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Biochemical and Biophysical Research Communications 295 (2002) 193–197

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Neonatal exposure to endocrine disruptors suppresses juvenile testis weight and steroidogenesis but spermatogenesis is considerably restored during puberty

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Received 22 May 2002

Abstract

Neonatal exposure to endocrine disruptors induces developmental abnormalities in the male reproductive system. As to investigate whether neonatal exposure affects spermatogenesis in juvenile and pubertal testes, Sprague–Dawley rat pups were given various endocrine disruptors by a single injection on the day of birth at concentrations ranging between 4 μM and 40 mM and sacrificed on day 21 (juvenile) or 50 (puberty). The testes were weighed and examined histologically at each stage. Further, the metabolites of steroidogenesis were analyzed using normal-phase high performance liquid chromatography. Neonatal exposure significantly reduced testis weights and steroid biosynthesis of juveniles, but they were highly restored at puberty.

Keywords: Endocrine disruptor; Testis; Steroid; Spermatogenesis; Steroidogenesis

Endocrine disruptors include pesticides, herbicides, polycyclic aromatic hydrocarbons, polychlorinated dibenzodioxins, and alkylphenolic compounds [1–5]. These compounds affect cellular function and specific gene expression [1] and may pose a risk to both human and animal lives by altering reproductive development and function. In fact, these compounds have been linked with recent increases in the incidence of testicular cancer as well as decreases in sperm counts in humans [6–8], although the endocrine disruptors have been documented less conclusively in males than in females [9,10]. However, the relationship of these trends to environmental endocrine disruptors has not been established [2,3]. Exposure of rats to endocrine disruptors during the neonatal period causes marked developmental abnormalities in the testis [3,21]. The neonatal developmental changes that occur within the testis are extremely sensitive to endocrine disruptors, including androgens and estrogens [15–20], but the mechanism of this neonatal endocrine disruptor imprinting is not known. In the present study, we examined the effects of neonatal exposure to endocrine disruptors at different stages, corresponding to juvenile and pubertal testes. Our results show that administration of endocrine disruptors resulted in a significant reduction in juvenile testis weight and steroidogenesis, whereas they were largely recovered compared with untreated control testis at puberty.

Materials and methods

Animals. Female adult Sprague–Dawley rats and rat pups were purchased from Japan SLC (Shizuoka, Japan). Rat pups were injected s.c. with 25 μl sesame oil containing 40 mM benzophenone, 0.8 mM or 4 mM bis(2-ethylhexyl)phthalate, 40 mM *p,p′*-DDE (1,1-dichloro-2,2-bis-(4-chlorophenyl)ethylene), 40 mM methoxychlor, 2 mM styrene, 40 mM 2, 4, 5-trichlorophenoxyacetic acid, and 4 μM tributyltin(IV)chloride or sesame oil alone on day 1. Rat pups in

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0006-291X/02/$ - see front matter
PII: S0006-291X(02)00644-7

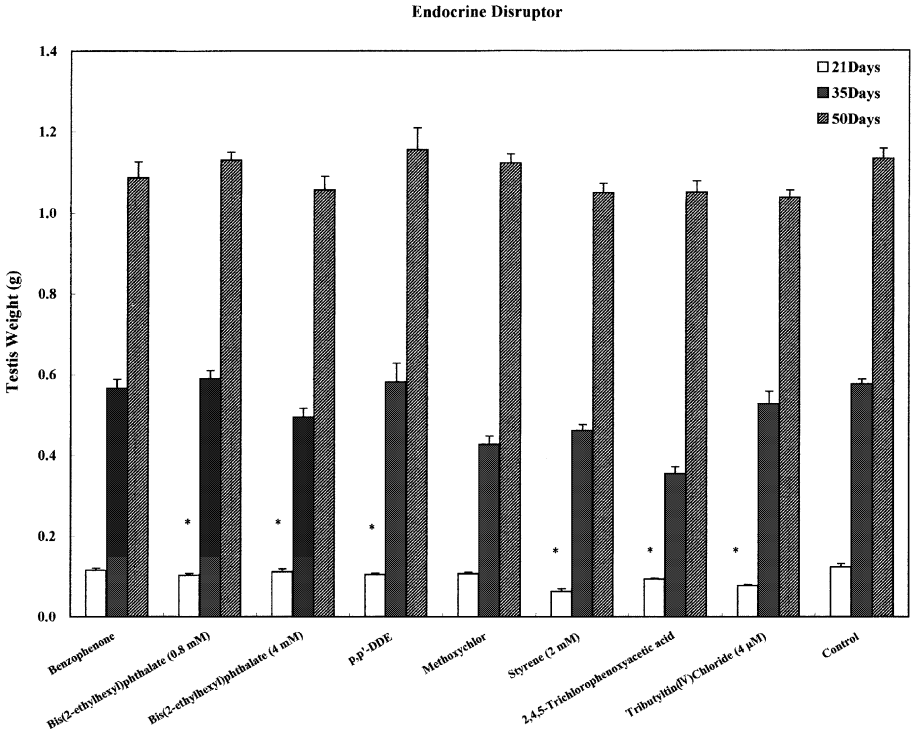

Fig. 1. All animals in a litter received a single injection of 25 μl sesame oil vehicle on the day of birth either alone (control) or containing 4 μM to 40 mM concentration of the various endocrine disruptors. The animals in various treatment groups ($n = 10$, 20, and 30) were killed at 21 days (juvenile), 35 days (prepuberty), and 50 days (puberty). Testes were collected. Each bar was the means ± SE. *, $P > 0.01$ (compared with control group).

various treatment groups ($n = 10$, 20, and 30) were killed on days 21 (juvenile), 35 (prepuberty), and 50 (puberty). Testes were isolated from the rats and their weights were recorded. Testosterone, 17-hydroxyprogesterone, and androstene-3,20-dione levels were also measured.

Preparation of testicular microsomes. Microsomes were prepared according to the method of Omura and Sato, with slight modification [15,16]. Fifty mM potassium phosphate buffer, pH 7.4, containing 250 mM sucrose, 100 μM EDTA, and 100 μM dithiothreitol was used throughout. Rat testes were decapsulated and sliced. The tissues were

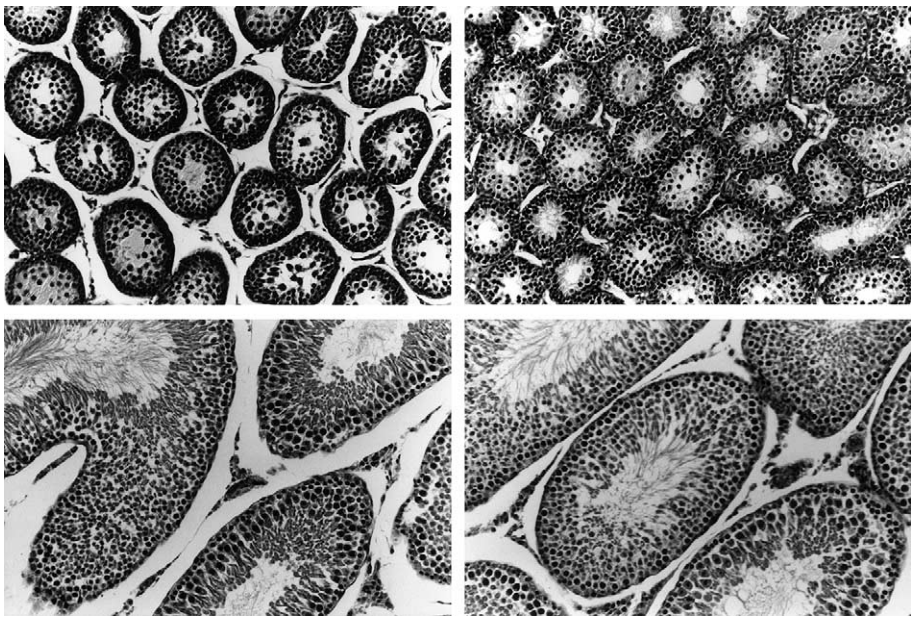

Fig. 2. Histology of rat testes killed at 21 days (upper panels) or 50 days (lower panels) of age. The animals were treated on the day of birth with either sesame oil (control: left panels) or 2 mM styrene (right panels). The photomicrographs were taken using ×20 objectives.

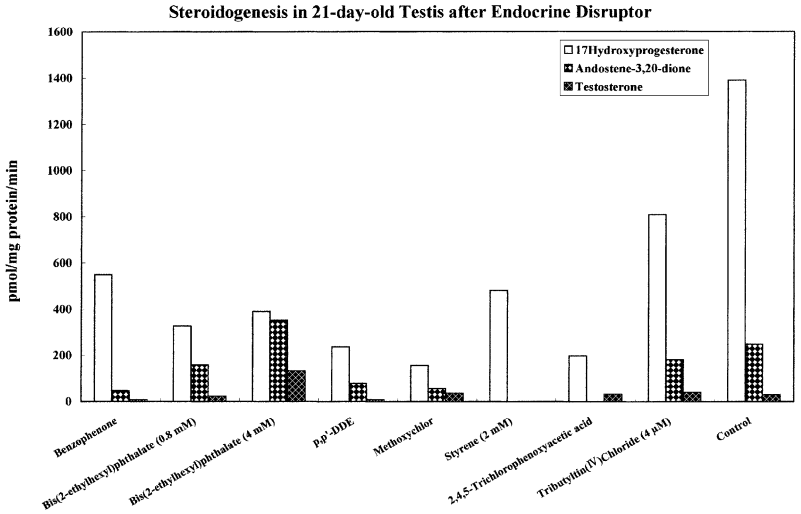

Fig. 3. Steroidogenesis in 21-day-old testis from rats neonatally treated with sesame oil (control) or one of the various endocrine disruptors. Testes catalyzed 17-hydroxyprogesterone of progesterone to androstenedione. Steroids were extracted, separated by HPLC, and analyzed as described in Materials and methods.

resuspended in 3 volumes of 50 mM potassium phosphate buffer and then homogenized with blender at 3000 rpm for 10 min in ice. The homogenate was centrifuged at 7000*g* for 20 min. This supernatant was centrifuged at 105,000*g* for 90 min in 4 °C. The precipitated microsomal pellets were resuspended in 2–3 volumes of 50 mM potassium phosphate buffer and kept at −80 °C until further use.

Incubation procedure. The addition of 100 nmol progesterone was introduced into 1 × 10 cm tubes and dissolved in 0.02 ml ethanol. To each tube, 0.5 ml incubation buffer solution was added. The buffer solution consisted of 300 mM potassium phosphate buffer, pH 7.4, 60 mM nicotinamide, 2 mM $MgCl_2$, 500 mM G6P, and G6PDH (0.5 U). The 0.5 ml suspension of the microsome fraction was then introduced to make the total volume of 1 ml. The mixture was incubated for 5 min at 35 °C before the addition of 200 nmol NADPH. The incubation was stopped at 30 min by adding 3.5 ml dichloromethane. Each sample was incubated in duplicate.

Histology. Tissue specimens from developing animals were examined for each treatment group. The sections were fixed in 10% formaldehyde. Fixed tissues were embedded in paraffin, mounted on slides, and stained with hematoxylin and eosin. The testicular tissue morphology was assessed by light microscopy.

Analytical methods. Each sample was extracted with 3.5 ml dichloromethane. The organic phase was washed with 2 ml of 0.1 N HCl, evaporated to dryness in a stream of nitrogen in a 40 °C water bath, and dissolved with 1 ml dichloromethane. Samples for high performance liquid chromatography (HPLC) were filtered with a Millipore

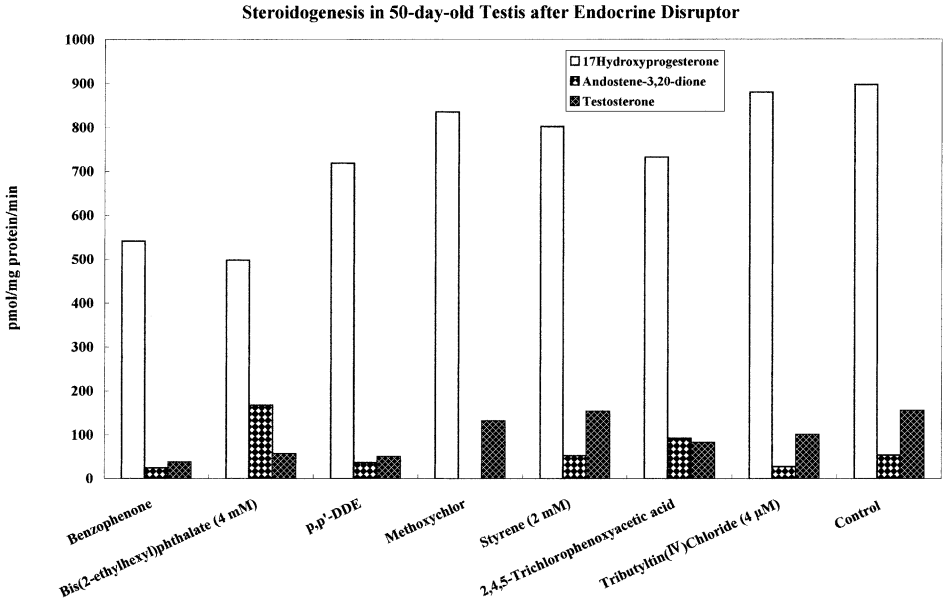

Fig. 4. Steroidogenesis in 50-day-old testis from rats neonatally treated with sesame oil (control) or one of the various endocrine disruptors. Testes catalyzed 17-hydroxyprogesterone of progesterone to androstenedione and further converted of androstenedione to testosterone. Steroids were analyzed as described in Materials and methods.

HV 0.45 μm disk filter. The extracted metabolites were finally dissolved in *n*-hexane, preparatory to HPLC. Metabolites were separated using normal-phase HPLC [17,18]. We used a TSK gel OH-120 (DIOL) obtained from Tosoh (Tokyo, Japan) with a mobile phase of solvent A:solvent B (1:5) at a flowrate of 1.5 ml/min. Components of solvents A and B were *n*-hexane and *n*-hexane:*i*-propanol = 70:30, respectively. We used a HPLC system (Shimadzu, Kyoto, Japan) equipped with LC-10 AD pump, an SPD-10 AUU-VIS detector, a TSK gel OH-120 column (4.6 mm × 250 mm), and a CTO-10A column oven. The column oven was maintained at 30 °C. The UV wavelength for detection was 246 nm. Sample volume was 20 μl.

Statistics. The testes weights were subjected to Student's *t* test to determine whether there were significant effects of treatment. Where these were indicated, subgroup comparisons between means for the control and individual treatment groups were then made using the variance from the experimental as a whole as the measure of error.

Results and discussion

Testis weight was affected by a variety of endocrine disruptors. Neonatal administration of six of the eight endocrine disruptors suppressed the testis weight in the juvenile (21 days) rats (Fig. 1). In particular, testis weight was extremely sensitive to tributyltin(IV)chloride (4 μM) and the higher concentration produced many deaths. Styrene (2 mM) and bis(2-ethylhexyl)phthalate (0.8 mM or 4 mM) also produced greater effects than the other endocrine disruptors at 40 mM concentration. At the pubertal stage (50 days), testis weights were similar in untreated control animals and all endocrine disruptor-treated animals (Fig. 1). Testis and prostate weights were significantly reduced in neonatally estrogen-treated rats, but during adulthood this decrease was restored to that of the control animals [19,20]. Testicular histology also revealed different susceptibilities to endocrine disruption of spermatogenesis and testicular development. Histological evaluation of testicular tissue from neonatally styrene-treated animals at the age of 21 days was different from that of untreated control animals. The Leydig cell morphology appeared normal, although the cells were organized as a sheath around the seminiferous tubules instead of as the single isolated clusters of cells seen in the control testis (Fig. 2). Neonatally styrene-treated animals and control animals at the age of 50 days revealed initiation of apparently normal spermatogenesis and the presence of germ cells in advanced stages of development (round and elongated spermatids). Leydig cells also appeared to be morphologically normal (Fig. 2). Steroidogenesis (androgen biosynthesis), which is essential for spermatid differentiation and maturation in the seminiferous tubules [21], occurred in the Leydig cells. These observations were supported by measurement of the metabolites of steroidogenesis (Figs. 3 and 4). Steroidogenesis in 21-day-old testis from neonatally treated animals was reduced with all endocrine disruptors, compared to untreated control animals (Fig. 3), but, steroidogenesis of many metabolites was considerably restored in 50-day-old testis (Fig. 4). Neonatal endocrine-disruptor exposure significantly reduced testis weights and steroid biosynthesis of 21-day-old testis (juvenile), but they were highly restored at 50 days (puberty). However, because the restoration of testis weight and steroid biosynthesis in 50 days did not equal the control levels, these data suggest that the mechanism of permanent neonatal estrogen imprinting is different from that of the endocrine disruptors in this experiment [22]. The postnatal developmental changes that occurred within the testis are extremely sensitive to several different hormones, including androgens and estrogens. To further understand the hypothalamic–pituitary–testicular axis and to provide insight into androgenic and estrogenic disruptor imprinting, investigation of spermatogenesis is now in progress.

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