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Endocrine effects of lifelong exposure to low-dose depleted uranium on testicular functions in adult rat



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ABSTRACT

Environmental toxicant exposure can induce disorders in sex steroidogenesis during fetal gonad development. Our previous study demonstrated that chronic adult exposure to a supra environmental concentration of depleted uranium (DU) does not impair testicular steroidogenesis in rats. In this study, we investigated the effects of lifelong exposure (embryo – adult) to low-dose DU (40 or 120 mg L⁻¹) on adult rat testicular steroidogenesis and spermatogenesis. A significant content of uranium was detected in testis and epididymis in the DU 120 mg L⁻¹ group and the assay in epididymal spermatozoa showed a significant content in both groups. No major defect was observed in testicular histology except a decrease in the number of basal vacuoles in the DU groups. Moreover, plasma Follicle-Stimulating Hormone [FSH] and Luteinizing Hormone [LH] levels were increased only in the DU 120 mg L⁻¹ group and intratesticular estradiol was decreased in both groups. Testosterone level was reduced in plasma and testis in the DU 40 mg L⁻¹ group. These modulations could be explained by an observed decrease in gene expression of luteinizing hormone receptor (*LHR*), and enzymes involved in steroid production and associated signal transduction (*StAR*, *cyp11a1*, *cyp17a1*, *3βhsd*, *17βhsd*, *TGFβ1*, *AR*). Several genes specific to germ cells and cell junctions of the blood-testis barrier were also modulated. In conclusion, these data show that fetal life is a critical window for chronic uranium exposure and that the endocrine activities of low-dose uranium could disrupt steroidogenesis through the hypothalamic-pituitary-testicular axis. Further investigation should be so useful in subsequent generations to improve risk assessment of uranium exposure.

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1. Introduction

In recent decades, observations of the decline of quality and quantity of human semen have been closely associated with increasing concern about the role of environmental pollutants on human male reproduction (Campion et al., 2012). Development of the male phenotype and adulthood reproductive function are highly dependent on androgens and estrogens (Carreau et al., 2008; Macleod et al., 2009). Consequently, disorders in sex steroid hormone balance during fetal development may interfere with male reproductive success.

In the environment, uranium may be present in drinking water contaminated naturally, by military activities or by human activity in nuclear power stations, mining sites, and farmland (use of phosphate fertilizers). For example, 90 water sources near mining sites in the Navajo Nation contained uranium above the World Health Organization (WHO) safe drinking water level (30 µg L⁻¹, (WHO, 2011)). Consequently, populations are exposed to unsafe levels, because they use water from the nearest source for household use (i.e. drinking water, cooking, laundering...) (Brugge, 2016). The scientific community and society in general are thus paying increasing attention to the consequences of low-dose uranium exposure.

Few reports have dealt with the effects of environmental uranium exposure on reproductive health outcomes. Some epidemiological studies report a significant decrease in the sex ratio of offspring of male uranium miners (Muller et al., 1967) and in serum testosterone levels in Namibian miners (Zaire et al., 1997).

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A significant relationship was also found between birth defects and the mother's proximity to uranium tailings in the Navajo Nation (Shields et al., 1992). In contrast, Squibb and McDiarmid showed no effect on reproductive hormone levels (testosterone, Follicle-Stimulating Hormone [FSH], Luteinizing Hormone [LH]), on Thyroid-Stimulating Hormone [TSH] level and sperm parameters (sperm production and motility, sperm chromatin stability) in the veterans wounded by depleted uranium fragments as a result of the First Gulf War (Squibb and McDiarmid, 2006).

Uranium toxicity is due to both its chemical and radiological properties (Katz, 2013), but depleted uranium (DU), which is a by-product of natural uranium enrichment, has a lower proportion of ^{235}U . The chemical toxicity effects of DU have been studied in kidney (Poisson et al., 2014), bone (Wade-Gueye et al., 2012) and brain (Dinocourt et al., 2015) after chronic exposure in experimental models. But data on the effects of DU on male reproductive function are scarce. Rodents chronically exposed to 10–80 mg/kg/day of uranium presented few testicular histopathological abnormalities and decreases in pregnancy rate and spermatid number (Linares et al., 2005; Llobet et al., 1991). Acute exposure resulted in embryotoxicity (Bosque et al., 1993; Kundt et al., 2000), though no effect was observed after chronic exposure to a low dose of uranium (Paternain et al., 1989). There is no evidence for steroidogenesis effects of low-dose [2.5 mg/kg/day] DU after adult male chronic exposure (Grignard et al., 2008), except after relatively low dose exposure at 4–40 mg/kg/day (Hao et al., 2012). An *in vitro* study has demonstrated that human fetal testes are more sensitive to low-dose uranium than mouse fetal testes, without any effect on testosterone production (Angenard et al., 2011). However, to our knowledge, there is no report on the effect of *in vivo* chronic exposure from the embryo until adulthood (*i.e.* lifelong) on male reproductive function. Low concentrations ($0.5\text{ }\mu\text{g L}^{-1}$) of DU have estrogenic effects in female mice (Raymond-Whish et al., 2007), and we hypothesized that low-dose DU could have endocrine effects in male rat exposed from gestation.

We aimed to elucidate the influence of uranium on steroidogenesis and spermatogenesis in mature rats subject to lifelong exposure to low-dose DU. We investigated the DU content in reproductive organs, and its effects on testis morphology, sex hormone levels, and mRNA expression of genes involved in sperm and steroid production and the blood-testis barrier, which protects the germ cells.

2. Materials and methods

2.1. Radionuclide

DU was from Areva NC (Areva-Cogema, France). The specific activity of DU is 14.103 Bq/g and its isotopic composition is ^{238}U : 99.73%, ^{235}U : 0.255%, ^{234}U : 0.01%. Uranium was administered as uranyl nitrate hexahydrate ($\text{UO}_2(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$), because uranyl ions (UO_2^{2+}) are the most stable types of uranium in solution and mammalian body fluids (ATSDR, 1999). Two concentrations of uranium were prepared, 40 and 120 mg L^{-1} , by dissolution in standardized mineral water (which naturally contains $1.42\text{ }\mu\text{g L}^{-1}$ uranium). These low concentrations were not nephrotoxic and were chosen from previous studies (Grignard et al., 2008; Legrand et al., 2016).

2.2. Animals and study design

Seventeen pregnant female 12-week-old Sprague-Dawley rats from Charles River (L'Arbresle, France) were housed alone under standard conditions with food and mineral water provided *ad libitum* (light on: 8.00 am/8.00 pm, temperature: $22^\circ\text{C} \pm 1^\circ\text{C}$,

relative humidity of 44.1%). Body weight, food (which naturally contains $1.46\text{ }\mu\text{g L}^{-1}$ uranium) and water consumption were measured weekly. Pregnant females were randomly divided into three groups ($n=9$ per group): control (CTRL), DU 40 mg L^{-1} (DU40) and DU 120 mg L^{-1} (DU120). Control rats were watered with mineral water. Pregnant females were exposed to DU *via* drinking water from the first day after mating (gestational day 1: GD1). At lactation day 21, all male pups were weaned and removed from the maternal cages. At 6 months old, ten rats from each group were used to measure content of DU in different organs. Twenty other rats were used to determine the effect of DU on reproductive function ($N=10$ per group for histology, and $N=10$ per group for blood and biochemical parameters and molecular analyses). All animal procedures were approved by the Animal Care Committee of the IRSN and were conducted in accordance with French legislation and European legal requirements (Decree 86/609/EEC) concerning the protection of animals used for experimental purposes. Scientists certified by the French Ministry of Agriculture performed all procedures in animals.

2.3. Uranium content

Kidney, testis and epididymis were removed in order to analyze uranium concentrations. All samples ($N=10$ per group) were weighed and stored at -20°C . Epididymal spermatozoa were extracted as described below.

For kidney, testis and epididymis, samples were prepared by adding 8 mL of ultrapure 69% nitric acid (ARISTAR, VWR, France) and 2 mL of hydrogen peroxide 30% and mineralizing them in a 1000 W microwave (Ethos Touch, Milestone Microwave Laboratory Systems, Italy) with a 20-min ramp to 180°C and then 10 min at 180°C . Uranium content of mineralized samples was determined with an inductively coupled plasma mass spectrometer (ICP-MS X-series 2, ThermoElectron, France) with bismuth (1 mg L^{-1}) as the internal standard. The ICP-MS limit of detection for uranium is 1 ng L^{-1} . Values were expressed as ng U g^{-1} tissue.

For epididymal spermatozoa, 3 mL of sample was added to 3 mL of 69% nitric acid. The sample was put in a muffle furnace (Nabertherm SARL, France) at 110°C until fully set and the mineral deposit was taken up in 6 mL of 11% nitric acid. Samples were diluted ten-fold before assay by ICP-MS. Values were expressed as ng U L^{-1} homogenate.

2.4. Blood sampling and biochemical parameter analysis

Rats were deeply anesthetized with isoflurane 5%/air 95% inhalation. Rats were terminally anesthetized by intraperitoneal injection of ketamine/xylazine. Blood was sampled by intracardiac puncture with a heparinized syringe and centrifuged ($950g$, 5 min), and plasma supernatants were immediately frozen at -80°C for subsequent analysis. Plasma biochemical parameters were determined with an automated spectrophotometric system (Konelab 20, Biological Chemistry Reagents, ThermoElectron Corporation, Cergy-Pontoise, France). Kits from Brahms (Asnières sur Seine, France) were used to measure triglycerides, alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine, urea, iron, calcium, phosphorus, chloride, potassium, sodium, magnesium, ferritin, and transferrin. Ferritin from Diagam (Lille, France) was adapted to the automated system.

2.5. Hormonal measurements

Testicular extracts were used to measure intratesticular testosterone and estradiol concentrations by radioimmunoassay (RIA) and ELISA, respectively. Testes were thawed and homogenized in cold phosphate-buffered saline (PBS) without Ca^{2+} and

Mg²⁺ by using an Ultra-turrax T25 (JANKE & KUNKEL, IKA® Labortechnik, Germany). After ultracentrifugation (20 625g, 15 min, 4°C), protein content was determined by the Bradford method (Bio-Rad Protein assay, Dye reagent concentrate, Bio-Rad, Marnes-la-Coquette France). All samples were assayed twice.

For testosterone measurement, samples (testicular extracts and plasma) were assayed as previously described (Hochereau-de Reviers et al., 1990). The sensitivity of the testosterone assay was 15 pg/tube and the inter-assay coefficient of variation was 5.3%.

For measurement of estradiol (testicular extracts and plasma) and FSH (plasma), a competitive inhibition enzyme immunoassay technique was used (estradiol – CEA461Ra and FSH – CEA830Ra, Euromedex, Souffelweyersheim, France). All procedures adhered to the standard protocols supplied. The minimum detectable concentrations were 4.75 pg.mL⁻¹ for estradiol and 0.92 ng mL⁻¹ for FSH. There is no cross-reactivity or interference between FSH or estradiol and analogues. The intra-assay coefficient of variation was <10% for both FSH and estradiol assay.

Plasma LH concentrations were determined by radioimmunoassay using reagents supplied by NIDDK (rat LH-RP-3 for standard calibration), rabbit polyclonal antiserum against rat LH, and reagents supplied by Dr. A. F. Parlow (NIDDK, Bethesda, MD), as previously described (Froment et al., 2002). The sensitivity of the LH RIA was 20 pg/tube. The inter-assay coefficient of variation was 18%.

2.6. Testicular histological evaluation

Rats were anesthetized with an intraperitoneal injection of 60 mg.kg⁻¹ sodium pentobarbital and were fixed by transcardial perfusion with paraformaldehyde (PFA) (4% in PBS pH7.4; Sigma-Aldrich, L'Isle D'Abeau Chesnes) at room temperature (RT). Testes were removed and post-fixed for 48 h in 4% PFA at RT. Samples were decapsulated, then dehydrated and embedded in paraffin. Sections (5 µm) were cut and placed on SuperFrost glass slides (Labonord, Templemars, France). The histological sections were deparaffinized and stained with periodic acid-Schiff reagent followed by a hemalum counterstain (PAS-H). Slides were mounted in Eukitt mounting medium (O. Kindler GmbH, VWR). Pictures were captured with a Leica DM 4000 B (Microvision, Evry, France), equipped with digital camera (SONY XCD-U100CR). Histolab software (Microvision Instruments, Evry, France) was used to analyze the histology of seminiferous tubules. Morphology and abnormalities of seminiferous epithelium were checked including vacuolization of Sertoli cells, depletion of germ cells, and multinucleated giant cell formation. Germ cells were identified and tubules were staged according to the morphological classification of Clermont and Perey et al. (Clermont and Perey, 1957). Seminiferous tubule vacuolization was defined as the presence of one or more vacuoles of greatest diameter ≥16 µm located within 1 cell layer of the seminiferous tubule basement membrane (Markelewicz et al., 2004). When a major:minor axis was less 1.5:1, tubules were counted and only the minor diameter axis was averaged for each animal and each group (100 tubules per animal).

2.7. Assessment of sperm production

Epididymis was immediately frozen at –80°C after weighing. After thawing at RT, the whole epididymis was homogenized in M199 medium (Sigma-Aldrich). Ten cycles of a manual Potter–Elvehjem homogenizer were used for each sample. The homogenate was observed under the microscope (×200) in a Malassez chamber to count the spermatozoa (epithelial cells <8%). Three counts per sample were averaged.

2.8. mRNA expression levels

Testicular total RNA was prepared with the RNeasy Mini Kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. After checking RNA quality, the cDNA was produced from 1 µg of total RNA by reverse transcription with High-Capacity cDNA Reverse transcription Kits (Applied Biosystems, Courtaboeuf, France). Real-time polymerase chain reaction (PCR) was performed with 10 ng of template DNA for each reaction on a QuantStudio™ 7 Flex Real-Time PCR (Applied Biosystems, Courtaboeuf, France). PCR amplification was performed by using Syber PCR master mix (Qiagen). All samples were normalized to three most stably expressed genes in rat testis: β actin (βact), β macroglobulin2 (βmg2) and hypoxanthine-guanine phosphoribosyltransferase (HPRT). The geometric mean of the three referenced genes was used to normalize the mRNA expression of target genes (Vandesompele et al., 2002). Relative quantification was calculated with real efficiency of each couple of primers (Pfaffl, 2001). All RT-qPCR results are expressed as mean ± standard deviation, and compared with expression levels of the CTRL group. When required, primers were designed using Primer-BLAST software (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). Several molecular markers were studied for their involvement in different functions: steroidogenesis, germ and Sertoli markers, blood-testis barrier (BTB) integrity, oxidative stress and apoptosis (**Supplemental method**, Table 1).

2.9. Statistical analysis

All data were represented as mean ± standard deviation (SD) and were statistically analyzed by Sigmaplot software (Systat Software Inc., San Jose, CA, USA). Homogeneity of variance was analyzed using the Shapiro-Wilk test. The homogeneous variables were analyzed by one-way analysis of variance (ANOVA) followed by the Holm-Sidak test for multiple comparisons. The non-homogeneous variables were evaluated using the Kruskal-Wallis test followed by Dunn's test for multiple comparisons. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. General observations

In order to estimate the exposure for dams and offspring, the quantity of uranium ingested by animals was calculated and based on their water consumption (Table 1). During the 41 days of exposure, dams were exposed to 97.8 ± 9.3 mg and 291.7 ± 18.5 mg, in DU40 group and DU120 group, respectively. 25% of the totality of the quantity was ingested during gestation and 75% during lactation. For the pups, the exposure was estimated at 67.2 ± 2.5 mg in DU40 group and at 227.9 ± 9.3 mg in DU120 group, with 41% before sexual maturity (*i.e* before 60 days) and 59%

Table 1
Estimation of the quantity of uranium (mg) ingested by drinking water, based on the water consumption in DU40 and DU120 groups.

	DU40	DU120
Dams		
During gestation	23.7 ± 4.54	71.4 ± 9.06
During lactation	74.1 ± 4.07	220 ± 14.4
Total	97.8 ± 9.28	292 ± 18.5
Pups		
Before sexual maturity	23.7 ± 1.82	77.5 ± 11.9
During adulthood	43.5 ± 5.11	25.2 ± 17.1
Total	67.2 ± 2.54	225 ± 9.27

Results are expressed as mean value ± S.D.

Table 2

Body weights (BW) and absolute and relative (/100 g BW) organ weights of DU-treated adult male rats after lifelong contamination.

Treatment	Body weight (g) N = 30 per group	Kidney (g) N = 10 per group		Testis (g) N = 20 per group		Epididymis (g) N = 20 per group	
		Absolute	Relative	Absolute	Relative	Absolute	Relative
CTRL	547.10 ± 70.13 ^b	1.59 ± 0.20 ^a	0.58 ± 0.08 ^a	1.96 ± 0.03 ^b	0.35 ± 0.02 ^b	0.69 ± 0.01 ^a	0.12 ± 0.01 ^b
DU40	563.23 ± 43.96 ^b	1.66 ± 0.22 ^a	0.59 ± 0.05 ^a	1.90 ± 0.08 ^b	0.31 ± 0.02 ^b	0.66 ± 0.03 ^a	0.11 ± 0.01 ^b
DU120	571.05 ± 68.82 ^b	1.72 ± 0.25 ^a	0.60 ± 0.04 ^a	2.28 ± 0.10 ^b **	0.38 ± 0.03 ^b	0.69 ± 0.04 ^a	0.12 ± 0.01 ^b

Results are expressed as mean value ± S.D. **p < 0.005 versus control.

^a One-way ANOVA and the Holm-Sidak test.^b Kruskal-Wallis test and Dunn's test.

during adulthood. During exposure, health parameters of dams and pups were analyzed. There were no between-group differences in food consumption, water intake, or body weight. No significant between-group difference was noted in the total number of live fetuses per litter at birth or in the sex ratio. No change was noted in the body weight of females (data not shown).

In pups, we observed no significant effect of DU on body weight or on relative kidney/epididymis weight, at any uranium concentration (Table 2). Absolute testis weight was significantly increased in the DU120 group, although relative testis weight was unchanged (Table 2).

Several toxicological endpoints were measured in plasma (Table 3). Plasma biomarkers for abnormal kidney function (urea, creatinine) were unchanged in all groups. AST, ALT/AST ratio and magnesium values were significantly increased in DU40- (p < 0.001 vs CTRL) and DU120-treated rats (p < 0.001 vs DU40). Iron and transferrin levels were modified in the DU40 (p < 0.05 vs CTRL) and DU120 groups (p < 0.001 vs CTRL or DU40), respectively. Each plasma markers, measured in rats and which are significantly different, fell outside the normal range for 6-month-old Sprague-Dawley rats.

3.2. Uranium concentrations

Uranium concentrations were measured in the kidneys of dams contaminated for 41 days (20 days of gestation and 21 days of lactation), and in the kidneys of their male pups (lifelong contamination, from GD1 to 6 months). A significant concentration of uranium was seen in the kidneys of rats in the DU120 group in comparison with the CTRL group (p < 0.05) (Table 4). Although statistical significance was missing, there is a trend to a dose dependent increase of DU in the kidney. Uranium content was similar in the kidneys of dams and male pups in both DU groups. Male reproductive organs (testis, epididymis) were significantly contaminated in the DU120 group in comparison with the CTRL group (p < 0.05) and between the two DU groups (p < 0.05) (Table 4). In these organs, the uranium concentration was 220–420 times less contaminated than the kidneys of male pups in DU groups. Interestingly, in both DU groups epididymal spermatozoa contained a significant uranium concentration (p < 0.05) (Table 4), which was twice that in the CTRL group. No dose-dependent effect was observed for uranium content in testis, epididymis and spermatozoa.

Table 3

Toxicological endpoints measured in plasma of DU-treated adult male rats after lifelong contamination.

	CTRL	DU40	DU120
ALT (32.01–59.22 U/L)	45.61 ± 21.95	63.84 ± 26.30 ^a	44.01 ± 18.24 ^a
AST (83.35–116.95 U/L)	100.2 ± 27.11	211.7 ± 59.16 ^{a***}	125.2 ± 36.58 ^{a###}
Ratio AST/ALT (1.96–2.83)	2.39 ± 0.7	3.63 ± 1.12 ^{a***}	3.00 ± 0.71 ^{a###}
Creatinine (51.25–48.27 µM)	49.76 ± 2.41	50.47 ± 2.31 ^a	51.16 ± 4.37 ^a
Urea (6.14–6.73 mM)	6.44 ± 0.48	6.48 ± 0.54 ^a	6.19 ± 0.48 ^a
Calcium (2.78–2.87 mM)	2.83 ± 0.07	2.81 ± 0.07 ^a	2.90 ± 0.11 ^a
Phosphorus (1.50–1.83 mM)	1.67 ± 0.27	1.50 ± 0.15 ^a	1.60 ± 0.24 ^a
Chloride (96.96–98.23 mM)	97.59 ± 1.02	97.46 ± 1.19 ^a	97.34 ± 1.33 ^a
Potassium (4.09–4.24 mM)	4.16 ± 0.13	4.46 ± 0.35 ^a	4.20 ± 0.31 ^a
Sodium (136.13–137.35 mM)	136.74 ± 0.98	136.54 ± 1.86 ^a	137.15 ± 1.40 ^a
Magnesium (0.66–0.70 mM)	0.68 ± 0.03	0.78 ± 0.03 ^{a***}	0.71 ± 0.05 ^{a###}
Iron (28.03–33.13 µM)	30.58 ± 4.11	37.55 ± 5.98 ^{a*}	32.78 ± 6.91 ^a
Ferritin (87.36–146.64 µg/L)	117.00 ± 47.82	89.58 ± 42.30 ^a	70.79 ± 39.62 ^a
Transferrin (1.34–1.45 g/L)	1.39 ± 0.08	1.44 ± 0.12 ^a	1.05 ± 0.25 ^{b****###}

Results are expressed as mean value ± S.D. N = 10 per group. In brackets, the ranges of plasma markers values correspond to MIN-MAX Interval Confidence calculated with normal values of CTRL group (SD rats aged of 6 months old). *p < 0.05, **p < 0.005 and ***p < 0.001 versus CTRL group. #p < 0.05, ##p < 0.005 and ###p < 0.001 versus DU40.

^a One-way ANOVA and the Holm-Sidak test.^b Kruskal-Wallis test and Dunn's test.**Table 4**Uranium concentration in kidney of dams (exposed for 41 days) and in kidney, testis, epididymis and epididymal spermatozoa in male pups (lifelong exposure), exposed to low doses of DU (40 and 120 mg mL⁻¹).

Uranium content	Kidney (ng U/g)		Testis (ng U/g)	Epididymis (ng U/g)	Epididymal spermatozoa (ng/L homogenate)
	Dam	Male pups	Male pups	Male pups	Male pups
CTRL	8.363 ± 3.336 ^a	11.68 ± 8.16 ^a	0.470 ± 0.780 ^a	0.256 ± 0.107 ^a	33.41 ± 10.99 ^a
DU40	317.1 ± 103.6 ^a	260.8 ± 93.24 ^a	0.710 ± 0.397 ^{a*}	0.734 ± 0.346 ^{a*}	88.53 ± 30.57 ^{a*}
DU120	718.3 ± 353.5 ^{a*}	654.9 ± 288.6 ^{a*}	2.320 ± 1.353 ^{a**}	1.566 ± 0.320 ^{a**}	90.04 ± 33.62 ^{a*}

Results are expressed as mean value ± S.D. *: p < 0.05 versus CTRL group. #: p < 0.05 versus DU40 group.

^a Kruskal-Wallis test and Dunn's test.

Table 5

Sperm concentration and testicular histopathological abnormalities of DU-treated adult male rats after lifelong contamination.

	CTRL	DU40	DU120
Spermatozoa counts			
No. X 10 ⁸ /g epididymis	4.82 ± 1.45	5.27 ± 1.11 ^a	5.19 ± 2.92 ^a
No. X 10 ¹² /g epididymis/g animal	8.89 ± 2.67	9.51 ± 2.01 ^a	9.12 ± 5.13 ^a
TESTIS EXAMINATION			
Tubular diameter (μm)	273.185 ± 18.41	257.023 ± 21.39 ^a	271.983 ± 29.02 ^a
Cytoplasmic vacuolization (% of seminiferous tubules [ST] with vacuoles per 100 ST counted)	12.05 ± 16.96	4.03 ± 6.92 ^{***}	5.84 ± 9.00 ^{#####}
Stage III (% of ST in stage III per 100 ST counted with vacuoles)	55.78 ± 93.20	58.33 ± 112.3 ^a	36.23 ± 82.45 ^{#####}
Stage V (% of ST in stage V per 100 ST counted with vacuoles)	22.45 ± 31.58	28.33 ± 61.44 ^a	37.68 ± 67.88 ^{#####}
Stage VII (% of ST in stage VII per 100 ST counted with vacuoles)	10.88 ± 33.31	10.00 ± 21.90 ^a	0.00 ± 0.00 ^{#####}
Stage VIII (% of ST in stage VIII per 100 ST counted with vacuoles)	2.72 ± 9.95	1.67 ± 7.45 ^a	11.59 ± 45.69 ^{#####}
Stage XI (% of ST in stage XI per 100 ST counted with vacuoles)	8.16 ± 20.52	1.67 ± 7.45 ^{***}	14.49 ± 46.55 ^{#####}
Sloughing germ cells (% of ST with sloughing germ cells per 100 ST counted)	0.12 ± 0.40	0.56 ± 1.22 ^a	0.12 ± 0.38 ^a
No lumen	1.42 ± 1.31	1.63 ± 1.20 ^a	2.32 ± 1.99 ^a

Results are expressed as mean value ± S.D. ***p < 0.001 versus CTRL group and #####p < 0.001 versus DU40 group.

^a One-way ANOVA and the Holm-Sidak test.

3.3. Sperm production and testicular histopathology

The results showed no difference in sperm production between the CTRL and DU groups (Table 5). Histopathological examination of the testes revealed few differences in the tubules and interstitial tissues between the CTRL and DU groups (Fig. 1A–D). All stages of the seminiferous epithelium appeared to be present, with normal cellular associations and no change was measured in tubule diameter and lumen. No significant retention of spermatids, Leydig

cell hyperplasia, or presence of sloughed testicular germ cells in the lumen (Fig. 1A) was observed in the DU groups in comparison with the CTRL group. Occasional tubules without lumen (Fig. 1B) and multinucleated giant cells (Fig. 1C) were detected in the DU120 group. Interestingly, the number of vacuoles decreased significantly in the DU40 and DU120 groups (–67% and –51%, respectively) in comparison with the CTRL group ((Table 4 & Fig. 1D). Analysis of seminiferous epithelium revealed this change in tubules at stages III, VII, VIII and XI. The number of vacuoles per

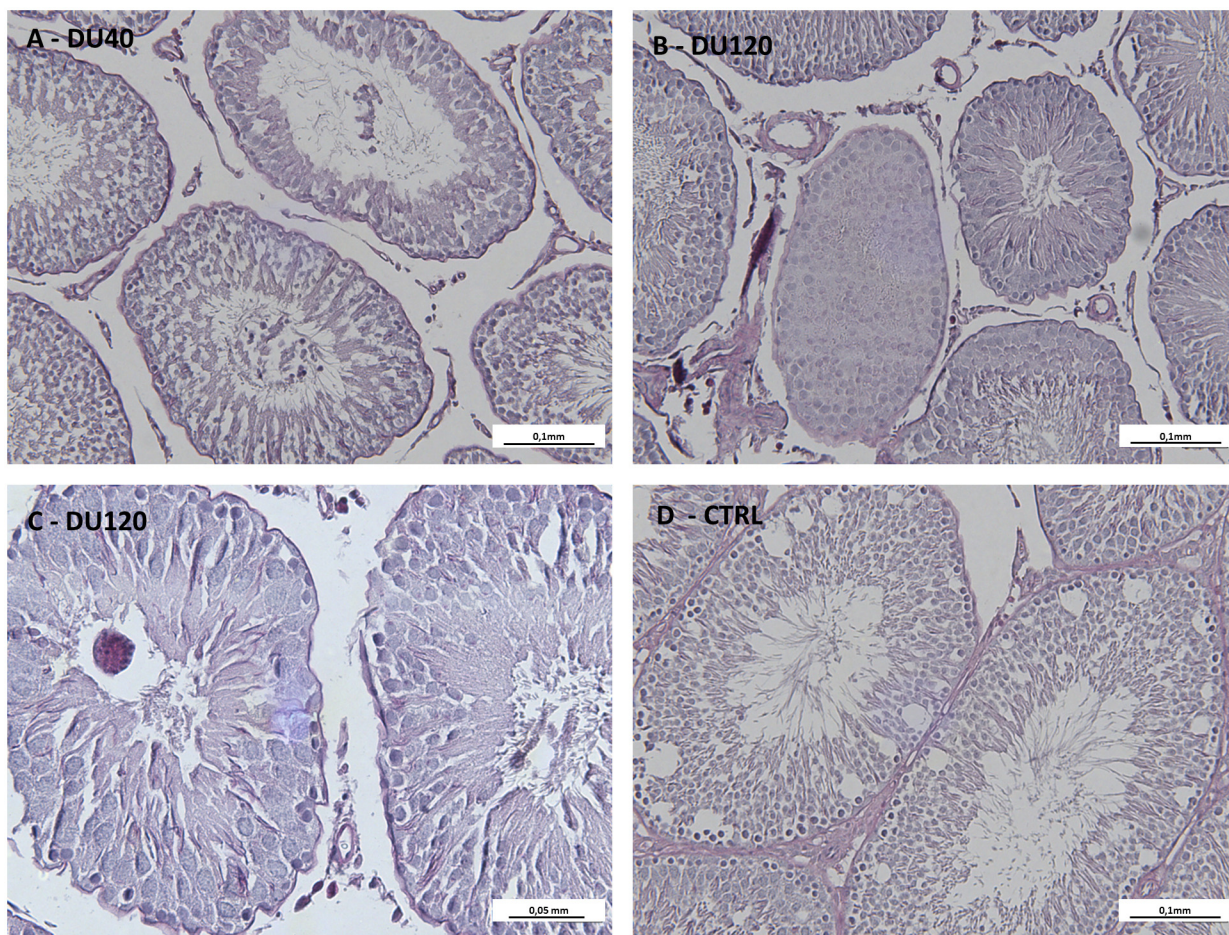


Fig. 1. Effects of DU exposure on testis histology. All stages of seminiferous epithelium appeared normal, and only occasional abnormalities were observed as germ cell sloughing (s), tubules without lumen (l), multinucleated giant cells (m). Basal vacuoles were observed in the CTRL group and the number of seminiferous tubules with vacuoles decreased in both DU groups. Corresponding bars are indicated in each figure.

Table 6

Hormone levels of DU-treated adult male rats after lifelong contamination.

	CTRL	DU40	DU120
Testosterone			
Plasma level (ng mL ⁻¹)	2.60 ± 1.37	1.93 ± 0.84 ^{b*}	2.74 ± 1.61 ^{b#}
Intratesticular level (ng mL ⁻¹)	1.70 ± 1.91	0.71 ± 0.26 ^{b*}	1.05 ± 0.57 ^{b#}
–			
Estradiol			
Plasma level (pg mL ⁻¹)	57.85 ± 17.89	50.48 ± 9.77 ^a	45.79 ± 15.51 ^a
Intratesticular level (pg mL ⁻¹)	15.53 ± 1.67	13.91 ± 1.11 ^{b*}	13.62 ± 0.41 ^{b*}
LH (pg mL ⁻¹)	2.28 ± 0.54	2.47 ± 0.92 ^a	3.39 ± 0.98 ^{a**}
FSH (pg mL ⁻¹)	29.02 ± 14.39	27.41 ± 15.38 ^a	52.60 ± 13.32 ^{a***}

Results are expressed as mean value ± S.D. N = 10 per group. *p < 0.05, **p < 0.005 and ***p < 0.001 versus CTRL group. #p < 0.05, ##p < 0.005 and ###p < 0.001 versus DU40 group, on Thyroid-Stimulating Hormone [TSH].

^a One-way ANOVA and the Holm-Sidak test.

^b Kruskal-Wallis test and Dunn's test.

tubule did not change (Table 4) and vacuoles were mainly localized near basement membranes of the seminiferous epithelium (Table 6).

3.4. Hormonal level of testicular hormones and gonadotropins

A significant decrease in intratesticular and plasma testosterone level was observed only in the DU40 group (p < 0.05) (Table 5). Whereas DU exposure did not seem to affect plasma estradiol levels significantly, the intratesticular estradiol concentration decreases significantly (–10% and –12% for DU40 and DU120, respectively, p < 0.05). LH and FSH levels were significantly higher in the DU120 group than in the CTRL group (+49% [p < 0.05] and +81% [p < 0.001], respectively) and were inversely correlated with steroid production.

3.5. Changes in mRNA expression levels

Molecular analyses were performed to evaluate the effects of DU on the mRNA expression of selected genes involved in steroid production, Sertoli cells and all types of germ cells, cell junctions of the BTB, and oxidative stress and the apoptosis pathway.

Steroidogenesis (Fig. 2) The testicular expression of *StAR* and of steroidogenic enzymes (*cyp11a1* and *cyp17a1*) was significantly lower in the DU40 and DU120 groups than in the CTRL group. The *hsd17b1* and *hsdβ3* mRNA expression levels were inhibited in the DU40 and DU120 groups (p < 0.05), respectively (Fig. 2A). No effect was observed on *FSHR* and *ERα* mRNA expression levels, although the expression of the *LHR* and *AR* genes was significantly lower (p < 0.001) in both treated groups than in CTRL group (Fig. 2B). No dose-dependent effect was observed.

3.6. Germ and Sertoli cell markers

No effect on *utfl* (spermatogonial stem cell marker) was detected. Expression levels of *c-kit receptor* (spermatogonial marker) and *tp2* (round spermatid marker) were significantly lower (p < 0.05) in the DU120 group and higher in the DU40 group, respectively, than in the CTRL group (Fig. 3A). The pachytene spermatocyte marker, *th2b*, was significantly less (p < 0.001) expressed in the DU40 group than in the CTRL group. mRNA levels of *gata6* and *transferrin*, two Sertoli cell markers, were unchanged after DU exposure, except that *gata6* expression was significantly inhibited (p < 0.05) in the DU120 group (Fig. 3B). No dose-dependent effect was observed.

Blood-testis barrier (Fig. 4) The mRNA expression of three types (i.e. tight junction, adherens junction and gap junction) of cell junctions constitutive of the BTB was evaluated. mRNA expression levels of *claudin 11* (tight junction), *N-cadherin* (adherens junction) and *connexin 43* (gap junction) remained unchanged after DU

exposure. Nevertheless, gene expression of the tight junction protein occludin was significantly and dose-dependently higher (p < 0.05) in the DU groups than in the CTRL group. The mRNA expression level of *beta-catenin* (adaptor of adherens junction) was inhibited in both the DU40 and DU120 groups (p < 0.005), without a dose-dependent effect. The expression level of *TGFβ1*, which is involved in cell junction dynamics and steroidogenesis regulation, was significantly lower (p < 0.001) in both DU groups than in the CTRL group (no dose-dependent effect).

Oxidative stress and apoptosis No effect was observed on markers of the intrinsic mitochondrial apoptosis pathway (anti- or pro-apoptotic markers) (Fig. 5A). Regarding the Fas system, gene expression of *fasl* (expressed by Sertoli cells) was significantly lower (p < 0.05) than in the CTRL group, but *fas* (expressed by germ cells) remained unchanged in both DU groups. Several markers of oxidative stress (mainly anti-oxidative) and nuclear transcription factor (*nrf2*) were tested, but only *gpx1* and *gst* were significantly lower (p < 0.001) in the DU120 group and in both DU groups, respectively, than in the CTRL group (Fig. 5B).

4. Discussion

Evidence has been mounting in recent decades of a potential relationship between exposure to environmental toxicants and adverse reproductive health outcomes (Campion et al., 2012). The fetal period is a critical and sensitive window as regards the potential effects of pollutants on the development of the reproductive tract in males and their fertility in adulthood (Welsh et al., 2008). No data are available on whether lifelong exposure to the environmental pollutant low-dose DU disrupts male reproductive function. In this study, we focused on the steroidogenesis effects of DU in the testis and hypothalamic-pituitary-testicular (HPT) axis and on the consequences for spermatogenesis.

There is some evidence that uranium translocates to the offspring during fetal development (Benson and McBride 1997; McClain et al., 2002) and via lactation during the neonatal period (Mullen et al., 1976). Despite the long period of exposure (fetal to adult stage), very low quantities of uranium (<5 ng/g) were measured in testes of rats exposed since fetal life, as already shown (Linares et al., 2005; Paquet et al., 2006). Moreover, our study showed for the first time the presence of uranium in other rat reproductive organs/cells. Although the uranium concentrations are low, epididymis in the DU120 group and epididymal spermatozoa in both DU groups were significantly higher than the control groups. In fact, the purity of the cell population in homogenates of epididymal spermatozoa increased the sensitivity of the ICP-MS assay of DU. As regards the study of Todorov et al., who detected uranium in human semen samples (Todorov et al., 2013), it is worth mentioning that uranium, like cadmium, another heavy metal, can pass through the BTB (Cheng et al., 2011) and is

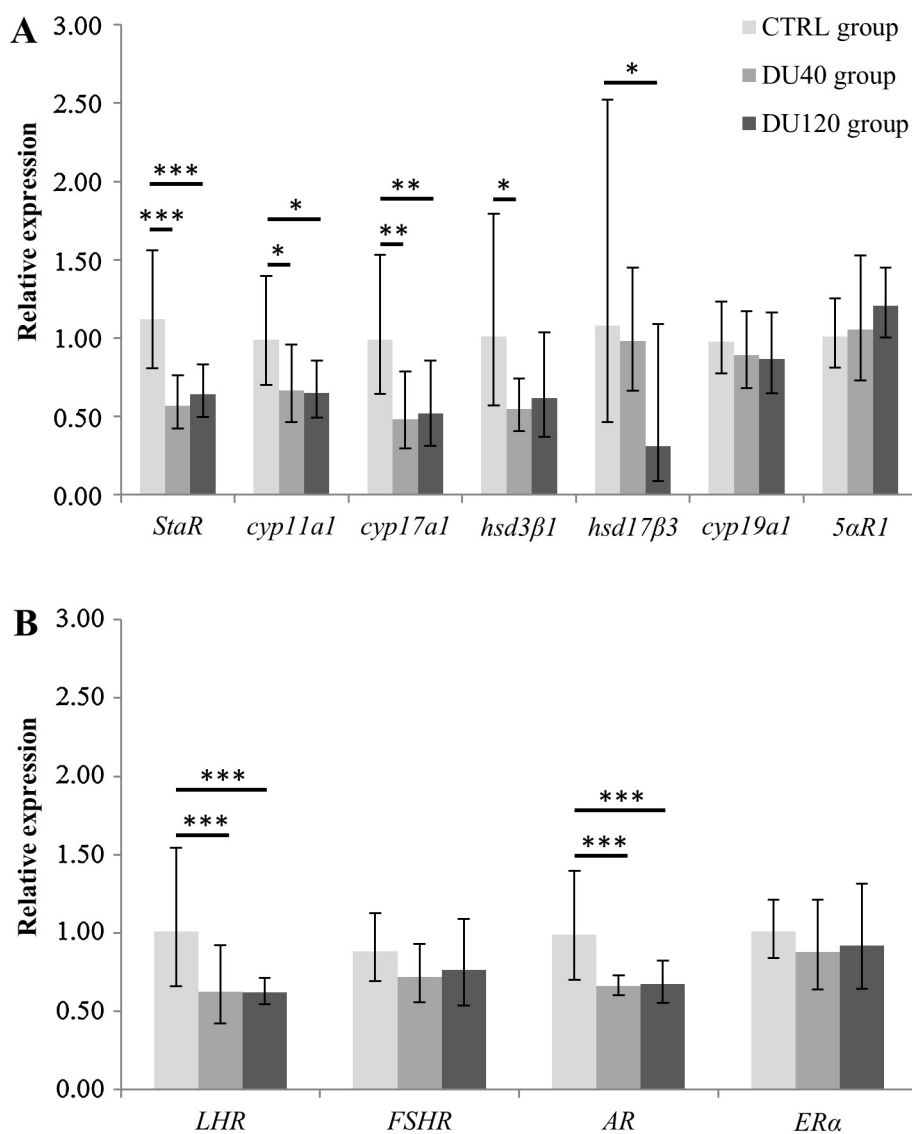


Fig. 2. Relative expression of **steroidogenesis-related genes as enzymes (A)** and **nuclear receptors (B)**. RT-QPCR results are expressed as the mean \pm S.D. (n = 5–10 per group). One-way ANOVA and the Holm-Sidak test. *: $p < 0.05$, **: $p < 0.005$, ***: $p < 0.001$ vs CTRL group.

more likely to target organs of the reproductive tract and to concentrate in the semen. In addition, because an environmental dose of cadmium has already been correlated with aberrant sperm motility in rats (Benoff et al., 2008), further research is needed to characterize sperm parameters (motility, morphology).

Furthermore, our results suggest that DU could come from testicular fluid (and then bypass the BTB) and/or bypass the blood-epididymal barrier. Both barriers are highly protective for male reproductive organs (Mital et al., 2011). Particularly, in testis, the normal function of the BTB is to protect developing germ cells against harmful agents and immunological influences (Yan et al., 2008). Several chemicals can disturb the cell junction between Sertoli cells (i.e. Sertoli barrier) and between Sertoli cells and germ cells (Cheng et al., 2011; Gao et al., 2015). We noted no effect on the mRNA level of the three types of junctions which compose the BTB: tight junction (*cln11*), adherens junction (*N-cdh*, β -cat) and gap junction marker (*Cx43*). Nevertheless, *occludin* gene expression (tight junction) was upregulated, whereas the adaptor of the adherens junction, β -cat, was downregulated. These data suggest for the first time that lifelong exposure to DU may modulate molecular expression of molecules involved in the BTB. Interestingly, our study suggests that DU is not only able to pass through,

but also could disrupt the BTB. Further investigations may be necessary to study in more detail expression and localization of proteins involved in BTB function. Furthermore, additional experimental endpoints (before and/or after 6 months) may be helpful to explore the potential “life-long” effect of DU on BTB markers. Particularly, the evaluation will be relevant at 21 dpn, when BTB is just established in rats, because some of chemicals are known to retard BTB establishment (Toyama et al., 2001).

In agreement with a study performed after adult rat exposure to water contaminated with 40 mgL^{-1} DU (Grignard et al., 2008), the presence of DU in testes was not correlated with spermatogenesis defects in our study. No difference was observed between the CTRL and DU groups, except for a decrease in basal vacuole formation. The nature and mechanisms of Sertoli cell vacuolization are not clearly understood. However, some studies have described Sertoli cell vacuoles as the spaces remaining after germ cell degeneration (Russell et al., 1990). Germ cell death by apoptosis is a part of the maintenance of testicular homeostasis in compliance with the supportive capacity of the Sertoli cells (Lagos-Cabre and Moreno, 2012) and may be related to vacuolization. The two divergent pathways of testicular apoptosis, namely the receptor-mediated Fas/FasL pathway and the mitochondrial-mediated pathway, play

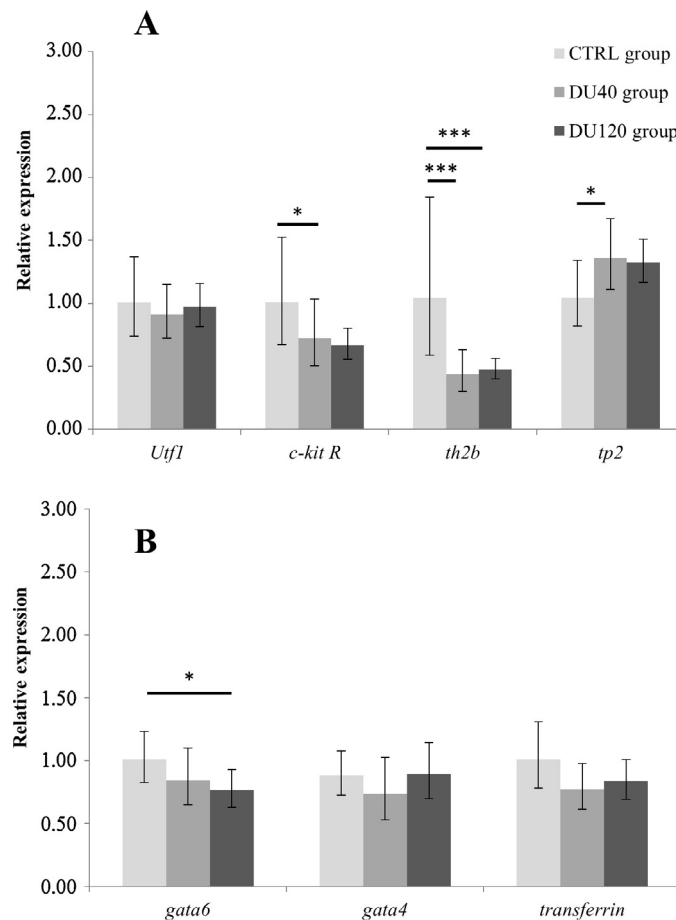


Fig. 3. Relative expression of testicular cell-related genes as germ cells (A) and Sertoli cells (B). RT-QPCR results are expressed as the mean \pm S.D. (n = 8–10 per group). One-way ANOVA and the Holm-Sidak test. *: $p < 0.05$, *: $p < 0.005$, *: $p < 0.001$ vs CTRL group.

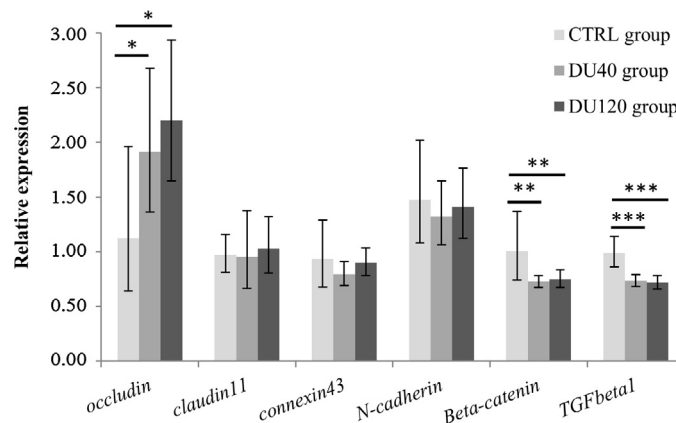


Fig. 4. Relative expression of genes involved in cell junctions of the blood-testis barrier and TGFβ1 gene. RT-QPCR results are expressed as the mean \pm S.D. (n = 8–10 per group). One-way ANOVA and the Holm-Sidak test. *: $p < 0.05$, *: $p < 0.005$, *: $p < 0.001$ vs CTRL group.

an important role in maintaining the germ cell population. Our analysis of mRNA expression levels of selected genes showed no major dysregulation of these two pathways in DU-exposed rat testis. Reactive oxygen species are also able to induce the mitochondrial apoptosis pathway and the Fas/FasL-mediated apoptotic signal transduction pathway (Chandra et al., 2000), but our molecular data showed no strong induction of oxidative stress. Together, these data are not sufficient to link the decrease of

vacuoles and apoptosis in the seminiferous epithelium of DU-exposed rats. Further studies are necessary to understand the mechanisms involved in this testicular change. Nevertheless, despite the absence of major testicular histology defects, we have evidenced differences in gene expression of specific germ cell markers in DU groups. In regard to gene expression of *ckitr* and *utf1*, DU may modulate spermatogonial proliferation and/or differentiation without targeting spermatogonial stem cells. Specific

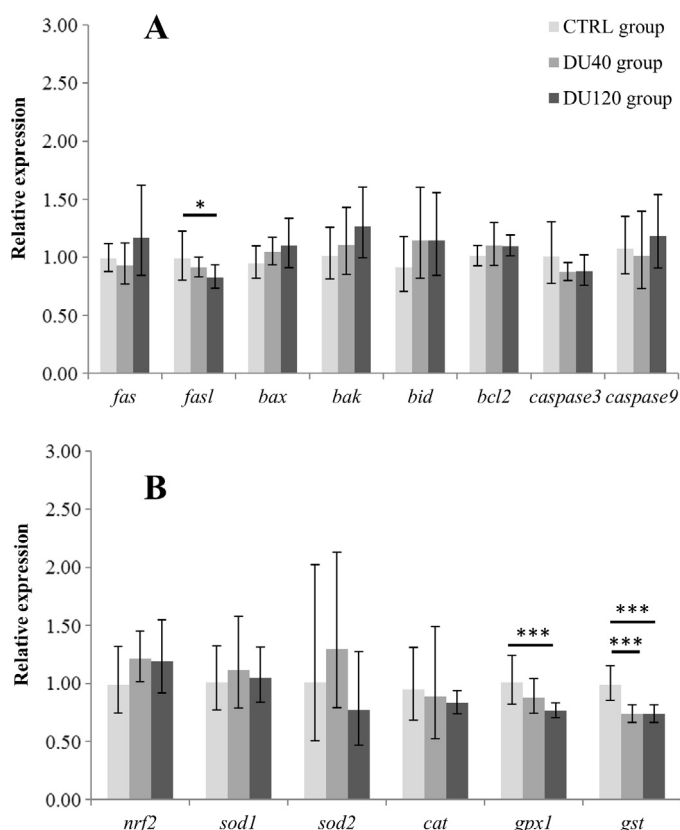


Fig. 5. Relative expression of genes involved in oxidative stress (A) and in the Fas system or mitochondrial pathway apoptosis (B). RT-QPCR results are expressed as the mean \pm S.D. (n = 8–10 per group). One-way ANOVA and the Holm-Sidak test. *: $p < 0.05$, *: $p < 0.005$, *: $p < 0.001$ vs CTRL group.

markers of pachytene cells (*th2b*) and round spermatids (*tnp2*) showed that uranium exposure could affect their mRNA expression. Together, in spite of the fairest interpretation of our gene expression data, the modulations showed that classic examination of testicular histology was insufficient to detect subtle effects on spermatogenesis induced by lifelong exposure to low-dose DU.

Hormonal balance involved in male reproductive function (as spermatogenesis and BTB integrity) is finely regulated by steroid hormones and gonadotropins. In contrast with previous studies (Angenard et al., 2011; Grignard et al., 2008), we observed a modification in sexual hormones after DU exposure. Although intratesticular level of estradiol was decreased in both DU groups, testosterone levels (intratesticular or plasma) diminished only in DU40 groups. Interestingly, we also showed statistically significant induction in gonadotropin levels (FSH, LH) only in the DU120 group. With respect to the induction of gonadotropin hormones, our results suggest firstly that uranium has an effect on the HPT axis in male rats exposed since gestation, like other chemicals with endocrine disrupting activities. This hypothesis is particularly assumed in DU120 group, where the secretion of LH and FSH seems to be modified, maybe in order to increase the testosterone level (plasma and testicular), which was too low in DU40 group. Indeed, the DU will act more at the testicular level at 40 mg L^{-1} , but at 120 mg L^{-1} the HPT axis will be stimulated to induce the sufficient production of intratesticular testosterone, essential for spermatogenesis. It would be interesting to study whether these modulations are detectable during fetal development of rat testis or at the end of the first cycle of complete spermatogenesis. Furthermore, the uranium effect on HPT is not surprisingly given its known effects on male rat brain (Dinocourt et al., 2015). Similarities and differences between uranium with other heavy metals, as cadmium, could increase the understanding of the effects on the HPT axis evidenced in our study. Sexual hormone levels and gonadotropine levels were modified but, as uranium, differently regarding the age of exposure and doses of cadmium (Lafuente,

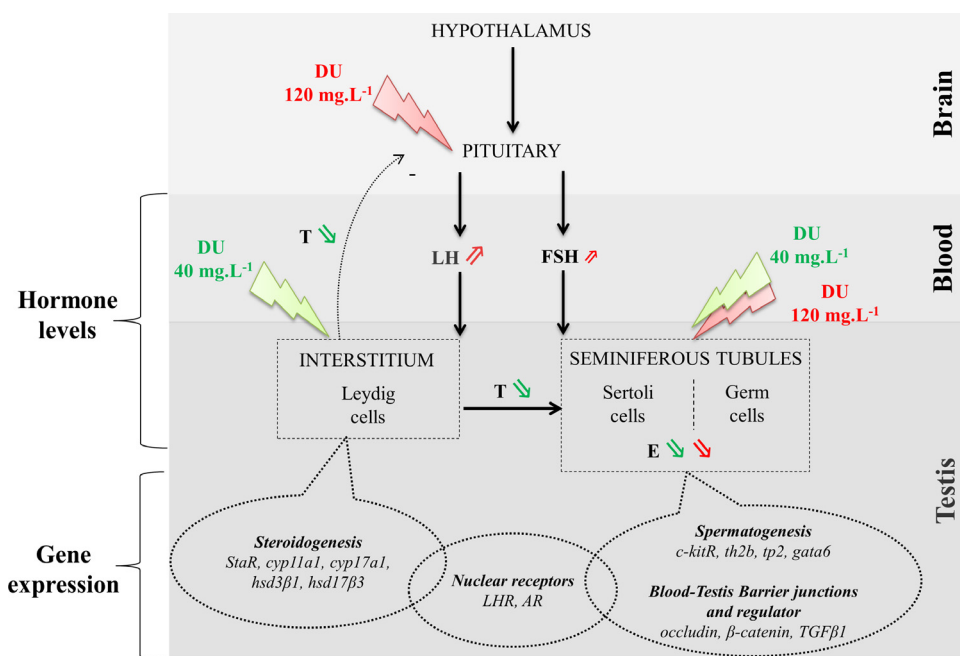


Fig. 6. DU effects after life-long exposure to DU by ingestion of drinking water: Uranium can affect the hormone concentrations by acting on testis and/or on pituitary. Uranium targets some mRNA expression involved in steroidogenesis and spermatogenesis. Gene expression of nuclear receptors and certain actors constitutive of the blood-testis barrier are also modulated. This model highlights the necessity to understand how the hypothalamic-pituitary-testis axis could be involved in DU effects. T: testosterone, E: estrogen.

2013). Particularly, after fetal exposure, cadmium inhibited the testosterone level in serum of male fetuses (Ji et al., 2011). In addition, cadmium accumulates in the adult rat hypothalamus (Márquez et al., 1998), but laboratory studies only shown significant uranium content after Enriched Uranium 4% adult exposure (Houpert et al., 2005). Nevertheless, DU, as cadmium, modifies secretion of hypothalamic biogenic amines, as dopamine (Bussy et al., 2006; Miller et al., 2005) and accumulates in the adult rat striatum (Houpert et al., 2005; Lafuente et al., 2002). Because of hypothalamus and striatum are in the same brain area, they can be affected together by endocrine activity of DU after prolonged exposure. Consequently, it may be necessary to elucidate the neuro-endocrinal mechanisms involved after uranium exposure, as the impact on GnRH and GnRH receptor.

In female mice, Raymond-Whish et al. have previously shown that uranium may act on reproductive function as a xenoestrogenic compound, suggesting that it could have direct effects by mimicking estradiol or indirect effects by interfering with estrogen receptors or estradiol production and metabolism (Raymond-Whish et al., 2007). In our study, despite intratesticular estrogen change, no effect was observed on testicular *ERα* and *Cyp19* mRNA expression, both involved in the estrogenic pathway. Nevertheless, as regards the mRNA levels of selected genes involved in androgen production, we observed that animals exposed since their fetal life are more sensitive than exposed adults (Grignard et al., 2008). Indeed, we found that DU exposure reduced mRNA levels of steroidogenesis enzymes and associated signal transduction (*StAR*, *cyp11a1*, *cyp17a*, *hsd3β1*, *hsd17β3*, *TGFβ1*). Interestingly, TGF-β1 has been reported to inhibit LH-mediated steroidogenesis by decreasing cAMP-induced testosterone production and the expression of steroidogenic genes such as *P450c17*, *StAR* and *hsdβ3* in mouse Leydig cells (Payne and Youngblood, 1995). With respect to the *AR* and *LHR* mRNA expression decrease we observed, our molecular results suggest that the androgenic pathway may be mainly targeted after lifelong exposure of male rats to low-dose DU.

In conclusion, we show here for the first time that, after lifelong exposure, uranium is present not only in rat testis but also in the epididymis and spermatozoa. Although no deleterious effects on testicular histology were observed, we found that low-dose DU has endocrine activity and can disrupt steroidogenesis by acting on the HPT axis. These hormonal effects also seem able to impact molecular mechanisms involved in spermatogenesis and BTB integrity, two functions highly regulated by hormones (Fig. 6). To elucidate the impact of these molecular changes and to contribute in risk assessment of uranium exposure, further experimental studies are needed to identify the effects on the next generations in experimental models. In conclusion, we should pay particular attention to human populations living close to contaminated areas, focusing on exposed pregnant women.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.tox.2016.08.014>.

Table 1 gives detailed information on the primers of target genes studied in our work.

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