1 **Reproductive toxicity of linuron following gestational exposure in rats and underlying**

2 **mechanisms**

3 Hongwei Ding[1], Wei Zheng[2], Hua Han[1], Xiyin Hu[1], Binli Hu[1], Feng Wang[1], Liyu Su[1], Hong Li[1],

4 and Yan Li[1,*]

5 [1]Department of Hygiene Toxicology, School of Public Health, Zunyi Medical College, Zunyi,

6 Guizhou, PRC

7 [2]School of Health Sciences, Purdue University, West Lafayette, Indiana, USA

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9 **Running Title**: Reproductive Toxicity of Linuron in Male Rats

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11 *To whom all correspondences shall be addressed:

12 Yan Li, Ph.D.

13 Department of Hygiene Toxicology

14 School of Public Health

15 Zunyi Medical College

16 Zunyi, Guizhou 563000

17 China.

18 E-mail: dinghw0222@163.com

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21 Submitted to *Toxicol Letters*

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Abstract

Linuron is a widely used herbicide in agriculture; its endocrine disruptive toxicity has recently received public attention. This study was designed to examine the developmental toxicity of linuron on the reproductive system of male offsprings following maternal exposure. Mother rats received oral gavages of linuron, once daily, at the dose of 0, 50, 100, 150 or 200 mg/kg, from gestational day (GD)13 to GD18; gonadal organs from GD20 fetuses were examined. Data indicated that exposed male offsprings had a significantly shortened anogenital distance. Pathological examination further revealed a lack of fusion in the urogenital fold in treated fetuses, the damaged seminiferous tubules, and the injured Leydig cell ultrastructure. Analysis of serum testosterone concentrations at postnatal day (PND)2 showed a significant dose-related reduction (about 33.7-58.75%, r=-0.838, $p<0.05$) as compared to controls. Immunohistochemical results demonstrated a significantly reduced expression of enzymes pertinent to the testosterone production including P450scc, 3β-HSD, and PCNA in Leydig cells ($p<0.05$). qPCR studies confirmed decreased levels of mRNAs encoding P450scc, 3β-HSD and PCNA ($p<0.05$). Taken together, these data suggest that maternal exposure to linuron hampers the male gonadal organ development; this appears to be due to linuron's direct action on the production of testosterone in fetal and postnatal offspring.

Key words: linuron; testosterone; offspring; reproductive toxicity; developmental toxicity; fetus; Leydig cells

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Introduction

Reproductive disorders due to environmental exposure to antiandrogenic pesticides, fungicides, and herbicides have been well recognized in literature (Auger et al., 2013; Klot et al., 2014; Monosson et al., 1999;). Particularly alarming is the increased incidence of developmental disorders in male reproductive system tract due to exposure to these toxicants (Sharpe and Skakkebaek, 1993; Storgaard et al., 2006). Toxic chemicals can interact with the androgen receptor (AR), alter the production and metabolism of testosterone, and/or directly damage the formation of male reproductive organs in the fetal stage. While studies on the reproductive toxicity of antiandrogenic substances are extensive, the mechanisms underlyiong these toxic actions in most cases remain elusive.

Linuron (also called methoxydiuron or afalon; CAS#330-55-2) is a widely used herbicide in the production of soybeans, corn, cotton, carrots, wheat, peanuts, sugar cane, fruit and other vegetables. Reports in literature suggest that linuron acts as an environmental endocrine disruptor; short-term exposures to linuron in rats result in a reduced production of testosterone (Lambright et al., 2000; Santos et al., 2014; Wilson et al., 2009). Exposure to linuron in mothers also causes the abnormal sexual differentiation and development in male offspring, such as hypospadias, cryptorchidism, prostate hyperplasia, and testicular atrophy (McIntyre et al., 2000; Hotchkiss et al., 2004; Sultan et al., 2001). The deformity of the male reproductive system is irreversible and persistent, and may last for lifetime. Limited data from in vivo and in vitro experiments suggest that linuron may compete with androgen for the AR binding (Gray et al., 2001; Lambright et al., 2000). Yet, the current understanding on how maternal exposure to this

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93 chemical may lead to the altered development of male reproductive system and what mechanism

94 underlies linuron toxicity is incomplete.

95 The purpose of this study was to explore and confirm the developmental toxicity of

96 linuron on male reproductive system following materal exposure by examining the changes in

97 male sex hormone levels (testosterone) as well as in male reproductive organs in fetal and

98 postnatal stages. It is known that the production of testosterone is regulated by P450scc, which

99 catalyzes the formation of pregnenolone from cholesterol, and by 3β-HSD, which converts

100 pregnenolone to progesterone (Arukwe, 2008, Rone et al., 2009, Issop et al., 2013). To

101 understand the mechanism of linuron toxicity, we further investigated the changes of P450scc,

102 3β-HSD and other male sex hormone-related enzymes in male offsprings after materal exposurfe.

103 The study, by offering a better understanding of linuron-induced reproductive toxicity, may

104 provide the useful information for better prevention and intervention.

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129 **Materials and methods**

130 **Chemicals and reagents**

131 Linuron was purchased from Chem Service Inc. (West Chester, USA); the enzyme-linked
132 immunosorbent assay (ELISA) kit to quantify serum total testosterone from Diagnostic Products
133 Corp. (Los Angeles, CA); all primary and secondary antibodies from Santa Cruz Biotechnology
134 (Santa Cruz, CA); and formaldehyde and other routinely used reagents from Sigma (St. Louis,
135 MO). All reagents were of analytical grade, HPLC grade or the best available pharmaceutical
136 grade.

137

138 **Animals and treatments**

139 Sprague-Dawley rats (4-8 week) of both sexes were obtained from the Animal Center of
140 the Third Military Medical University (Chongqing, China). Upon arrival, the animals were
141 housed in a temperature (21 ± 1°C)- and humidity (55 ± 5%)-controlled room under a 12-hr
142 light/dark cycle and allowed for acclimatization for two weeks prior to experimentation. At the
143 time of experiment, rats were 9 weeks old weighing 240 ± 10 g for males (n = 60) and 180 ± 10
144 g for females (n = 60). Rats had access to food and tap water at libitum. The study was
145 conducted in compliance with the Animal Care and Use Guidelines in China and approved by the
146 Animal Care and Use Committee of Zunyi Medical College.

147 After acclimatization, each male rat was caged with two females. Vaginal smears were
148 performed daily; the sperm-positive smear in female rats was considered as Gestational Day (GD)
149 0. The pregnant dams were housed individually and were randomly assigned to one of the

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155 following exposure conditions. Linuron was dissolved in groundnut oil. At GD13, mother rats

156 received the oral gavage (2 mL/kg body weight), once daily, at the dose of 0 (control), 50, 100,

157 150 or 200 mg/kg, for 5 consecutive days. The daily equivalent volume of oil vehicle was given

158 to the animals in the control group. These dosage levels were selected based on the previous

159 report in literature (Lambright et al., 2000). Each exposure group had 10 dams. At the designated

160 time, mother and/or fetal male rats were dissected to determine reproductive toxicity.

161 Daily oral gavage caused the minimal stress, as animals were gradually accustomed to the

162 procedure. The dams were weighed daily before and after dose administration. After parturition

163 (PND0), the pups were counted and weighed. Pups were then caged with their biological mother

164 thereafter.

165

166 **Pathological examination**

167 At 20 days into pregnancy (GD20), four mother rats from each group were sacrificed to

168 collect fetuses; the genital tubercle and testis of male fetal rats were isolated and prepared for

169 pathological examination. Each sample was fixed in 4% formaldehyde for 24 hr; the samples

170 were then dehydrated and embedded in paraffin according to the routine pathological sample

171 preparation procedures. The tissues were cut into 5-mm section with a microtome and stained

172 with haematoxylin and eosin (H&E). Each of tissue samples was made in triplicates. During the

173 tissue dissection, the position of testis and the development of prostate were also observed.

174 The tissue samples from the same dams described above were used for electron

175 microscopic examination. The testes were fixed in 3% glutaraldehyde solution for 6 hr and

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193 treated with 1% osmium tetroxide for 45 min for tissue preparation prior to electron microscopic

194 analysis.

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196 **Determination of serum testosterone levels in postnatal male rats**

197 Blood samples were collected from 2-3 PND2 male pups from 4-5 litters in each

198 treatment group by decapitation and allowed to coagulate on ice. Serum samples were prepared

199 by centrifuging the whole blood samples at approximately l000×*g* at 4°C for 30 min and stored

200 at -20°C until analysis. Serum total testosterone was determined in duplicates by using an

201 enzyme-linked immunosorbent assay (ELISA) kit (Diagnostic Products Corp, Los Angeles, CA).

202

203 **Immunohistochemical examination**

204 Bilateral testes from male fetuses of GD20 rats (2 pups from 4 litters in each treatment

205 group) were extracted as described above and prepared in 4% formaldehyde solution for 48 hr.

206 Conventional immunohistochemical procedure (including dehydration in gradient ethanol,

207 xylene clearance, and paraffin embedding) was followed to prepare the sections. Sections were

208 heated to 62°C to remove wax and then rehydrated prior to treatment with blocking serum

209 (phosphate-buffered saline/0.5% Triton X-100 with 5% serum) for 1 hr at room temperature.

210 Primary antibodies, i.e., mouse Mab against 3β-HSD (1:300 dilution), mouse Mab against

211 P450scc (1:200 dilution), anti-proliferating cell nuclear antigen (PCNA) (1:100 dilution) and

212 mouse Mab against AR (1:200 dilution) were incubated overnight at 4°C. After washes, the

213 sections were incubated at room temperature with the secondary antibodies for 1 hr. Secondary

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215 antibodies included mouse anti-biotin (1:75 dilution) and goat anti-rabbit immunoglobulin G

216 (IgG) rhodamine (1:300 dilution). Samples were examined under a light microscope; an

217 IPWIN60 software was used to analyze the optical density (OD) value for the signals from

218 Leydig cells. Each group had 8 sections and each section had 3 visual fields.

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220 **Real-time RT-qPCR analysis**

221 The expression levels of mRNAs encoding P450c17, 17β-HSD and AR in bilateral testes

222 collected from male GD20 fetuses were quantified using qPCR. The total RNA was isolated by

223 using TRIzol reagent. An aliquot of 0.5 μg RNA was reverse-transcribed into cDNA. The iTaq

224 Universal SYBR Green Supermix (Bio-Rad, CA) was used for qPCR analyses. The amplification

225 was run in the FTC-2000A Real-Time PCR Detection system (Funglyn Biotech, CHN). After

226 initial 2-min denaturation at 94 °C, the amplification program was set at 45 cycles of 20 sec

227 denaturation at 94°C, 30 sec gradient 55.0 to 60.0°C and 40 sec extension at 72°C. Each

228 real-time RT-PCR reaction was run in triplicates. The forward and reverse primers for tested

229 genes were designed by Takara Biotechnology (Shiga, Japan). Primers sequences for these genes

230 are listed in Table 1. The rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as

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231 an internal control. To compare the interest gene expression levels in different groups, the

232 comparative $2^{-\Delta\Delta Ct}$ method was used (Livak and Schmittgen, 2001).

233

234 **Statistical analysis**

235 The statistical analyses were performed with SPSS software version 21.0 for Windows

238 (SPSS Inc., Chicago, IL). Values of all variables are presented as mean ± standard deviation.

239 One-way analysis of variance (ANOVA) with Tukey's HSD as post-hoc test and LSD-t test were

240 used to determine the differences between different treatment groups. The differences between

241 two means were considered significant if *p* values were equal or less than 0.05.

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Results

Systemic effects of linuron on postnatal male rats following maternal exposure

To understand the developmental toxicity of linuron on male reproductive system, the mother rats were orally exposed to various doses of linuron from GD13 to GD18; the male offspring were then subjected to testing serum testosterone level at postnatal day 2 (PND2), and to measurement of body weight and anogenital distance (AGD) at PND28. Linuron exposure in mothers caused a dose-related reduction in male offspring in their serum testosterone concentrations, about 33.7%, 46.0%, and 58.8% reduction in 100-mg/kg, 150-mg/kg and 200-mg/kg groups, respectively, as compared to controls (p<0.05) (Table 2). A linear regression analysis of this dose-effect relationship established a correlation coefficient of r = -0.838 (*p*<0.05).

At PND28, while the body weights were not significantly changed in male offsprings, the AGD, as measured between the anus and penis, were significantly shortened in male pups of the linuron-exposed groups, about 17.7-24.6% reduction in 100-200 mg/kg groups, as compared to controls (Table 2). Further linear regression analysis revealed a dose-related correlation efficiencient of r = -0.873 (*p*<0.05). Clearly, the maternal exposure to linuron caused the damage to offspring male reproductive functions.

Pathological changes of fetal male reproductive system following maternal linuron exposure

Oral exposure to linuron in maternal rats for five consecutive days caused a significant

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280 pathological damage to offspring male reproductive structure (Fig. 1). In control fetuses, the HE
281 staining showed that the genital tubercles possessed a well-developed urethra; the urogenital fold
282 became confluent; and no epithelial gaps were observed (Fig.1A). In contrast, the samples from
283 the linuron-exposed fetuses showed that the genital tubercles were opened with continuous
284 urethral groove; there was no fusion in the urogenital fold, but the epithelial gaps were evident
285 (Fig.1B). Noticeably also, the location of urethral opening was unusual. It is known that an
286 incompletely developed urogenital fold will ultimately lead to hypospadias (Baskin et al., 2006).

287 Further examination of testicular cord under microscope revealed four typical cell types
288 in the seminiferous tubules, including Leydig cells, spermatogonia, supporting Sertoli cells and
289 peritubular myoid cells. In controls, spermatogonia had clear nuclear staining and lined against
290 the basement membrane; there were abundant Sertoli cells in lumen of tubules (Fig. 2A).
291 Treatment with linuron appeared to reduce spermatogonia, disrupt the normal arrangement of
292 cell layers in tubule lumens, and cause karyopyknosis (Fig. 2B). There were obvious vacuoles in
293 nearly all of the cell types. With the increase of linuron dose, these morphological changes
294 became more severe (Fig. 2C,D) and significant cell loss could be seen in Fig. 2D.

295 The Leydig cells distributing in interstitial space function to secrete male sex hormone.
296 Under the electron microscope, a distension of rough endoplasmic reticulum in Leydig cells
297 became evident (Fig. 3B). At the high dose (200 mg/kg), the swollen mitochondria in Leydig
298 cells could be observed in most cases (Fig. 3C).

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Effect of maternal linuron exposure on enzymes and proteins participating in male reproduction

The apparent reproductive toxicity following maternal linuron exposure could be due to the altered production of male sex hormones or androgen receptor (AR) in fetuses. To test this hypothesis, we used the immunohistochemistry to examine the expression of a host of selected enzymes and proteins in fetal Leydig cells from GD20 dams. Data presented in Fig. 4 showed that PCNA, a marker for DNA synthesis during cell proliferation, was apparently reduced, so were the enzymes involving in synthesis of testosterone, i.e., P450scc and 3β-HSD. By quantitation of expression signals, there were 18.3%, 15.5%, and 16.9% reductions in PCNA, P450scc and 3β-HSD, respectively, as compared to controls ($p<0.05$) (Table 3), while the signal levels for AR in Leydig cells were not changed (Fig. 4 and Table 3).

To verify the results from immunohistochemical studies, we further employed qPCR to quantify the mRNAs encoding these proteins. Data presented in Table 4 demonstrated that the gene expressions of PCNA, P450c17 and 17β-HSD in fetal testes at GD20 were significantly lower, about 47.0%, 50.8% and 39.9%, respectively, than those in controls ($p<0.05$), while the gene expression of AR was not changed (Table 4).

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Discussion

Results of these experiments demonstrate that oral exposure to linuron in mother rats can profoundly alter the reproductive organ development in male fetal and neonatal rats. The alteration can be observed at morphological, biochemical, and molecular levels. Under normal physiological condition, the urogenital folds have two fusions; one is on the surface of the genital tubercle and the other is positioned in the middle. Upon the formation of complete urethral, the chamber is absorbed to form the epidermis. Urogenital groove and urogenital fold can integrate together to form a cavernous body of urethra tube (Lambright et al., 2000). Following maternal exposure to linuron, however, the fusion of urogenital groove and urogenital fold was incomplete, resulting in an abnormal location of the urethral orifice. The failure to form a complete urogenital groove may lead to a shortened anogenital distance in postnatal rats. Our evidence supports the view that maternal linuron exposure hampers sexual differentiation and development in male offspring.

The formation and differentiation of the gonadal organs during the embryonic development are regulated by a host of hormones through the hypothalamus-pituitary-gonad axis in mother as well as in fetus (Barlow et al., 2003; Hotchkiss et al., 2004; Kroupova et al., 2014); the process is highly sensitive to variation of hormone levels in the fetal stage (Vukusic et al., 2013). Reports by Fitch et al. (1990) show that from the embryonic day GD17 to the postnatal day 6-8, the secretion of testosterone in offspring testes reaches the peak, which determines the early differentiation and development of testis (Sinha et al., 1999; Russell et al., 2008). Other reports also show that impaired testosterone secretion during this stage usually causes the

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357 irreversible and permanent damage to testicular development, which in turn directly affects the
358 reproductive function in adulthood (Han et al., 2004; Wu et al., 2010; Esteves et al., 2011). Our
359 data clearly show that exposure to linuron in mother rats caused a dose-related decline of
360 testosterone in offspring testes. Thus, it appears that linuron's reproductive toxicity is directly
361 associated with the production of testosterone.

362 What is the targeted cell type underlying linuron developmental, reproductive and
363 toxicity? Results from electron microscopic studies demonstrated that at GD20, the Leydig cells
364 in fetal rats appeared to be significantly injuried by linuron treatment. The rough endoplasmic
365 reticulum in Leydig cells became dilated and the mitochondria were swollen. Since the
366 endoplasmic reticulum in Leydig cells is a known intracellular location where testosterone is
367 synthesized, it is likely that the damage on endoplasmic reticula in Leydig cells, in combination
368 with the distorted energy supply in malfunctioned mitochondria, may reduce the production of
369 testosterone from the fetal to postnatal stage.

370 Synthesis of testosterone requires numerous enzymes that transport cholesterol across the
371 mitochondrial membrane and convert cholesterol to pregnenolone; the latter reaction is catalyzed
372 by mitochondrial P450scc. Pregnenolone is then further catalyzed by 3β-HSD to form
373 progesterone. These reactions are the rate-limiting steps in the synthesis of testosterone. (Arukwe,
374 2008; Rone et al., 2009; Issop et al., 2013). It is also known that Leydig cells in fetal mouse
375 differentiate rapidly during GD12 to GD14, followed by synthesis of testosterone. To investigate
376 the mechanism of linuron toxicity, we determined a number of key enzymes and proteins
377 involving testosterone production in fetal GD20 rats. Our IHC data demonstrated a significant

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392 reduced expression of P450scc and 3β-HSD in Leydig cells of fetal GD20 rats. The qPCR

393 experiments confirmed these findings and further indicated a 40-51% reduction of these two

394 critical enzymes in fetal gonad organs. It is interesting to notice that linuron exposure had no any

395 significant effect on AR gene expression. Recent data in literature have also suggested that

396 exposure to other gonadotoxicants such as polychlorinated biphenyls or acteoside can inhibit

397 P450scc activity and decrease testosterone synthesis, leading to hypospadias and cryptorchidism

398 (Mcglynn et al., 2009; Liu et al. 2015). Thus, our results establish that linuron exposure in

399 mothers inhibits the key enzymes indispensable to male sex hormone production. The exact

400 mechanism on how linuron interacts with these enzymes at molecular and/or genetic levels

401 remains unknown and deserves further in-depth investigation.

402 In summary, the present study confirms the developmental toxicity of linuron on male

403 reproductive system. Our data show that maternal exposure to linuron results in an altered

404 development of male gonadal organs, damaged seminiferous tubules, and abnormal Leydig cell

405 ultrastructure. The mechanism underlying linuron toxicity appears to be associated with the

406 direct action of the chemical on the production of testosterone in fetal and postnatal offspring.

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414 **Acknowledgments**

415 This study was partly supported by the Natural Science Foundation of Guizhou

416 Provincial Scientific and Technology Department Grant (J[2007]2125) (Li Yan), Education

417 Department of Guizhou Province ([2008]033) (Li Yan), International Scientific and Technology

418 cooperation project of Guizhou Province (G[2014]7012) (Li Yan), Innovative talent team training

419 project of Zunyi city ([2015]42) (Li Yan) and the Scientific and Technology Foundation of

420 Health and Family Planning Commission of Guizhou Provincial (D-424) (Li Yan).

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Figure Legends

Fig.1 Pathological changes of the genital tubercle following maternal exposure to linuron. Mother rats received oral gavage at 150 mg/kg from GD13 to GD18; fetal male gonadal organs at GD20 were dissected for examination. (A). A typical genital tubercle from a control fetus. Arrowhead indicates a normal urogenital fold. (B). A typical genital tubercle from a linuron-exposed fetus. Arrowhead indicates no fusion in the urogenital fold and the epithelial gaps. (×100)

Fig. 2 Pathological changes in seminiferous tubules following maternal exposure to linuron. Mother rats received oral gavage at 0 (A), 100 mg/kg (B), 150 mg/kg (C), or 200 mg/kg (D) from GD13 to GD18. Fetal male dams at GD20 were dissected for examination. Arrowheads indicate karyopyknosis and vacuoles. (×400).

Fig. 3 Ultrastructure of Leydig cells. Mother rats received oral gavage from GD13 to GD18; fetal male gonadal organ at GD20 were dissected for electron microscopic study. (A) A typical sample from a control rat. Arrowhead indicates a normal mitochondrion. (B) A typical sample from a rat treated with 150 mg/kg linuron. Arrowheads indicate a dilated endoplasmic reticulum. (C) A typical sample from a rat treated with 200 mg/kg linuron. Arrowhead indicates swollen mitochondrion. (×12500)

Fig. 4 Immunohistochemical analysis of PCNA, P450scc, 3β-HSD and AR expression in Leydig cells. Mother rats received oral gavage at 200 mg/kg from GD13 to GD18; fetal male gonadal organ at GD20 were dissected for IHC study. (×400)

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546 **Tables**

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548 Table 1. Forward and reverse primer sequence for selected genes in qPCR study

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)	
PCNA	AAGAGGAAGCTGTGTCCATAGA	CTTCATCTTCGATCTTGGGA	204bp
P450c17	GGAGAAGCTAATCTGTCAGGAA	GCATCCACGATACCCTCAGT	198bp
17β-HSD	CAGAAGAGATTGAGAGGACCA	CAGGAAATGACTTGGGAGCA	158bp
AR	GGACATGCGTTTGGACAGTA	ACTTCTGTTTCCCTTCCGCA	173bp
GAPDH	TGGGTGTGAACCACGAGAA	GGCATGGACTGTGGTCATGA	141bp

549 The forward and reverse primer sequences for selected genes were designed with the ABI Primer Express
550 software (Foster City, CA)

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553 Table 2. Comparison of testosterone (Tes), anogenital distance (AGD) and body weight

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Group (mg/kg)	PND2		PND28		
	n	Tes (ng/ml)	n	AGD (mm)	Body weight (g)
0 (control)	10	4.63±0.72	20	20.3±0.65	77.5±0.65
50	10	4.43±0.62	20	20.3±0.78	77.3±0.56
100	10	3.07±1.22*	20	16.7±1.11*	77.3±0.44
150	10	2.50±0.95*	20	16.5±0.69*	77.3±0.52
200	10	1.91±0.82*	20	15.3±0.84*	76.3±0.42

554 For PND2 experiments, male pups (2-3) from 4-5 litters in each treatment group underwent experimentation.
555 Animal numbers were doubled for PND28 study. Data represent mean ± SD. *: p<0.05 as compared to
556 controls. PND: postnatal day.

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561 Table 3. Quantitation of signal density of PCNA, P450scc, 3β-HSD and AR expression in GD20

562 testicular tissue by immunohistochemical analysis

Group (mg/kg/day)	n	Optical Density			
		PCNA	P450scc	3β-HSD	AR
control	8	1931±39	1683±21	1714±21	1238±68
200	8	1578±18 *	1422±14*	1424±28*	1277±62

563 Mother rats received oral gavage at 200 mg/kg from GD13 to GD18. Fetal male pups (2 from 4 litters in each

564 treatment group) at GD20 were dissected for IHC examination. The optical density for each protein was

565 analyzed using IPWIN60 software. Data represent mean ± SD, n=8 different fetal rats. *: p<0.05 compared to

566 controls.

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569 Table 4. Relative mRNA expression of PCNA, 17β-HSD, P450c17 and AR in GD20 testes

group	n	$2^{-\Delta\Delta Ct}$			
		PCNA	17β-HSD	P450c17	AR
control	10	1.64±0.98	1.87±0.26	1.53±0.50	1.51±0.43
exposure	10	0.87±0.32*	0.92±0.26*	0.92±0.42*	1.35±0.56

570 Mother rats received oral gavage at 200 mg/kg from GD13 to GD18. Fetal gonadal organs (2 from 5 litters in

571 each treatment group) at GD20 were dissected for qPCR analysis. Data represent mean ± S.D. *: p<0.05

572 compared to controls.

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