# Alterations of Sertoli cell activity in the long-term testicular germ cell death process induced by fetal androgen disruption

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## Abstract

Fetal androgen disruption, induced by the administration of anti-androgen flutamide (0·4, 2, and 10 mg/kg day) causes a long-term apoptosis in testicular germ cells in adult male rat offspring. One of the questions raised by this observation is the role of the Sertoli cells in the adult germ cell apoptotic process. It is shown here that Sertoli cells originating from 15-day-old rats treated *in utero* with the anti-androgen (10 mg/kg d) did no longer protect adult germ cells against apoptosis. Indeed, untreated spermatocytes or spermatids exhibited increased ($P<0·0001$) active caspase-3 levels when co-cultured with Sertoli cells isolated from rat testes exposed *in utero* to the anti-androgen. This alteration of Sertoli cell functions was not due to modifications in the androgen signal in the adult (90-day-old) animals, since plasma testosterone and estradiol, androgen receptor expression, and androgen-targeted cell number (e.g., Sertoli cells in the seminiferous tubules) were not affected by the fetal androgen disruption. In contrast, this inability of Sertoli cells to protect germ cells against apoptosis could be accounted for by the potential failure of Sertoli cell functions. Indeed, adult testes exposed *in utero* to anti-androgens displayed decreased levels of several genes mainly expressed in adult Sertoli cells (anti-Mullerian hormone receptor type II (AMHR2), Cox-1, cyclin D2, cathepsin L, and GSTα). In conclusion, fetal androgen disruption may induce alterations of Sertoli cell activity probably related to Sertoli cell maturation, which potentially leads to increased adult germ cell apoptosis.
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## Introduction

It has been reported that the fetal hormonal disruption induced either by chemical anti-androgens such as flutamide or by different environmental anti-androgen compounds (e.g., vinclozolin, phthalates) gives rise to a wide range of reproductive system abnormalities such as hypospadias, undescended testes, developmental testicular alterations (Fisher 2004, Crews & McLachlan 2006, Gray *et al.* 2006, Newbold *et al.* 2006), and particularly to a long-term apoptosis of germ cells in the adult testis (Omezzine *et al.* 2003, Bozec *et al.* 2004, Uzumcu *et al.* 2004, Florin *et al.* 2005) transmitted by male through at least four generations (Anway *et al.* 2005). These observations support the concept of a fetal and transgenerational programming of adult testicular germ cell apoptosis. Although it was shown that this apoptotic process was related to a long-term increase in the expression and activation of effector caspases-3 and -6 (Omezzine *et al.* 2003), resulting from changes in the ratio of Bcl-2 family peptides in favor of the pro-apoptotic members (Bozec *et al.* 2004), the upstream cellular and molecular mechanisms still remain to be investigated. Indeed, one of the major questions raised by these observations is related to the potential role of the Sertoli cells in the apoptotic process that affects germ cells in the adult rat testes exposed *in utero* to anti-androgens. Although apoptosis occurred in germ cells, in the seminiferous tubules, it is Sertoli cells (and peritubular myoid cells) that are the direct target cells of testosterone action as they express androgen receptor (AR; You & Sar 1998). These observations would suggest that Sertoli cells (in cooperation with peritubular myoid cells) might primarily exhibit altered functions that result in increased germ cell death process.

The aim of the present study was to determine the potential role of Sertoli cells in the adult germ cell death through the evaluation of plasma hormones that target Sertoli cells, AR expression, Sertoli cell number, and functions via the evaluation of the levels of specific transcript expressed in these adult somatic cells.


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## Materials and Methods

### *Ethics*

All studies on animals were conducted in accordance with the current regulation and standards approved by the INSERM (French Institute for Health and Medical Research) Animal Care Committee.

### *Materials*

Flutamide obtained from Sigma was dissolved in an aqueous solution of 0·5% w/v methylcellulose 400 (Fluka, Mulhouse, France) and stored for a maximum of 1 week at ~5 °C (±3 °C). Dulbecco's Modified Eagle (DME)/Ham's F12 (1:1) medium, reverse transcriptase (Moloney-murine leukemia virus (M-MLV)), polyacrylamide 37·5:1 solution, Ponceau Red, and TRIzol were obtained from Life Technologies. Collagenase/dispase and protease cocktail inhibitor were obtained from Roche. Gentamicin, 3-cyclohexylamino-1-propanesulfonic acid (CAPS), Kodak XOMAT films, antibody raised against actin, diaminobenzidine (DAB), sodium bicarbonate, HEPES, DNase I, 3-N-morpholinopropane-sulfonic acid (MOPS), SDS, random primers, and BSA were purchased from Sigma. Rabbit polyclonal anti-human Glutathione-S-transferase (GST)α antibody (Mehta *et al.* 1994, Murray *et al.* 1995) was purchased from Novocastra-Tebu (Le Perray en Yvelines, France). Peroxidase-conjugated goat anti-rabbit immunoglobulin G and Covalight chemiluminescence detection kit were obtained from CovalAb (Lyon, France). AR rabbit polyclonal antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody diluent, Harris hematoxylin, and Envision$^{+}$ kit were obtained from Dako (Copenhagen, Denmark). Bradford reagents and Pierce detection kit were purchased from Bio-Rad Laboratories. Hybond-C membranes [$\alpha$-$^{33}$P] dATP and [$\alpha$-$^{32}$P] dCTP were purchased from Amersham Biosciences. Proligo SA (Paris, France) was the source for oligonucleotide primers. Random priming DNA labeling kit and *Taq* polymerase were purchased from Promega Life Science, and Schleicher & Schuell polyvinyl difluoride (PVDF) membrane from Merck Eurolab (Strasbourg, France).

### *Methods*

**Animals: *in utero* exposure to anti-androgens** Fifteen pregnant rats (Sprague–Dawley rats from Charles River Laboratories, St Aubin les Elbeuf, France) per group were administered vehicle (methylcellulose) or flutamide by daily gavage from day 10 of gestation (GD 10) up to the day before delivery (GD 21 or 22). This period includes the period of male internal and external sex differentiation. The experimental protocol used was based on the previous data reported by McIntyre *et al.* (2001). However, lower doses of the anti-androgen were chosen to avoid or minimize important germ cell loss, i.e., before the occurrence of major histological lesions. Indeed, alterations in testicular cellularity (i.e., the ratio of somatic cells to germ cells) may confound the interpretation of the effects of the anti-androgen on gene expression in the different testicular cell types (for reference see Ivell & Spiess 2002). Consistently, at the doses used here, there were no changes in adult testicular weight and epididymis sperm counts (untreated: 472·4±186·9 vs 10 mg/kg d flutamide: 352·9±263·4; $P>0·05$). At the maximal dose (10 mg/kg per day) used for flutamide, anti-androgenic effects of the compound were observed including i) increased cryptorchid testes rate (Bozec *et al.* 2004) ii) reduced ventral prostate weight (dose 0: 0·683±0·119 versus dose 10: 0·07±0·053; $P<0·0001$) and iii) increased malformations of *vas deferens* (dose 0: 0% versus dose 10: 18·75%; $P<0·05$). Animals were given flutamide at doses of 0, 0·4, 2, and 10 mg/kg body weight per day (mg/kg d) adjusted daily based on body weight. Dams were weighed daily from GD 10 up to the day of delivery. After birth, male pups were grown without treatment until they were killed at 15 or 90 days by $CO_2$ inhalation. Each testis was weighted before being fixed, used for Sertoli cell isolation, or frozen. The position of the testes was carefully observed to detect cryptorchidism. Cryptorchid testes were most often located ectopically in the peritoneal cavity or inguinal area. Fully descended testes are the ones positioned in the scrotum. In the present study, we have used only descended testes. At least seven rats from three different litters were used per condition. The experiment was repeated at least three times.

**Cell isolation and culture** Seminiferous tubules were isolated from testes of adult (90-day-old) rats exposed *in utero* to methylcellulose (control) or flutamide (0, 0·4, 2, and 10 mg/kg d). Once the albuginea was removed, the testicular tissues were mechanically dispersed with forceps in DME/F12 medium (supplemented with 1·2 mg/ml sodium bicarbonate, 15 mM HEPES, and 20 μg/ml gentamicin) containing DNase I (0·05 mg/ml). Testicular tissues were then dispersed by collagenase/dispase treatment (0·5 mg/ml) in DME/F12 medium through mild stirring. After enzymatic dissociation, testicular cells were washed three times by gravity sedimentation (3–5 min). The seminiferous tubules were washed at least three times to remove potential contaminating Leydig cells. Seminiferous tubules were dry frozen and kept at −70 °C until use for GSTα expression.

In the co-culture Sertoli cell–germ cell model, Sertoli cells were isolated, by collagenase dissociation, from pre-pubertal (15-day-old) testes of rats exposed *in utero* to methylcellulose (control) or the anti-androgen flutamide (10 mg/kg d). Mature germ cells (spermatocytes and spermatids) were isolated and purified from adult untreated rat testes by the centrifugation–elutriation method as reported previously (Boussouar *et al.* 2003). The Sertoli cells were seeded onto Petri dishes (Falcon, Los Angeles, CA, USA) 6 cm in diameter, at a density of 500 000 cells/cm$^2$ and cultured in standard conditions (Florin *et al.* 2005). Two days after Sertoli cell isolation and culture, germ cells were added to Petri dishes generating three groups of cultured cells: Sertoli cells cultured (alone) without germ cells, Sertoli cells co-cultured with spermatocytes (500 000 cells/cm$^2$), and Sertoli cells co-cultured with spermatids (500 000 cells/cm$^2$). After 72 h of co-culture, the cells were either collected in an ice-cold lysis buffer (25 mM Tris, 0·1% SDS, 1% vol/vol protease inhibitor cocktail) for western

blotting analysis or in 1 ml TRIzol for RT-PCR analysis. The purity of the isolated pre-pubertal Sertoli cell was checked by western blotting (tubulin-β3 and GATA-6). The resulting Sertoli cells were free of Leydig (assessed by 3-β-hydroxysteroid dehydrogenase) and germ cells (assessed by c-kit for spermatogonia, poly (ADP-ribose) polymerase (PARP)-1 for spermatocytes, protamine-1 for round spermatids, and MCT2 for elongated spermatids) and contained between 2% and 5% peritubular myoid cells, as evaluated by fibronectin and alkaline phosphatase immunostaining (data not shown). The purity of spermatocytes and round spermatids was assessed by the presence of PARP-1 and protamine-1 respectively. The absence of somatic cells was assessed by the markers mentioned above.

**Cell type counting** For Sertoli and Leydig cell counting, ten 90-day-old rats (originating from three different litters) treated *in utero* with flutamide (0 or 10 mg/kg d) were used for each experimental condition. After the animals were killed by $CO_2$ inhalation, the testes were rapidly dissected in order to remove the connective tissue and the epididymis and weighed. The left testis of each animal was fixed overnight in Bouin's solution by immersion. These were weighed and then sampled in a random systematic manner. The testes were sliced transversally into six pieces and the slices 1, 3, and 5 or 2, 4, and 6 were randomly processed in graded ethanol and infiltrated with JB4 resin (TAAB, Berkshire, UK). After full polymerization, 25 μm sections were cut on an Ultracut microtome (Reichert-Jung Inc., Wien, Austria) using glass knives, mounted onto adhesive glass slides, and stained with Harris hematoxylin. Sertoli and Leydig cells were then counted using the optical dissector method as described by Wreford (1995) and Sharpe *et al.* (1998), (2000). The thick sections were viewed at high magnification and optically sectioned with a microscope equipped with a microcator to measure stage movement in the *z*-axis. The top of the section was focused and a small guard area of 3 μm was traversed, the microcator was zeroed, and the section was then viewed at a series of 1·5 μm intervals for a further 15 μm. The images of each plane were acquired using a video camera coupled with a Leitz Quantimet 570 image analyzer system (Milton Keynes, UK). Sertoli and Leydig cell nuclei were then counted in an unbiased counting frame of 6400 $\mu m^2$ area giving a 96 000 $\mu m^3$ volume for each field. More than 100 Sertoli or Leydig cells were counted for each animal representing at least 1000 counted nuclei for each experimental condition. The counting results gave a direct estimation of the number of Sertoli or Leydig cells per testis.

**mRNA quantification** Co-amplification RT-PCR with an endogenous control: total RNA (2 μg), from whole testis, was reverse transcribed into cDNA. PCR was then performed on 1 μl reverse transcription product as described previously (Bozec *et al.* 2004). PCR mixtures were submitted to an initial denaturing step at 95 °C, followed by *n* cycles consisting of 30 s at 95 °C, 30 s at hybridization temperature, and 30 s at 72 °C, and the reaction ended with a final extension step of 5 min at 72 °C (Table 1). PCR products were subsequently resolved on 8% polyacrylamide gel, which were then exposed to a Storage Phosphor Screen and the signals analyzed using the Cyclone OptiQuant Software (Packard, Meriden, CT, USA). Results from at least three separate experiments were used for statistical analysis. PCR analyses were carried out from the logarithmic phase of amplification. PCRs with different cycle numbers were realized for each primer pair to determine the minimum number of cycles necessary to detect the PCR product. Primers were designed inside separate exons to avoid any bias caused by residual genomic contamination. Moreover, for all primers, no

**Table 1** Primer list and PCR conditions

| Primer name | Primer sequence | Hybridization temperature (°C) | Amplicon size (bp) |
|---|---|---|---|
| Androgen receptor | ATTGTCCATCGTGTCGTCTCCG<br>GAGTTGACATTAGTGAAGGACC | 60 | 447 |
| HPRT | CCTGCTGGATTACATTAAAGC<br>GTCAAGGGCATATCCAACAAC | | 354 |
| Cox-1 | CGAAGCAAGCTATGATGGCGAATA<br>ATGTAGACACCCGAGCCTACTTTA | 62 | 288 |
| Cystatin TE | ACCCTCTAGAGGAAAGG<br>GAGATCGACAAGAATGAAGAGGAG | 62 | 370 |
| Cathepsin L | TTTACAAGATCCATCCTTTGCTTC<br>CCCCAAGACTGTGGACTGGAGAGA | 62 | 295 |
| AMHR2 | AGCAATGGCTGTAGAGGCAG<br>GGGTAGCTGTGCTGGTGTGT | 62 | 228 |
| Cystatin SC | GTGGCATCAGTAGAGTTTGCTGTGG<br>GCATTAGCACCACACAGACCTTGG | 58 | 378 |
| β-Actin | TTGCTGATCCACATCTGCTG<br>GACAGGATGCAGAAGGAGAT | | 146 |
| GSTα2 | GGACGTCCACCTGCTGGAAC<br>TGGCTCCATCAATGCAGCCA | 60 | 224 |
| Cyclin D2 | CGATGATTGCAACTGGAAGC<br>TTCAGCAGCAGAGCTTCGAT | 58 | 169 |

amplification was observed when PCR was performed on RNA preparations.

**Northern blotting** The GSTα probe was prepared by PCR and labeled as described previously (Benbrahim-Tallaa *et al.* 2002*a*). Total RNAs from adult rat seminiferous tubules were size fractionated by electrophoresis on 1·2% agarose and 2·2 M formaldehyde gels. The northern blotting analysis was performed as described previously (Benbrahim-Tallaa *et al.* 2002*a*). The intensities of the autoradiographic bands were estimated by densitometric scanning using Intelligent Quantifier Software (Bio-Image, Cheshire, UK). The data are expressed as the ratio of GSTα to 18S mRNA.

**Western blotting analysis** Total proteins were extracted from rat whole testes, seminiferous tubules, or cultured testicular cells treated under different conditions. Protein extracts (80 μg/well for GSTα, 100 μg/well for AR) were size fractionated on SDS-polyacrylamide gel (Benbrahim-Tallaa *et al.* 2002*b*). The membranes were incubated with the rabbit polyclonal anti-GSTα antibody diluted 1:1000 or the rabbit polyclonal anti-AR antibody diluted 1:100 in a solution of tris buffered saline (TBS) with 0·5% nonfat dry milk for 2 h at room temperature. After washing with TBS, the membranes were then incubated with the goat anti-rabbit peroxidase-conjugated antibody diluted at 1:2000 in TBS buffer with 0·5% nonfat dry milk. The antibody–antigen complexes were detected by chemiluminescence using CovalAb detection kit. The protein loading was checked by reprobing the blot with a rabbit IgG anti-actin antibody (1:500).

### *Data analysis*

Data are expressed as the mean ± S.D. Three to seven animals from different litters were used. For statistical analysis of data generated in *in vivo* and *in vitro* models, one-way ANOVA was performed to determine whether there were differences between all groups ($P<0·05$), and then the Bonferroni post-test was performed to determine the significance of the differences between the pair of groups. $P<0·05$ was considered significant. The statistical tests were performed on StatView software version 5.0 (SAS institute Inc., Cary, NC, USA).

## Results

### *Effects of* in utero *exposure to flutamide on Sertoli cell activity in adult germ cell survival*

As fetal androgen disruption induced a long-term apoptosis in adult germ cell characterized by caspase-3 activation in spermatocytes and spermatids (Omezzine *et al.* 2003) and Sertoli cells are targeted by androgens in the adult seminiferous tubules, we addressed the question as to whether the increased adult germ cell death might be related to altered Sertoli cell activity. For this purpose, purified post-natal (PND 15) Sertoli cells from rat testes exposed *in utero* to 10 mg/kg d of flutamide were co-cultured with adult purified germ cells (i.e., spermatocytes or spermatids) not treated with flutamide. While active caspase-3 levels were not affected in Sertoli cells isolated from testes treated *in utero* with flutamide and cultured alone compared with Sertoli cells originating from sham-treated (methylcellulose) testes (Fig. 1A), increased active caspase-3 levels were detected in spermatocytes (Fig. 1B; $P<0·0001$) or spermatids (Fig. 1C; $P=0·037$) when co-cultured with Sertoli cells treated *in utero* with the anti-hormone (10 mg/kg per day). Together, these observations would suggest a possible alteration in the functions of (flutamide-treated) Sertoli cells related to their role in promoting adult germ cell survival.

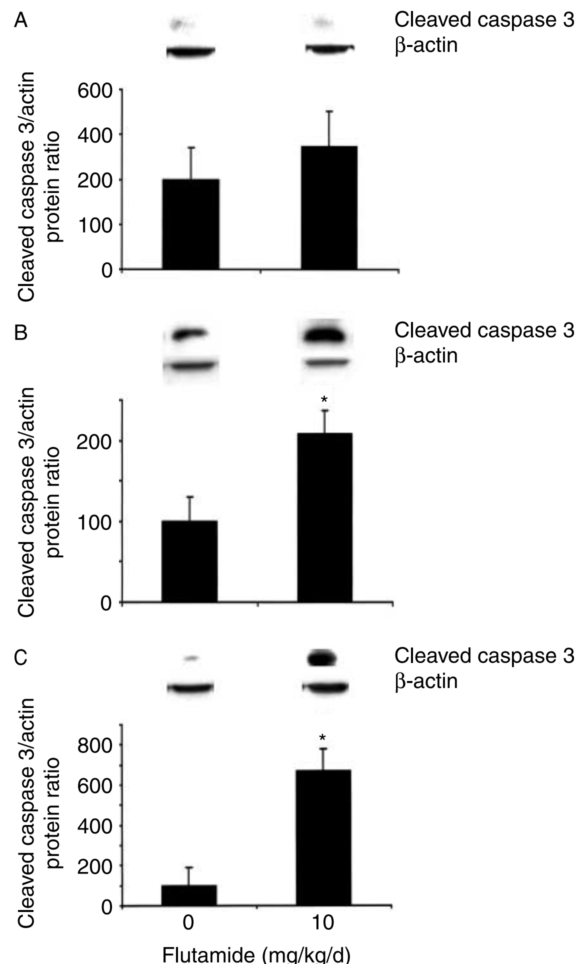


**Figure 1** Effects of *in utero* treatment with flutamide on cleaved caspase-3 protein levels in Sertoli cell–germ cell co-cultures. Cleaved caspase-3 protein levels were determined in Sertoli cell isolated from (15-day-old) rat untreated (methylcellulose) (0) or treated (10) *in utero* with flutamide (10 mg/kg day) and cultured either alone (A) or in the presence of spermatocytes $*P<0·0001$ (B) or spermatids $*P<0·037$ (C) purified from adult (90-day-old) untreated rats. Representative autoradiograms and histograms were shown in the upper and lower panel respectively. The experiment was carried out at least three times.

*Effects of fetal androgen disruption on androgen signal in the adult animals*

As the germ cell survival has been linked to androgen action, which is mainly exerted through Sertoli cells (De Gendt *et al.* 2004, Tan *et al.* 2005), we addressed the question as to whether the long-term apoptosis observed in germ cells might be related to alterations in the androgen signal in the adult animals. The androgen signal could be evaluated, at least, through three components including the plasma levels of testosterone, its metabolite estradiol, and the number of Sertoli cells that are targeted by androgens in the adult seminiferous tubules and the AR expression. First, fetal androgen disruption following an *in utero* treatment with flutamide (0·4, 2, and 10 mg/kg per day) was shown to have no effect on the plasma levels of hormones including testosterone, estradiol, gonadotropins, follicle-stimulating hormone (FSH), and luteinizing hormone (LH) in adult animals (Table 2). Secondly, with regard to the number of somatic cells targeted by androgens, no change in Leydig cells (Fig. 2A) and Sertoli cell number (Fig. 2B) was observed in adult rat testes treated *in utero* with flutamide. It is noteworthy that the testicular weight is not affected by the anti-androgen treatment at the doses used here (Fig. 2D; see also Bozec *et al.* 2004). Thirdly, in terms of AR expression, immunohistochemical experiments revealed that AR nuclear staining occurred, as expected, in Sertoli, peritubular myoid, and Leydig cells from adult rat testes (Fig. 3A). Fetal treatment by the anti-hormone flutamide (10 mg/kg d) did not affect AR immunolocalization in the adult rat testes (Fig. 3B). Interestingly, the AR expression remained unaffected in the adult testis fetally treated by the anti-androgen, as evaluated through mRNA (Fig. 3C) and protein levels (Fig. 3D). Altogether, the data obtained suggest that the altered Sertoli cell activity leading to increased adult germ cell apoptotic process (Fig. 1) appears not to be linked to the androgen signal when evaluated through plasma hormone levels, Sertoli cell number, and AR expression.

*Effects of* in utero *exposure to flutamide on Sertoli cell gene expression*

As the germ cell death process specifically affects adult germ cells (spermatocytes and spermatids), we hypothesized that the potential alterations in Sertoli cell functions in terms of protecting adult germ cells against death might be related to a possible lack of Sertoli cell maturation. To test such a hypothesis, we evaluated the levels of several transcripts reported to be specifically present in adult Sertoli cells (O'Shaughnessy *et al.* 2003): AMH type II receptor, Cox-1, cathepsin L, cystatin SC and cystatin TE, cyclin D2, and GST$\alpha$. *In utero* flutamide treatment (10 mg/kg d) resulted in a decrease in AMH type II receptor (Fig. 4A; $P=0{\cdot}0021$), cathepsin L (Fig. 4B; $P=0{\cdot}0029$), Cox-1 (Fig. 4C; $P=0{\cdot}0017$), cyclin D2 (Fig. 4D; $P=0{\cdot}0055$) mRNA levels. In contrast, cystatin TE (Fig. 4E) and cystatin SC (Fig. 4F) mRNA levels were not affected.

In addition to these different genes expressed in somatic testicular cells, we have further focused on GST$\alpha$ expression. This gene is expressed in both Leydig and Sertoli cells (Benbrahim-Tallaa *et al.* 2002*b*) and was reported to be under the control of androgens in Sertoli cells (Benbrahim-Tallaa *et al.* 2002*b*). *In utero* flutamide treatment resulted in a decrease in GST$\alpha$ mRNA levels in seminiferous tubules with a significant decrease observed at 2 mg/kg d ($P=0{\cdot}01$) and 10 mg/kg d ($P=0{\cdot}001$) flutamide (Fig. 5A). The decrease in GST$\alpha$ mRNA levels was associated with a parallel decrease in GST$\alpha$ protein levels in the seminiferous tubules (Fig. 5B). Such a decrease in GST$\alpha$ protein levels was further confirmed in cultured Sertoli cells isolated from rats exposed *in utero* to flutamide (Fig. 5C; $P=0{\cdot}0006$).

## Discussion

The experimental model used here is based on the previous data from McIntyre *et al.* (2001), in terms of both the window of exposure (fetal period) and the doses used. These authors have shown a significant decrease in adult testes weight from 12·5 mg/kg per day of flutamide. In our present study, we used relatively lower doses (0·4, 2, and 10 mg/kg per day) in order to avoid, in the adult testis, massive germ cell loss, as our objectives are mainly related to the identification of the upstream genes/mechanisms involved in the increased germ cell death, i.e., the main phenotype we observed in the adult testes. Especially, we addressed here the question as to whether Sertoli cell number/activity could be involved in this cellular germ cell apoptosis. At the maximal dose of flutamide used (10 mg/kg day), we observed an androgen fetal disruption shown by increased cryptorchid testes rate

**Table 2** Plasma hormonal status of adult rats treated *in utero* with flutamide. Data were obtained from 16 animals from 8 litters and are expressed as the means ± S.E.M.

| | **Flutamide** (mg/kg d) | | | |
|---|---|---|---|---|
| **Dose** (mg/kg per day) | 0 | 0·4 | 2 | 10 |
| Testosterone (ng/ml) | 4·52 ± 2·18 | 3·64 ± 2·60 NS | 4·41 ± 3·71 NS | 3·24 ± 1·71 NS |
| LH (ng/ml) | 0·785 ± 0·290 | 0·705 ± 0·212 NS | 0·675 ± 0·118 NS | 0·579 ± 0·25 NS |
| FSH (ng/ml) | 21·20 ± 4·53 | 21·84 ± 5·24 NS | 19·44 ± 3·82 NS | 21·33 ± 7·28 NS |
| Estradiol (pg/ml) | 22·89 ± 4·99 | 21·87 ± 3·30 NS | 24·11 ± 5·36 NS | 19·97 ± 3·57 NS |

Testosterone, LH, FSH, and estradiol plasma levels were evaluated using specific RIA assay. NS, not significant (control (0) versus the different doses of flutamide).

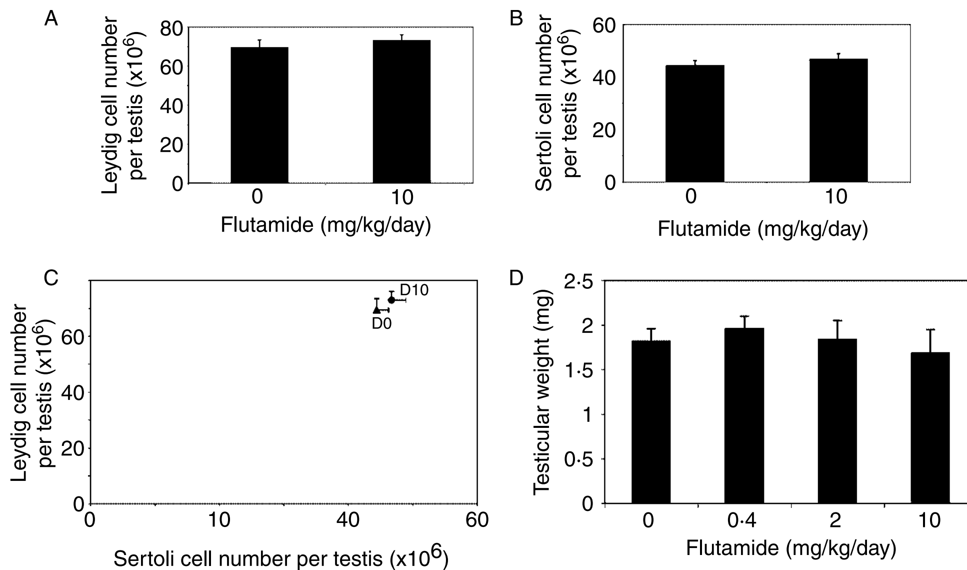


**Figure 2** Morphological parameters in 90-day-old rats treated *in utero* by flutamide. (A) Leydig or (B) Sertoli cells per testis were evaluated in 90-day-old rats, which were untreated (methylcellulose) (0) or treated (10) *in utero* with 10 mg/kg d of flutamide. (C) Ratio of Sertoli to Leydig cells ($10^6$ cells per testis). (D) Testicular weight was evaluated in 90-day-old rats that were untreated (0) or treated *in utero* with 0·4, 2, and 10 mg/kg d of flutamide. The data are obtained from ten different animals per condition.

(Bozec *et al.* 2004), reduced ventral prostate weight, and increased malformations of *vas deferens*. Fetal exposure to flutamide induces a long-term apoptosis in testicular germ cells in adult male rat offspring (Omezzine *et al.* 2003, Bozec *et al.* 2004, Florin *et al.* 2005), particularly in spermatocytes and spermatids at the level of stages VII–VIII of the seminiferous epithelium androgen-dependent seminiferous tubules (Bozec *et al.* 2004). As in the adult seminiferous tubules, it is Sertoli cells that are primarily targeted by the androgens; we have further addressed the question as to whether the long-term germ cell death process might be related to altered Sertoli cell functions. The increased activation in caspase-3 in spermatocytes or spermatids when co-cultured with Sertoli cells originating from testes with a fetal androgen disruption clearly supports the existence of possible alterations in the functions of Sertoli cell related to their promoting role in germ cell survival. As the adult germ cell death process has been linked to altered androgen action exerted through Sertoli cells (De Gendt *et al.* 2004, Tan *et al.* 2005), one should consider, at least, three possible mechanisms that could affect Sertoli cell response to androgens leading to germ cell death, i) modifications in Sertoli cell number, ii) androgen signal including plasma hormone levels and AR expression/activity, and iii) Sertoli cell androgen-dependent transcriptional machinery. Although the long-term germ cell apoptosis in the adult animals exposed *in utero* to flutamide could be linked to an altered androgen signal, we found no modification in the plasma hormonal balance, in the number of Sertoli cells, and in the AR expression, suggesting that the androgen signal is compromised at a receptor or post-receptor level, particularly at the level of the androgen-targeted transcription machinery. However, in terms of AR, there are at least two aspects that should be discussed. First, with regard to testicular AR localization, while in the adult testes, AR is expressed in the different somatic cells (i.e., Leydig, Sertoli, peritubular myoid cells), in the fetal testes, i.e., at the time when the androgen disruption occurs through exposure to flutamide, AR is expressed in the interstitial (Leydig) and peritubular myoid cells (You & Sar 1998), and in gonocytes (Merlet *et al.* 2007) but not in Sertoli cells (You & Sar 1998). These observations would suggest that alterations of adult Sertoli cell functions following flutamide exposure should be taken into the context of the interactions between at least peritubular myoid cells and Sertoli cells. Secondly, although the AR expression appears not to be affected in the adult testis, its activity might be compromised at a post-receptor step(s), i.e., at AR cofactor expression and/or recruitment levels or at androgen-dependent gene transcriptional activity level. Indeed, AR activity could be reduced in view of recent findings showing that *in utero* treatment with the anti-androgen resulted in the adult testes in a decrease in Smad3 and an increase in p-c-Jun protein levels (Maire *et al.* 2005), which represent AR co-activator (Kang *et al.* 2001) and AR co-repressor (Sato *et al.* 1997) respectively. Although the alterations of AR activity in terms of changes in the balance between AR co-activators and co-repressors clearly support alterations at the level of the androgen-dependent transcriptional machinery, the Sertoli cell androgen-targeted genes whose expression is especially involved in adult germ cell

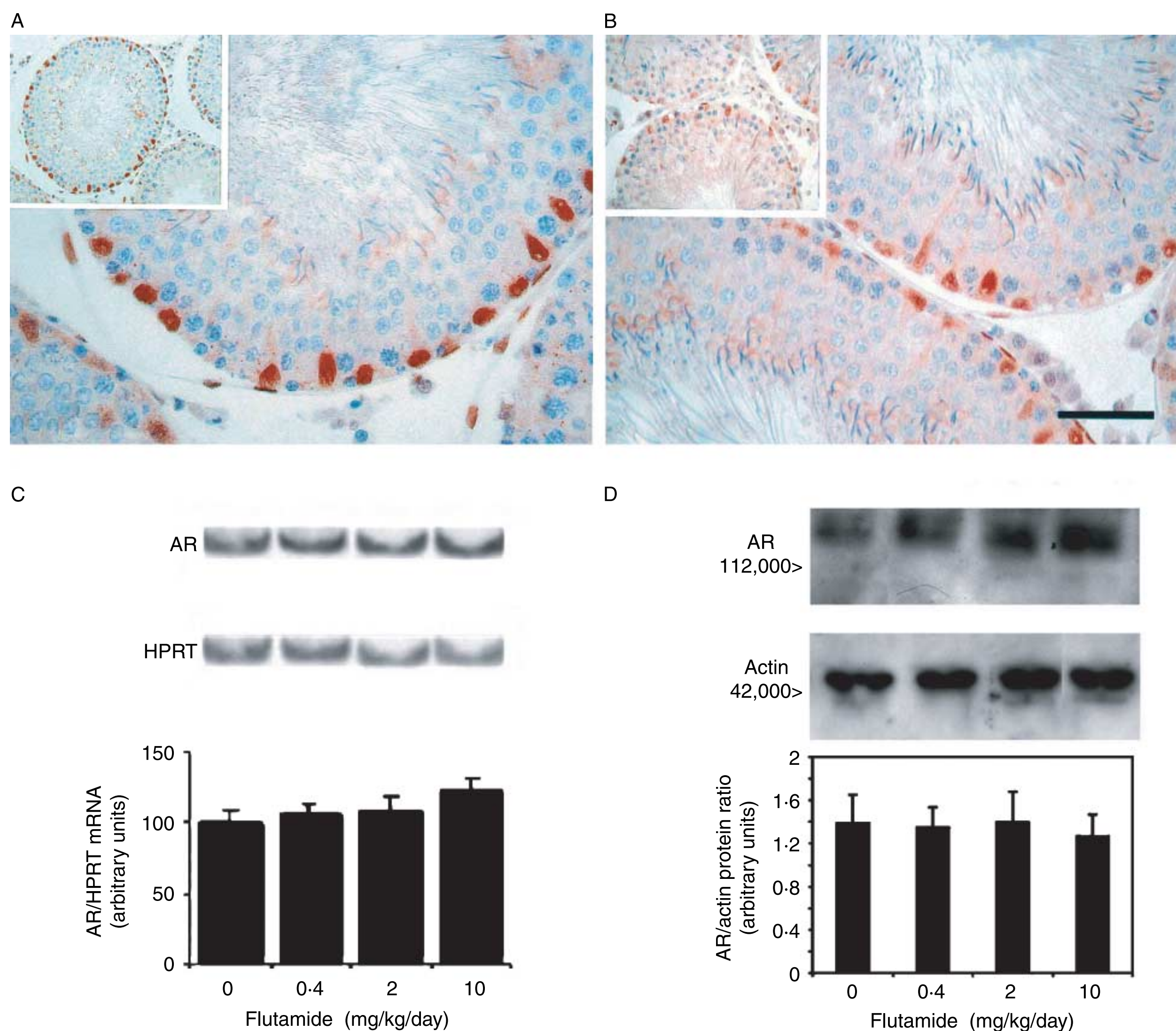


**Figure 3** Androgen receptor expression in the rat testes treated *in utero* with flutamide. Testes were obtained from 90-day-old untreated (methylcellulose) rats (A) or rats treated *in utero* with flutamide 10 mg/kg d (B). Immunohistochemistry experiments were carried out with antibodies directed against AR (scale bar=50 μm). Androgen receptor (AR) levels were determined in adult (90-day-old) rat testes untreated (0) or treated *in utero* with 0·4, 2, and 10 mg/kg d of flutamide through (C) mRNA levels and through (D) protein levels. AR protein is evidenced as a band about 112 kDa and actin protein is evidenced as a band about 40 kDa. Representative autoradiograms and histograms are shown in the upper and lower panel respectively. Data are obtained from seven animals (per condition) originating from at least three different litters. The experiment was repeated at least three times.

death remain yet to be identified. However, as the germ cell death process specifically affects adult germ cells, we hypothesized that the inability of Sertoli cells to protect adult germ cells against apoptosis might be related to an alteration of Sertoli cell maturation process. For this purpose, we have tested the possibility as to whether *in utero* exposure to the anti-androgen may affect the expression of several genes normally expressed in adult Sertoli cells. Most of the genes expressed in Sertoli cells have been selected according to their differentiated expression in adult Sertoli cells (O'Shaughnessy *et al*. 2003). Interestingly, the expression of these genes including AMHR2, cyclin D2, cathepsin L, Cox-1, cystatin SC, and GSTα was found to be down-regulated in adult testes exposed *in utero* to the anti-androgen flutamide. This altered pattern of expression may suggest that following fetal exposure to the anti-androgen, Sertoli cells remained in a relative immature state. Such a relative lack of Sertoli cell maturation may explain the loss of their protective role in terms of adult germ cell survival. However, there are still two points that need more clarification. First, the relative alteration of Sertoli cell maturation needs still to be confirmed by the approaches related to 'omic' genome-wide approaches.

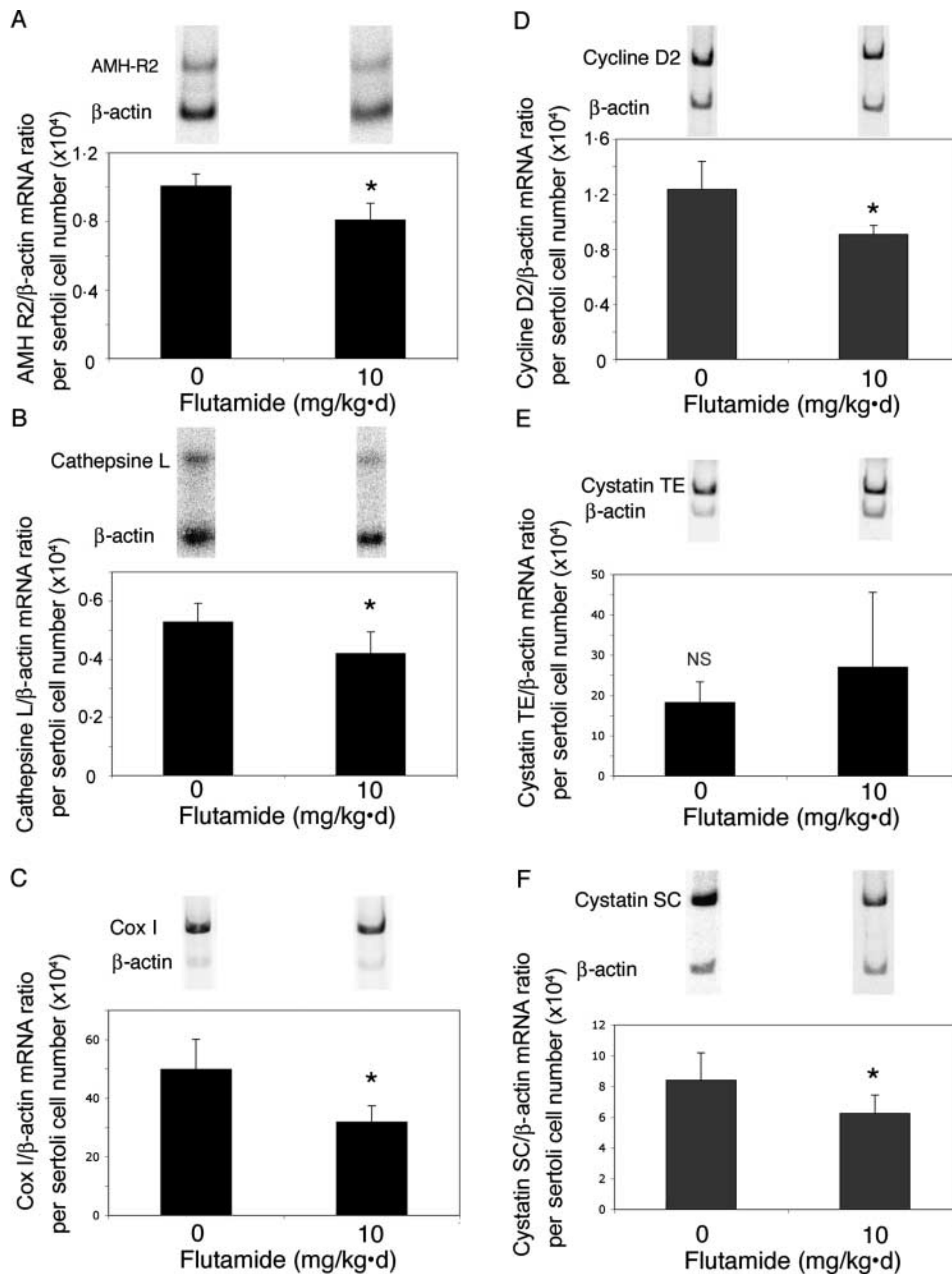


**Figure 4** mRNA levels of Sertoli cell genes in the testes from rats treated *in utero* by flutamide. mRNA levels for AMH type II receptor $*P=0{\cdot}0021$ (A), cathepsin L $*P=0{\cdot}0029$ (B), Cox-1 $*P=0{\cdot}0017$ (C), cyclin D2 $*P=0{\cdot}0055$ (D), cystatin TE (E), or cystatin SC $*P=0{\cdot}04$ (F) were determined in the testes from 90-day-old rats untreated (methylcellulose) (0) or treated (10) *in utero* with flutamide (10 mg/kg d) by RT-PCR. The data are obtained from ten different animals per condition. The experiment was repeated at least three times.

Secondly, while we suggest that the decrease in adult Sertoli cell gene expression leading to this potential lack of Sertoli cell maturation may result in increased germ cell death, it still remains to identify adult Sertoli cell androgen-targeted genes whose altered expression may directly affect germ cell survival. Among these Sertoli cell genes, GSTα is a detoxification enzyme that protects the adult testicular germ cells against apoptosis (Rao & Shaha 2000) by decreasing the cytotoxic effects of oxidants (Yang *et al.* 2001). As such, the decrease in GSTα levels in adult Sertoli cells could represent also one of the possible mechanisms supporting the long-term apoptosis in germ cells from animals exposed *in utero* to

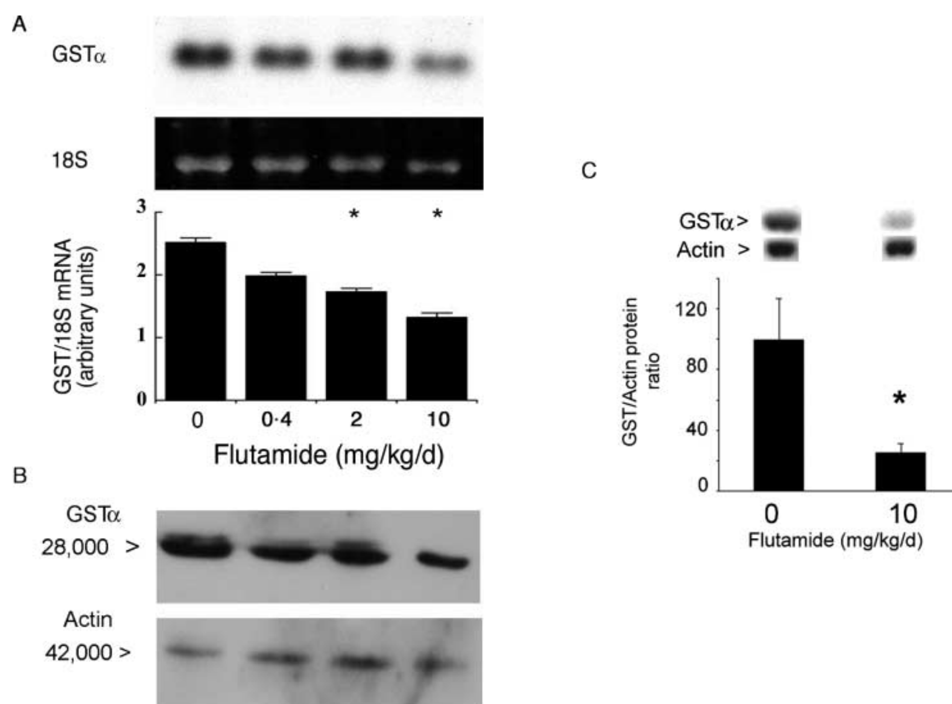


**Figure 5** Effects of *in utero* treatment with flutamide on GSTα mRNA and protein levels in rat testicular seminiferous tubules. Rats (90-day-old) were treated *in utero* with increasing doses of flutamide (0, 0·4, 2, and 10 mg/kg d). Representative autoradiograms and histograms were shown in the upper and lower panel respectively. Evaluation of GSTα mRNA; *$P$=0·0006 (*significance level at 2 and 10 mg/kg d was $P$=0·01 and $P$=0·001 respectively) (A) and protein (B) were realized on seminiferous tubules. (C) GSTα protein levels were determined in Sertoli cells isolated from rat (15-day-old) untreated (0) or treated (10) *in utero* with flutamide (10 mg/kg d). The data are obtained from seven to ten different animals per condition. The experiment was repeated at least three times.

anti-androgen. This could impair the Sertoli–germ cell interactions leading to a long-term apoptosis of germ cells.

One of the major questions raised by the present findings is related to the causal link between the fetal exposure to the anti-androgen flutamide and the adult testicular phenotype characterized by increased germ cell death process probably related, as suggested here, to alteration in Sertoli cell functions. First, although we are still lacking in parameters (or molecular biomarkers) to identify the anti-androgenic effects of flutamide in the fetal testis (i.e., in the AR-expressing fetal testicular cells such as Leydig, peritubular myoid cells (You & Sar 1998), and gonocytes (Merlet *et al.* 2007), there are extratesticular parameters that clearly indicate the androgen action disruption after fetal exposure to flutamide at the doses used (maximum 10 mg/kg per day). These parameters include decreased prostate weight, increased malformations in *vas deferens*, and increased cryptorchid testis number. One should note that the recently reported fetal testicular parameters to be affected following fetal exposure to environmental endocrine disruptors such as phthalates appear to be more related to their toxic rather than to their anti-androgenic activities (Scott *et al.* 2007). One of the possible approach to identify parameters (or molecular biomarkers) AR-targeted genes related to androgen action disruption could be based on the use of omic tools in isolated and purified fetal testicular cells expressing AR. Secondly, in the adult testes, among the arguments supporting that the testicular phenotype, i.e., the increased germ cell death process, might be linked to androgen action disruption is that the death process occurred in spermatocytes and spermatids at androgen-dependent VI–VIII stages (Bozec *et al.* 2004). Such a germ cell death process occurred despite normal plasma testosterone levels, normal (androgen targeted) Sertoli cell number, and normal testicular AR protein and mRNA levels. It is possible that the androgen action disruption occurred at post-AR steps, as yet mentioned, in terms of AR activity, fetal exposure to flutamide may induce an alteration in AR cofactor balance in the adult testes (Maire *et al.* 2005). With regard to alterations of androgen-targeted gene expression, it is quite possible that these alterations might be related to epigenetic changes involving, for example, the methylation/acetylation pattern of the androgen-dependent gene promoters, as yet suggested, for example, for lactotransferrin (Li *et al.* 1997) and c-fos (Li *et al.* 2003) in adult uterine mice tissues from neonatally treated animals with diethylstilbestrol (Ruden *et al.* 2005) and more recently for phosphodiesterase type 4 in adult rat prostate neonatally exposed to estradiol (Ho *et al.* 2006).

In summary, we showed here that fetal androgen disruption induced a long-term alteration of Sertoli cell functions, which may result in a long-term increased adult germ cell death process in the context of Sertoli cell–germ cell interactions.

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