staining. The presence of BrdU-SCP3–labeled cells until the last day of culture is explained by the differentiation of BrdU-labeled spermatogonia in spermatocytes throughout the culture (Perrard *et al.*, 2003).

The sequential appearance of the different types of SCP3 and/or BrdU-labeled meiotic cells in culture from preleptotene to diplotene spermatocytes (Fig. 3) extends previous results from Perrard *et al.* (2003) as it shows that the kinetics of differentiation of the germ cells throughout the meiotic prophase *ex vivo* is close to that observed *in vivo*.

An important result of the present study is that the meiotic prophase that develops under our *ex vivo* conditions is similar

on many aspects to what happens in the testis *in vivo*. Different percentages of leptotene, zygotene, pachytene, and diplotene spermatocytes were observed *in vivo* between the 23-, 42-, and 100-day-old rats (Table 2). The percentages of leptotene and zygotene stages decreased inversely to the age of the rats, whereas the percentage of the pachytene stage increased with age (Table 2). A peculiar profile was found for the diplotene stage, whose frequency in the 42-day-old rats was higher than that in the 20- and 100-day-old rats; this could be explained by the progressive development of the spermatogenic process in the immature rats. At this age, spermatogenesis is not as efficient as that in the adult, which likely explains the accumulation of diplotene spermatocytes (Yang *et al.*, 1990). We showed here that there was no significant difference between the percentages of leptotene, zygotene, pachytene, and diplotene stages in the 42-day-old rats and on day 16 of culture of seminiferous tubules from 23-day-old rats. This reinforces the view that the development of the meiotic step in testes of pubertal rats is rather similar *in vivo* and *ex vivo* (Perrard *et al.*, 2003).

Another interesting point is that the cultures of seminiferous tubules revealed two short and specific transitional stages that are rarely observed *in vivo*: (1) several bouquet form zygotenes (Fig. 4a) observed on day 7 and (2) an early diplotene stage displaying partial separation of homologous chromosomes and long regions of bivalents still paired (Fig. 4b). Thus, not only do the conditions of seminiferous tubule cultures not modify the chronology of meiosis, the development of spermatocytes I *ex vivo* accurately reproducing their development *in vivo* but also the culture reveals stages too transitory to be easily seen *in vivo*.

Qualitative analysis of meiosis has shown that the same SC abnormalities are observed in rat and human meiosis (Geoffroy-Siraudin *et al.*, 2007; Tassistro *et al.*, 2009): asynapsis and fragmentation of SC and dotted SC. The percentage of asynapsed nuclei was the same on the first days of culture and during *in vivo* spermatogenesis for the three ages of rats studied. Then, from day 9 of the culture, this percentage increased significantly. This may be because of the *ex vivo* conditions, which may differ somewhat from the physiological conditions. It has been shown that a high number of asynapsed nuclei may result from a defective testicular environment (Guichaoua *et al.*, 2005; Tassistro *et al.*, 2009). The frequency of fragmented nuclei on the very first days of culture was significantly less than *in vivo* (Tables 3 and 5). This could be related to the decrease in the number of cells between days 2 and 5 (Hue *et al.*, 1998). Indeed, it could be hypothesized that spermatocytes with fragmented nuclei are rapidly lost during the first days of culture, as Sertoli cells are highly phagocytic (Byers *et al.*, 1993; Shiratsuchi *et al.*, 1999). The proportion of fragmented nuclei increased significantly with time of culture to become significantly higher than for *in vivo* spermatogenesis, reflecting likely some alterations of the spermatogenic process and/or Sertoli cells at the end of the culture. Dotted nuclei were the less frequent abnormality in rats, both *in vivo* and *ex vivo*, and there was no significant increase at the end of the culture. It stands to reason that culture of seminiferous tubules does not play a role in this SC defect interpreted as defective condensation of bivalents during the first stages of meiotic prophase. All these results should be taken into account for the use of this model in toxicological studies. Nevertheless, in toxicological studies, the results obtained with a toxicant are to be compared with those of the corresponding control cells.

Usefulness of This System of Culture for Toxicological Studies

Previous studies have shown that chromium exerts deleterious effect on spermatogenesis. Indeed, in the rat and in human, Cr(VI) reduces sperm counts and increases sperm abnormalities (Li *et al.*, 2001). Disruption in germ cell arrangement and spermatogenesis was described in the rat by Li *et al.* (2001) and in a nonhuman primate (*Macaca radiata* Geoffroy) by Aruldhas *et al.* (2005). Pereira *et al.* (2004) have demonstrated that Sertoli cells are affected by Cr(V) and these cells showing a large number of vesicles and vacuoles and that Cr(V) leads to the disruption of the blood-testis barrier. A significant reduction in semen quality is also observed in male welders occupationally exposed to chromium: decrease in sperm count, mobility and vitality, large number of morphologically abnormal spermatozoa, and these semen changes are dose dependent (Danadevi *et al.*, 2003).

Our study confirms and extends, by a new approach, these previous studies. Indeed, our system of culture allowed a better understanding of the mechanisms by which chromium impairs spermatogenesis, thanks to a precise quantitative and qualitative analysis of the first meiotic prophase. The presence of Cr(VI) in the cells exposed to the three concentrations indicates that this metal was able to run through the membrane partitioning the culture device and through the *ex vivo*–maintained blood-testis barrier (Perrard *et al.*, 2009; Staub *et al.*, 2000) because the metal was added only in the basal compartment of the bicameral chamber. As expected, the cellular chromium concentration increased with those of Cr(VI) in the culture medium.

Flow cytometric analysis revealed that the number of Sertoli cells and young spermatocytes appeared not greatly, if any, affected by either concentration of Cr(VI) (Figs. 6a and 6b), whereas the numbers of more differentiated cells, from middle spermatocytes to round spermatids were somewhat decreased (Figs. 6c–e). These results confirm the observations of Aruldhas *et al.* (2005) in electron microscopy, which also revealed that spermatogonia and preleptotene spermatocytes exhibited little pathological changes in response to Cr(VI), whereas pachytene spermatocytes had fragmented and discontinuous chromatin. In our study, immunostaining of SC with an anti-SCP3 antibody revealed that the chronology of the prophase I stages was not affected by Cr(VI) whatever its concentration but that there was a significant increase of total

abnormalities of the SC, in parallel with the concentrations of chromium because of increased numbers of asynapsis and fragmented SC. Thus, SC abnormalities visible at the pachytene stage demonstrate that alteration of the spermatogenic process was already present in pachytene exposed to Cr(VI). SC analysis is a highly sensitive indicator of potentially heritable effects of the genotoxic agents (Backer *et al*., 1988). In previous studies, all tested agents caused dose-dependent SC damages: breakage, asynapsis, and synapsis irregularities, which varies among the chemical (Allen *et al*., 1988a,b; Backer *et al*., 1988). Thus, this study shows that Cr(VI) is toxic for the meiotic cells even at low concentrations.

It appears therefore that quantitative studies are not sufficient to estimate the effects of toxicants on the germ cells and that a fine qualitative analysis is essential to approach the mechanisms by which toxicants may act on spermatogenesis. The arsenal of specific antibodies that permits detection of individual proteins of the meiotic prophase may be applied to this model, which also lends itself to other tests of mutagenesis and toxicity (Perrin *et al*., 2007) for a better understanding of the impacts of our environment on fertility.

FUNDING

CNRS, INRA, the University Claude Bernard Lyon I, the Ecole Normale supérieure de Lyon, and the University of Méditerranée.

ACKNOWLEDGMENTS

The authors thank Dr O. Sabido, Centre Commun de Cytométrie en Flux, Faculté de Médecine, Université Jean Monnet St-Etienne France, for help with cytometry analysis.

REFERENCES

Allen, J. W., Gibson, J. B., Poorman, P. A., Backer, L. C., and Moses, M. J. (1988a). Synaptonemal complex damage induced by clastogenic and antimitotic chemicals implications for non-disjunction and aneuploidy. *Mutat. Res.* **201,** 313–324.

Allen, J. W., Poorman, P. A., Backer, L. C., Gibson, J. B., Westbrook-Collins, B., and Moses, M. J. (1988b). Synaptonemal complex damage as a measure of genotoxicity at meiosis. *Cell Biol. Toxicol.* **4,** 487–494.

Aruldhas, M. M., Subramanian, S., Vengatesh, G., Chandrahasan, G., Govindarajulu, P., and Akbarsha, M. A. (2005). Chronic chromium exposure induced changes in testicular histoarchitecture are associated with oxidative stress: study in a non-human primate (Macaca radiata Geoffroy). *Hum. Reprod.* **20,** 2801–2813.

Backer, L. C., Gibson, J. B., Allen, J. W., and Moses, M. J. (1988). Synaptonemal complex damage in relation to meiotic chromosome aberrations after exposure of male mice to cyclophosphamide. *Mutat. Res.* **203,** 317–330.

Barceloux, D. G. (1999). Chromium. *J. Toxicol. Clin. Toxicol.* **37,** 173–194.

Byers, S., Jegou, B., MacCalman, C., and Blaschuk, O. (1993). Sertoli cell adhesion molecules and the collective organization of the testis. In *Sertoli Cell* (L. D. Russell and M. D. Griswold, Eds.), pp. 461–476. Cache River Press, Clearwater, FL.

Carlsen, E., Giwercman, A., Keiding, N., and Skakkebaek, N. E. (1992). Evidence for decreasing quality of semen during past 50 years. *BMJ* **12,** 609–613.

Chardin, B., Dolla, A., Chaspoul, F., Fardeau, M. L., Gallice, P., and Bruschi, M. (2002). Bioremediation of chromate: thermodynamic analysis of the effects of Cr(VI) on sulphate reducing bacteria. *Appl. Microbiol. Biotechnol.* **60,** 352–360.

Clermont, Y. (1972). Kinetics of spermatogenesis in mammals: seminiferous epithelium cycle and spermatogonial renewal. *Physiol. Rev.* **52,** 198–236.

Clermont, Y., and Perey, B. (1957). Quantitative study of the cell population of the seminiferous tubules in immature rats. *Am. J. Anat.* **100,** 241–267.

Danadevi, K., Rozati, R., Reddy, P. P., and Grover, P. (2003). Semen quality of Indian welder occupationally exposed to nickel and chromium. *Reprod. Toxicol.* **17,** 451–456.

Fouchécourt, S., Godet, M., Sabido, O., and Durand, P. (2006). Glial cell-line-derived neurotropic factor and its receptors are expressed by germinal and somatic cells of the rat testis. *J. Endocrinol.* **190,** 59–71.

Geoffroy-Siraudin, C., Aknin-Seiffer, I., Metzler-Guillemain, C., Ghalamoun-Slaimi, R., Bonzi, M. F., Levy, R., and Guichaoua, M. R. (2007). Meiotic abnormalities in patients bearing complete AZFc deletion of Y chromosome. *Hum. Reprod.* **22,** 1567–1572.

Godet, M., Damestoy, A., Mouradian, S., Rudkin, B., and Durand, P. (2004). Key role for cyclin dependant kinases in the first and the second meiotic divisions of rat spermatocytes. *Biol. Reprod.* **70,** 1147–1152.

Godet, M., Sabido, O., Gilleron, J., and Durand, P. (2008). Meiotic progression of rat spermatocytes requires mitogen-activated protein kinases of Sertoli cells and close contacts between the germ cells and the Sertoli cells. *Dev. Biol.* **315,** 173–188.

Godet, M., Thomas, A., Rudkin, B., and Durand, P. (2000). Developmental changes in cyclin B1 and cyclin-dependant kinase 1 (CDK) levels in the different populations of spermatogenic cells of the post-natal rat testis. *Eur. J. Cell Biol.* **79,** 816–823.

Guichaoua, M. R., Perrin, J., Metzler-Guillemain, C., Sais-Magnan, J., Giorgi, R., and Grillo, J. M. (2005). Meiotic anomalies in infertile men with severe spermatogenic defects. *Hum. Reprod.* **20,** 1897–1902.

Hartung, T., and Rovida, C. (2009). Chemical regulators have overreached. *Nature* **460,** 1080–1081.

Hue, D., Staub, C., Perrard-Sapori, M. H., Weiss, M., Nicolle, J. C., Vigier, M., and Durand, P. (1998). Meiotic differentiation of germinal cells in three-week cultures of whole cell population from rat seminiferous tubules. *Biol. Reprod.* **59,** 379–387.

Irvine, S., Cawood, E., Richardson, D., Macdonald, E., and Aitken, J. (1996). Evidence of deteriorating semen quality in the United Kingdom: birth cohort study in 577 men in Scotland over 11 years. *BMJ* **312,** 467–471.

Jensen, T. K., Sobotka, T., Hansen, M., Tonnes Pedersen, A., Lutz, W., and Skakkebaek, N. (2008). Declining trends in conception rates in recent birth cohorts of native Danish women: a possible role of deteriorating male reproductive health. *Int. J. Androl.* **31,** 81–92.

Kawanishi, S., Inoue, S., and Sano, S. (1986). Mechanism of DNA cleavage induced by sodium chromate in the presence of hydrogen peroxide. *J. Biol. Chem.* **261,** 5952–5958.

Lackner, J., Schatzl, G., Waldhor, T., Resh, K., Kratzik, C., and Marberger, M. (2005). Constant decline in sperm concentration in infertile males in a urban population: experience over 18 years. *Fertil. Steril.* **84,** 1657–1661.

Li, H., Chen, Q., Li, S., Yao, W., Li, L., Shi, X., Wang, L., Castranova, V., Vallyathan, V., Ernst, E., *et al*. (2001). Effect of Cr(VI) exposure on sperm quality: human and animal studies. *Ann. Occup. Hyg.* **45,** 505–511.

Martin, R. (2006). Meiotic chromosome abnormalities in human spermatogenesis. *Reprod. Toxicol.* **22,** 142–147.

Metzler-Guillemain, C., and Guichaoua, M. R. (2000). A simple and reliable method for meiotic studies on testicular samples used for intracytoplasmic sperm injection. *Fertil. Steril.* **74,** 916–919.

Morris, B. W., Griffiths, H., and Kemp, G. J. (1988). Effect of glucose loading on concentrations of chromium in plasma and urine of healthy adults. *Clin. Chem.* **34,** 1114–1116.

Pedersen, F., de Bruijn, J., Munn, S., and Van Leeuwen, K. (2003). Assessment of additional testing needs under REACH_effects of (Q)SARs, risk based testing and voluntary industry initiatives. *JRC Report EUR* **20863,** 33.

Pereira, M. L., Santos, T. M., and Garcia e Costa, F. (2004). Pedrosa de Jesus, J. (2004). Functional changes of mice Sertoli cells induced by Cr(V). *Cell Biol. Toxicol.* **20,** 285–291.

Perrard, M. H., Hue, D., Staub, C., Le Vern, Y., Kerboeuf, D., and Durand, P. (2003). Development of the meiotic step in testes of pubertal rats: comparison between the in vivo situation and under ex vivo conditions. *Mol. Reprod. Dev.* **65,** 86–95.

Perrard, M. H., Prisant, N., Geoffroy-Siraudin, C., Segretain, D., Pointis, G., Guichaoua, M. R., and Durand, P. (2009). Analysis on the intra-testicular control of spermatogenesis by ex-vivo approaching. *Folia. Histochem. Cytobiol* **47,** S89–S94.

Perrin, J., Lussato, D., De Meo, M., Durand, P., Grillo, J. M., Guichaoua, M. R., Botta, A., and Bergé-Lefranc, J. L. (2007). Evolution of DNA strand-breaks in cultured spermatocytes: the comet assay reveals differences in normal and irradiated germ-cells. *Toxicol Ex Vivo* **21,** 81–89.

Scherthan, H., and Schönborn, I. (2001). Asynchronous chromosome pairing in male meiosis of the rat (rattus norvegicus). *Chromosome Res.* **9,** 273–282.

Shiratsuchi, A., Kawasaki, Y., Ikemoto, M., Arai, H., and Nakanishi, Y. (1999). Role of class B scavenger receptor type I in phagocytosis of apoptotic spermatogenic cells by Sertoli cells. *J. Biol. Chem.* **26,** 5901–5908.

Staub, C., Hue, D., Nicolle, C., Perrard-Sapori, M. H., Segretain, D., and Durand, P. (2000). The whole meiotic process can occur ex vivo in untransformed rat spermatogenic cells. *Exp. Cell Res.* **260,** 85–95.

Tassistro, V., Ghalamoun-Slaimi, R., Sais-Magnan, J., and Guichaoua, M. R. (2009). Chronology of meiosis and synaptonemal complex abnormalities in normal and abnormal spermatogenesis, a new index in the evaluation of spermatogenesis quality. *Indian J. Med. Res.* **2009,** 268–278.

Von Burg, R., and Liu, D. (1993). Chromium and hexavalent chromium. *J. Appl. Toxicol.* **13,** 225–230.

Yang, Z. W., Wreford, N. G., and de Kretser, D. M. (1990). A quantitative study of spermatogenesis in the developing rat testis. *Biol. Reprod.* **43,** 629–635.

Zickler, D., and Kleckner, N. (1998). The leptotene-zygotene transition of meiosis. *Annu. Rev. Genet.* **32,** 619–697.