Onset of Spermatogenesis Is Accelerated by Gestational Administration of 1,2,3,4,6,7-Hexachlorinated Naphthalene in Male Rat Offspring

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We treated pregnant rats with 1 µg/kg body weight/day 1,2,3,4,6,7-hexachlorinated naphthalene (1,2,3,4,6,7-HxCN) on days 14–16 of gestation and examined the effects on the reproductive systems of their male offspring at various phases of sexual maturation. Sperm count in the cauda epididymidis did not change in 1,2,3,4,6,7-HxCN-treated rats on postnatal day 89, the age of sexual maturity, but the sperm count in the cauda epididymidis did increase to approximately 180% of the control value on postnatal day 62. In addition, homogenization-resistant testicular spermatids increased to approximately 160% of the control value on postnatal day 48, and the percent of postmeiotic tubules increased to approximately 190% of the control value on postnatal day 31 in this group. These results indicate that the onset of spermatogenesis was accelerated in the 1,2,3,4,6,7-HxCN rats. Serum concentrations of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) had already reached the maximum level on postnatal day 31 in the 1,2,3,4,6,7-HxCN group, suggesting that the onset of LH and FSH secretions from the pituitary gland was also accelerated and that this endocrine disruption was the cause of early onset of spermatogenesis in this group. In the fat of 1,2,3,4,6,7-HxCN-treated dams, 5.75 ± 2.81 ppb 1,2,3,4,6,7-HxCN was detected when offspring were weaned. This concentration was 5–10 times higher than that found in human adipose tissue. *Key words*: endocrine disruption, gonadotropins, 1,2,3,4,6,7-hexachlorinated naphthalene, *in utero* and lactational exposure, onset of spermatogenesis, polychlorinated naphthalenes, rats. *Environ Health Perspect* 108:539–544 (2000). [Online 26 April 2000]

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Polychlorinated naphthalene (PCN) is one of the major halogenated aromatic hydrocarbons formed during municipal solid waste incineration; others include polychlorinated dibenzo-*p*-dioxin (PCDD) and polychlorinated biphenyl (PCB) (*1*). PCNs are widely detected in the environment and biota (*2–4*). PCNs were detected in the adipose tissue of a person intoxicated by consumption of a commercial rice oil contaminated with technical grade PCBs (Yusho rice oil) (*5*), and PCNs were also detected in the adipose tissue of individuals in the general population (*6,7*). The toxic effects of PCNs are comparable to those of PCDDs and PCBs (*8,9*). PCNs cause acnelike skin lesions, liver damage, and thymus atrophy in animals (*8,9*), and chloracne and liver dysfunction were reported in workers occupationally exposed to PCNs (*10–12*). Toxic effects of dioxins are mediated by the aryl hydrocarbon receptor (AhR), and AhR ligands induce cytochrome P-450 1A1-dependent enzyme activities such as ethoxyresorufin *O*-deethylase (EROD) and aryl hydrocarbon hydroxylase (AHH). Highly chlorinated naphthalenes induce these enzyme activities *in vivo* (*13–15*), and some hexachlorinated and heptachlorinated naphthalenes are as potent as certain coplanar PCBs (*16*). Male rats born to dams treated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) during gestation have various types of damage in their reproductive function and androgenic status (*17–22*). These adverse effects include decreases in epididymal sperm count and sex accessory organ weights, altered sexual behaviors, decreased anogenital distance, and delayed puberty. To our knowledge, adverse effects of PCNs on reproduction have not been examined. PCNs probably cause adverse effects that are mediated by AhR; the adverse effects of PCNs are comparable to those of dioxins. Therefore, PCNs may cause adverse effects on reproductive function of male rats born to dams given those compounds during gestation.

In this study, we treated pregnant rats with 1,2,3,4,6,7-hexachlorinated naphthalene (1,2,3,4,6,7-HxCN) and examined the effects on the reproductive system of their male offspring at various phases of sexual maturation. In addition, we analyzed 1,2,3,4,6,7-HxCN concentrations in the fat of dams and offspring in order to compare the exposure levels of 1,2,3,4,6,7-HxCN between the rats in this study and humans. We chose 1,2,3,4,6,7-HxCN as a test material because it is one of the most potent inducers of EROD and AHH among PCNs (*16*), and it is also the most strongly bioaccumulating PCN in animals and humans (*5,6,23*).

Materials and Methods

Animals. We purchased 14 pregnant Wistar rats (Kud:Wistar; Kyudo Co., Ltd., Tosu, Japan) on day 12 of gestation (the day after mating is day 0) and housed the animals individually in aluminum cages with wood shavings as bedding. The rats were randomly divided into a 1,2,3,4,6,7-HxCN group and a control group, each comprising 7 rats. Animals were maintained in a room with a temperature of 20–24°C and with a relative humidity of 30–60%; rats were provided with CE-2 feed (Clea Japan Inc., Tokyo, Japan) and tap water *ad libitum*. We analyzed concentrations of 1,2,3,4,6,7-HxCN, 2,2(4-chlorophenyl)-1,1-dichloroethene (DDE), and PCBs in the feed six times during the experiment with the following results: 1,2,3,4,6,7-HxCN, below the detection limit (0.01 ppb); DDE, 0.21 ± 0.03 ppb; and PCBs, 0.15 ± 0.01 ppb. This experiment was reviewed by the Committee of Ethics on Animal Experiments in the Faculty of Medicine, Kyushu University, and was carried out under the Guideline for Animal Experiment in the Faculty of Medicine, Kyushu University, and the Law (No. 105) and Notification (No. 6) of the Government of Japan.

Treatment. We purchased 1,2,3,4,6,7-HxCN in a *n*-nonane solution (100 µg/mL *n*-nonane) from Cambridge Isotope Laboratories (Woburn, MA). We administered 1 µg/kg body weight (bw)/day 1,2,3,4,6,7-HxCN in corn oil (2.0 mL/kg bw) by gavage to pregnant rats in the 1,2,3,4,6,7-HxCN group between 1600 and 1700 hr on days 14, 15, and 16 of gestation. We administered 0.01 mL/kg bw/day of *n*-nonane (purity > 99%; Tokyo Kasei Organic Chemicals, Tokyo, Japan) in corn oil to control rats in the same manner. We chose the daily dose of 1.0 µg/kg bw 1,2,3,4,6,7-HxCN because this dose has been frequently used in the same type of experiments with TCDD (*17–21*). We chose the administration period, days 14–16 of gestation, because sexual differentiation begins on those days.

On the day of birth [postnatal day 0 (PND 0)], offspring were counted, sexed, and examined for gross malformations, and litters were randomly reduced to five males and five females, when possible. Body weight and anogenital distance (AGD) were recorded on PNDs 1, 4, and 21, and offspring were examined for eye opening on PNDs 14–18. Offspring were weaned on PND 21 and were housed by sex, based on litters. On PNDs 31, 48, 62, and 89, one male offspring from each litter was chosen at random and killed by CO_2 inhalation; blood was collected from the posterior vena cava. Serum was separated by centrifugation and was stored at -80°C. The testes, epididymides, ventral prostate, and seminal vesicles were removed and weighed. From each litter, we removed the whole body of one female offspring on PND 0, and we removed the abdominal fat from one female offspring killed on PNDs 21 and 89; these were stored at -80°C until analysis for 1,2,3,4,6,7-HxCN, DDE, and PCBs. We also collected and stored the abdominal fat of dams killed at weaning for analysis.

Homogenization-resistant spermatid and sperm counts. We homogenized the decapsulated testes in saline containing 0.05% (v/v) Triton X-100 in a blender and counted homogenization-resistant spermatids using a hemocytometer. We also counted sperm collected from the cauda epididymides in the same manner.

Sperm motility. The epididymis was clamped at the corpus-cauda junction, and the vas deferens was clamped as well. The distal cauda epididymidis was then cut with a razor. Sperm was allowed to diffuse into medium 199 (M199) with Hanks' salts and L-glutamine (GIBCO, Grand Island, NY) containing 0.5% bovine serum albumin (Katayama Chemical, Osaka, Japan). The sperm sample was incubated in a glass chamber for 15 min at 37°C and then diluted with M199 solution (1:3). We observed the motility of approximately 100–150 sperm using a microscope equipped with a stage warmer set at 37°C. We counted sperm with progressive and nonprogressive motility and immotile sperm and calculated percentages of motile sperm and progressively motile sperm.

Tissue preparation and histopathology. The testes were fixed in Bouin's solution, embedded in paraffin, sectioned at 3 μm, and stained with periodic acid Schiff (PAS) reagent and hematoxylin. In the histopathologic examination, we classified seminiferous tubules into 14 stages (stages I–XIV), but we combined stages II and III according to the method of Russell et al. (24). We examined 300–500 seminiferous tubules in each rat to calculate the percentage of postmeiotic seminiferous tubules at PNDs 31 and 48. We defined postmeiotic seminiferous tubules as

those seminiferous tubules containing spermatids, regardless of the number of spermatids. We calculated the percentage of seminiferous tubules containing step 7 or more advanced spermatids on PND 31, and we calculated the percentage of seminiferous tubules containing step 19 spermatids on PND 48.

Hormone determinations. We measured serum concentrations of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) by radioimmunoassay. We used the Biotrak rat luteinizing hormone (rLH) [125I] assay system (Amersham Life Science Ltd, Buckinghamshire, UK) and the Biotrak rat follicle stimulating hormone (rFSH) [125I] assay system (Amersham Life Science Ltd). We used lyophylized rat FSH and LH from each testkit as standards for these assays. For each assay, we randomized all samples before analysis.

Measurement of 1,2,3,4,6,7-HxCN, DDE, and PCB concentrations. We obtained 1,2,3,4,6,7-HxCN, PCB congeners, and DDE from Cambridge Isotope Laboratories, Woburn, Massachusetts; Analabs Inc., North Haven, Connecticut; and Kanto Chemical Co., Inc., Tokyo, Japan, respectively. We purchased n-hexane, acetone, and ethanol (special grade for PCB analysis) from Kanto Chemical Co., Inc. Wako gel S-1 for PCB analysis (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and anhydrous sodium sulfate (special grade for PCB analysis; Kanto Chemical Co., Inc.) were heated at 450°C for 24 hr before use. We cleaned all water used for analysis with n-hexane twice before use.

Fat tissues from dams (0.7–2.6 g) and offspring (0.09–4.3 g), new born offspring (5.1–6.8 g) sliced to 1–2 mm thickness with scissors, and CE-2 animal feed (3.2–3.6 g) soaked in 5 mL water were mixed with acetone:n-hexane (2:1; 15 mL total solution) and sonicated for 10 min. The acetone/n-hexane layer was separated and the remainder was

extracted again with acetone/n-hexane (2:1; 15 mL solution) and sonicated. The acetone/n-hexane extracts were combined and mixed with 10 mL water. We separated the upper n-hexane layer and extracted the lower layer again with 5 mL n-hexane. We combined the n-hexane layers and dried them over anhydrous sodium sulfate. We then transferred the dried n-hexane solution to a weight-determined vial (30 mL). We held the vial for 1 or 2 days on a metal block, which was kept at 50°C; the vial was then weighed to determine the fat content of the tissue samples. We added 4–14 mL 1N KOH, 4–14 mL ethanol, and 5 ng 2,2′,3,4,5,5′,6-heptachlorobiphenyl for an internal standard. The mixture was heated to 50°C, held for 2 hr, and stored at room temperature overnight; 5–16 mL water was then added. The solution was extracted with 5 mL n-hexane two or three times. The combined n-hexane solution was washed with 2 mL water and dried over 2 g anhydrous sodium sulfate. The dried n-hexane was charged on a column of 1 g silica gel, which was later eluted with 20–30 mL n-hexane. We concentrated the n-hexane eluate to ≤ 1 mL. We analyzed an aliquot of the concentrated solution using gas chromatographic analysis. For blank analyses, we used a mixture of water, 1N KOH ethanol solution, and 5 ng internal standard and followed the same procedures.

We used a gas chromatograph with an electron capture detector and an auto injector (Shimadzu GC-14A, AOC-14; Shimadzu, Kyoto, Japan), which was equipped with a 0.25 mm × 60 m DB5MS capillary column (J & W Scientific, Folsom CA). The column temperature was kept at 70°C for 2 min, programmed to 240°C by 20°C/min and to 290°C by 4°C/min, and maintained at 290°C for 30 min. Pure nitrogen (99.999%) was used as carrier gas at a flow rate of 0.7 mL/min. We determined the concentrations of 1,2,3,4,6,7-HxCN, individual PCBs, and

Table 1. Effects of gestational (days 14–16) administration of 1.0 μg/kg bw/day 1,2,3,4,6,7-HxCN on growth of offspring during lactation.

	Male offspring		Female offspring	
	Control	1,2,3,4,6,7-HxCN	Control	1,2,3,4,6,7-HxCN
Body weight (g)				
PND 1	6.7 ± 0.7	6.9 ± 0.7	6.5 ± 0.8	6.7 ± 0.8
PND 4	9.7 ± 1.6	10.7 ±1.4	9.4 ± 1.8	10.5 ± 1.4
PND 21	44.1 ± 4.3	47.1 ± 6.7	43.2 ± 3.2	46.2 ± 6.5
AGD (mm)				
PND 1	5.1 ± 0.4	4.9 ± 0.3	2.8 ± 0.2	2.7 ± 0.2
PND 4	6.6 ± 0.5	6.6 ± 0.2	3.8 ± 0.3	3.9 ± 0.4
PND 21	14.7 ± 0.7	15.6 ± 0.1	9.9 ± 0.4	10.1 ± 1.1
AGD (mm/g$^{1/3}$)[a]				
PND 1	2.7 ± 0.2	2.6 ± 0.2	1.5 ± 0.1	1.5 ± 0.1
PND 4	3.1 ± 0.3	3.0 ± 0.2	1.8 ± 0.2	2.0 ± 0.5
PND 21	4.2 ± 0.1	4.3 ± 0.1	2.8 ± 0.1	2.8 ± 0.2
Eye opening (PND)	16.2 ± 0.5	15.9 ± 0.5	16.0 ± 0.5	15.7 ± 0.4

All values were calculated and analyzed using litter means. Results are expressed as mean ± SD ($n = 7$).
[a]AGD(mm/g$^{1/3}$) = AGD (mm)/[bw(g)]$^{1/3}$.

DDE by the internal standard method, using relative peak areas of individual peaks and the internal standard peak determined by a Shimadzu Chromatopac C-R7A automatic peak area determinator.

Statistical analysis. We analyzed statistical difference using the Student's *t*-test. Results were interpreted as significant at $p < 0.05$.

Results

Mothers and offspring during lactation. All dams delivered their offspring. There were 12.6 ± 1.9 control offspring/litter and 13.9 ± 2.3 1,2,3,4,6,7-HxCN offspring/litter; all offspring were born live in the 1,2,3,4,6,7-HxCN group and 2 of 15 offspring were born dead in one control litter. We found no apparent gross malformations in offspring. The male:female ratios (% male) were 48.4 ± 14.9% in the control group and 49.1 ± 12.9% in the 1,2,3,4,6,7-HxCN group.

Body weights on PNDs 1, 4, 21; the percentage of survival during lactation; and the

Table 2. Effects of gestational (days 14–16) administration of 1.0 µg/kg bw/day 1,2,3,4,6,7-HxCN on organ weights, sperm count, and sperm motility of male offspring.

	Control	1,2,3,4,6,7-HxCN
Body weight (g)		
PND 31	99.4 ± 13.6	99.8 ± 13.2
PND 48	229.9 ± 23.4	242.7 ± 18.1
PND 62	332.9 ± 29.4	332.9 ± 19.3
PND 89	453.1 ± 29.7	458.5 ± 14.3
Testis weight (g)		
PND 31	0.613 ± 0.175	0.758 ± 0.197
PND 48	2.234 ± 0.274	2.427 ± 0.242
PND 62	3.129 ± 0.209	3.207 ± 0.303
PND 89	3.599 ± 0.343	3.522 ± 0.237
Epididymis weight (g)		
PND 31	0.080 ± 0.013	0.086 ± 0.014
PND 48	0.263 ± 0.045	0.279 ± 0.037
PND 62	0.609 ± 0.067	0.653 ± 0.075
PND 89	1.148 ± 0.167	1.077 ± 0.051
Seminal vesicle weight (g/100 g bw)		
PND 31	0.019 ± 0.003	0.023 ± 0.003
PND 48	0.067 ± 0.015	0.089 ± 0.021*
PND 62	0.172 ± 0.033	0.205 ± 0.033
PND 89	0.241 ± 0.066	0.261 ± 0.035
Ventral prostate weight (g/100 g bw)		
PND 31	0.036 ± 0.007	0.043 ± 0.014
PND 48	0.052 ± 0.014	0.052 ± 0.011
PND 62	0.065 ± 0.013	0.079 ± 0.012*
PND 89	0.088 ± 0.021	0.086 ± 0.017
Caudal sperm count ($\times 10^6$)		
PND 62	24.7 ± 8.2	45.6 ± 10.3**
PND 89	170.0 ± 20.9	178.7 ± 11.3
Sperm motility		
Percent motile		
PND 62	82.5 ± 10.8	87.0 ± 2.6
PND 89	86.6 ± 5.5	88.4 ± 3.3
Percent progressively motile		
PND 62	49.8 ± 27.5	46.1 ± 23.7
PND 89	63.6 ± 26.8	48.2 ± 31.1

Results are expressed as mean ± SD ($n = 7$). Statistical significance was analyzed with Student's *t*-test.
*$p < 0.05$; **$p < 0.01$.

day of eye opening were not significantly different between the control group and the 1,2,3,4,6,7-HxCN group (Table 1). The AGD was expressed by the absolute AGD value and by the absolute AGD value/body weight$^{1/3}$, which was used as an index of AGD to correct the influence of body size on absolute AGD value. Irrespective of the indices, the AGD values on PNDs 1, 4, and 21 were not significantly different between the two groups.

Effects of 1,2,3,4,6,7-HxCN on the male reproductive system. Body weight was not significantly different between the two groups at any age (Table 2). Neither the testis weight nor the epididymis weight was different between the two groups. In the 1,2,3,4,6,7-HxCN group, the testis weight increased on PNDs 31 and 48 and the epididymis weight increased on PND 62, but the increases were not significant. In the 1,2,3,4,6,7-HxCN group, there was a significant increase in the seminal vesicle weight on PND 48 and an increase, although not significant ($p = 0.10$), on PNDs 31 and 62. The ventral prostate weight increased nonsignificantly ($p = 0.07$) on PND 62 in the 1,2,3,4,6,7-HxCN group. On PND 89, the age of sexual maturity, the caudal sperm count was not significantly different between the two groups, but on
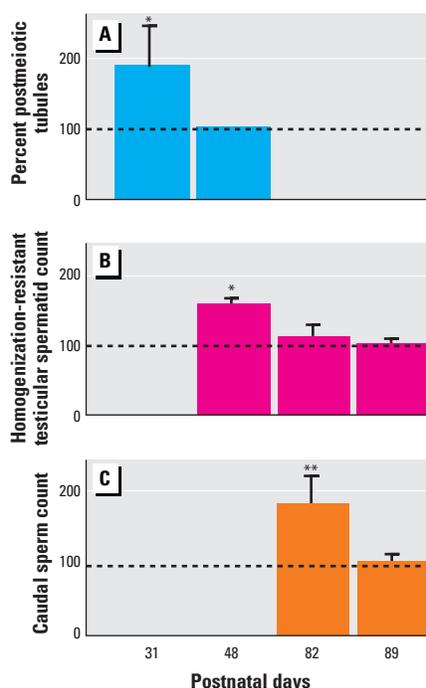

Figure 1. Effects of 1.0 µg/kg bw/day 1,2,3,4,6,7-HxCN administered on gestation days 14–16 on the percentage of postmeiotic tubules, the homogenization-resistant testicular spermatid count, and the caudal sperm count. Data are expressed as the percent of mean control value ($n = 7$); error bars indicate SD. Statistical significance was analyzed with Student's *t*-test.
*$p < 0.05$; **$p < 0.01$.

PND 62, the caudal sperm count in the 1,2,3,4,6,7-HxCN group increased to approximately 180% of the control value. Sperm motility was not different between the two groups.

Testicular spermatid count and the percentage of postmeiotic tubules. Advanced spermatids have a highly condensed nucleus and are resistant to homogenization. Therefore, the number of homogenization-resistant testicular spermatids reflects the number of advanced spermatids (steps 17–19) in the testis. On PNDs 62 and 89, the number of homogenization-resistant testicular spermatids was not significantly different between the 1,2,3,4,6,7-HxCN group (Figure 1) and the control group (Table 3). On PND 48, testicular spermatids in the 1,2,3,4,6,7-HxCN group increased to approximately 160% of the control value. In the histopathologic examination, almost all seminiferous tubules reached the postmeiotic phase in the two groups; the percentage of postmeiotic tubules was not significantly different between the two groups on PND 48, although the percentage of seminiferous tubules containing step 19 spermatids increased to approximately 160% of the control value in the 1,2,3,4,6,7-HxCN group (29.0 ± 2.7% vs. 18.3 ± 11.7% in controls). On PND 31, the percentage of postmeiotic tubules in the 1,2,3,4,6,7-HxCN group increased to approximately 190% of the control value and the percentage of seminiferous tubules containing step 7 or more advanced spermatids increased to 700% of the control value (2.8 ± 3.5% vs. 0.4 ± 0.8% in the control group). Figure 2 and Figure 3 show representative cross-sections of rat seminiferous tubules on PND 31 in the control and 1,2,3,4,6,7-HxCN groups, respectively. Figure 2 shows seven complete or partial tubular cross-sections, three of which are postmeiotic tubules. The most advanced spermatids observed in Figure 2 are step 2–3. Figure 3 shows seven complete or partial tubular cross-sections, five of which are
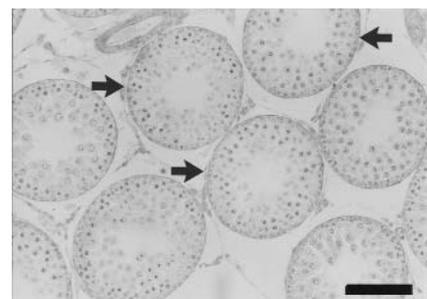
Figure 2. Cross sections of seminiferous tubules on PND 31 in control rats. Three of the seven complete or partial tubular cross-sections are postmeiotic tubules (indicated by arrows). The most advanced spermatids are step 2–3 spermatids. Bar indicates 100 µm.

postmeiotic tubules. Step 7 spermatids are present in Figure 3A.

Results of the evaluations of the percentage of postmeiotic tubules on PND 31, the testicular spermatid count on PND 48, and the caudal sperm count on PND 62 indicated that the onset of spermatogenesis was accelerated in the 1,2,3,4,6,7-HxCN group. The onset of spermatogenesis in this group could be affected by litter size and by the intrauterine position of fetus; therefore, we tried to evaluate these effects on spermatogenesis. Because the offspring were not delivered by caesarean section in this study, it was impossible to determine the position of offspring in the uterus. If the male:female ratio is high in the uterus, there is a high possibility that many male fetuses are beside one male (1M) or between two males (2M). Therefore, we used the male:female ratio at birth as a representative value of intrauterine position of the fetus. Figure 4 shows the relationship between litter size at birth and the percentage of postmeiotic tubules on PND 31, the testicular spermatid count on PND 48, and the caudal sperm count on PND 62; Figure 5 shows the relationship between the male:female ratio at birth and these evaluation values. The more progressed spermatogenesis in the 1,2,3,4,6,7-HxCN group was not due to small litter size and a high male:female ratio on any postnatal day.

Serum concentrations of LH and FSH. In the control group, serum concentrations of LH and FSH increased from PND 31 to PND 48 (Table 4); afterward, the LH concentration reached a plateau and the FH concentration decreased. In the 1,2,3,4,6,7-HxCN group, the serum concentrations of

LH and FSH reached the plateau (or the maximum value) on PND 31.

1,2,3,4,6,7-HxCN, DDE, and PCBs in body fat. DDE and PCBs were detected in the fat of the dams and offspring in both groups; the concentrations of these compounds were not significantly different between the groups (Table 5). The 1,2,3,4,6,7-HxCN concentration was below the detection limit in all fat samples in the control group. In the 1,2,3,4,6,7-HxCN group, the concentration of 1,2,3,4,6,7-HxCN in fat was 22.18 ± 6.59 ppb at birth (PND 0), 9.78 ± 2.86 ppb at weaning (PND 21), 0.45 ± 0.08 ppb on PND 89 in female offspring, and 5.75 ± 2.81 ppb in dams at weaning. In this group, the total amount of 1,2,3,4,6,7-HxCN in fat was 1.48 ± 1.64 ng/pup at birth and was estimated to be 13.1–26.2 ng/pup at weaning. To calculate the amount of 1,2,3,4,6,7-HxCN per pup at weaning, we calculated the percentage of fat (relative to body weight) to be 3–6%, as described previously (*25,26*).

Discussion

The toxicities of PCNs are comparable to those of dioxins (*8,9*); TCDD damages reproductive functions of male rats whose mothers were exposed to TCDD during gestation (*17–22*). Therefore, we administered 1,2,3,4,6,7-HxCN to pregnant rats and examined the effects on the reproductive system of male offspring at various phases of sexual maturity, based on the hypothesis that 1,2,3,4,6,7-HxCN causes reproductive damages to male offspring.

On PND 89, the age of sexual maturity, male reproductive functions were not affected by 1,2,3,4,6,7-HxCN. On PND 62 in the

1,2,3,4,6,7-HxCN group, the sperm count in the cauda epididymidis increased to approximately 180% of the control value. There are two mechanisms that can explain this phenomenon. In one mechanism, gestational 1,2,3,4,6,7-HxCN exposure increased the sperm production capacity of male offspring and increased caudal sperm count on PND 62. Because the caudal sperm count in the 1,2,3,4,6,7-HxCN group did not increase on PND 89, this mechanism is improbable. The other mechanism involves 1,2,3,4,6,7-HxCN acceleration of the onset of sperm production in the testis so that sperm begin to fill the cauda epididymidis earlier than usual; thus, the caudal sperm count temporarily increases on PND 62. Mature spermatids (step 19) first appear in the testis on PND 50 (*27*) and it takes 6–7 days for the sperm to pass through the epididymis and reach the cauda (*28*); thus, PND 62 is only a few days after the first appearance of sperm in the cauda epididymis.

If an early onset of sperm production actually occurred in the 1,2,3,4,6,7-HxCN group, the first spermatogenic wave had to proceed more than usual on PNDs 31 and 48. According to earlier studies (*27,29,30*), PND 31 is approximately 1 week after the first appearance of postmeiotic germ cells

Table 3. Percent postmeiotic tubules and the number of homogenization-resistant testicular spermatids in the control group.

	PND 31	PND 48	PND 62	PND 89
Percent postmeiotic tubules	24.9 ± 18.6	99.9 ± 0.1	NE	NE
No. homogenization-resistant testicular spermatids ($\times 10^6$/testis)	NE	36.3 ± 19.3	98.8 ± 15.0	150.2 ± 18.0

NE, not examined. Results are expressed as mean ± SD; n = 7 except on PND 62 (n = 6).
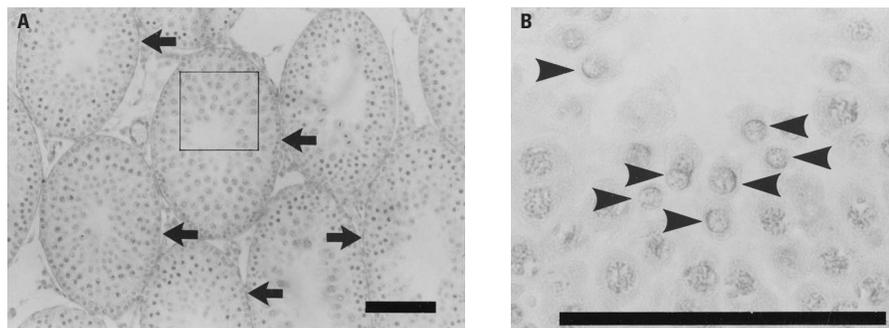
Figure 3. Cross-sections of seminiferous tubules on PND 31 in the 1,2,3,4,6,7-HxCN group. (*A*) Five of the seven complete or partial tubular cross-sections are postmeiotic tubules (indicated by arrows). (*B*) Enlargement of the postmeiotic tubule indicated by the square in (*A*). Step 7 spermatids are indicated by arrow heads. Bars indicate 100 μm.
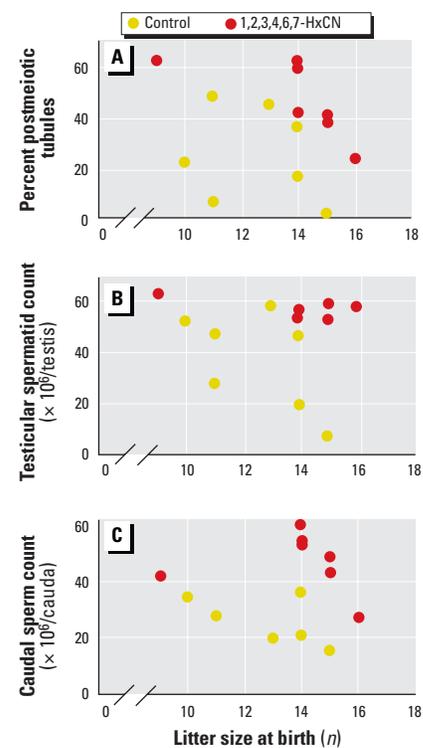
Figure 4. Relationship between litter size at birth (*n*) and (*A*) percent of postmeiotic tubules on PND 31, (*B*) homogenization-resistant testicular spermatid count on PND 48, and (*C*) caudal sperm count on PND 62 in control and 1,2,3,4,6,7-HxCN-treated rats.

(step 1 spermatid), and PND 48 is approximately 1 week after the first appearance of step 15–18 spermatids in the testis. Therefore, we expected that the percentage of postmeiotic tubules would be increased on PND 31 and that homogenization-resistant testicular spermatids, which reflects the number of advanced spermatids, would be increased on PND 48 in the 1,2,3,4,6,7-HxCN group. As shown in Figure 1, this is what actually occurred. The percentage of postmeiotic tubules increased to approximately 190% of the control value on PND 31, and homogenization-resistant testicular spermatids increased to approximately 160% of the control value on PND 48. We did not confirm the age of spermatogenesis onset in this study, but the results of this study strongly suggested that the onset of spermatogenesis was accelerated in male rats born to dams administered 1,2,3,4,6,7-HxCN during gestation.

vom Saal and colleagues (31–33) showed that the intrauterine position of a fetus influences fetal hormone levels and affects pubertal development after birth. Litter size (the number of pups in the uterus) also affects pubertal development after birth. The acceleration of the onset of spermatogenesis in the 1,2,3,4,6,7-HxCN group could be caused by

small litter size and/or a high ratio of 1M and 2M male fetuses; therefore, we tried to evaluate these effects on spermatogenesis. We could not determine the position of offspring in the uterus because they were not delivered by caesarean section; therefore, we used the male:female ratio at birth as a representative value of the intrauterine position of fetus. As shown in Figures 4 and 5, the more progressed spermatogenesis in the 1,2,3,4,6,7-HxCN group was not attributible to small litter size and a high male:female ratio at birth. Therefore, we believe that the acceleration of the onset of spermatogenesis was caused by gestational and lactational exposure to 1,2,3,4,6,7-HxCN. Contrary to our expectation, 1,2,3,4,6,7-HxCN did not cause adverse effects on the reproductive system, such as a decrease in caudal sperm count, in male offspring. But this compound certainly affected the reproductive system of male offspring.

Why was the onset of spermatogenesis accelerated in the 1,2,3,4,6,7-HxCN group? In adult rats, spermatogenesis is maintained primarily by testosterone secreted from Leydig cells, and LH secreted from the pituitary gland stimulates testosterone secretion (34). In immature rats, FSH plays an important role in the control of Sertoli cell function and the regulation of Leydig cell development. In addition, spermatogenesis is initiated as a consequence of increased secretion of FSH (34). In this study, serum concentrations of LH and FSH were still increasing on PND 31, and they reached the plateau level (LH) or the maximum level (FSH) on PND 48 in the control group. These results were consistent with those reported previously (27,29). In the 1,2,3,4,6,7-HxCN group, the

serum concentrations of LH and FSH had already reached the plateau level or maximum level on PND 31. From these results, we estimated that the onset of LH and FSH secretions from the pituitary gland was accelerated in the 1,2,3,4,6,7-HxCN group and that this was the cause of the early onset of spermatogenesis in this group. Almirón et al. (35) reported that when testosterone propionate alone was subcutaneously injected into immature rats from PND 5 to PND 35, the progression of spermatogenesis was suppressed. Nonetheless, when human menopausal gonadotropin (a mixture of FSH and LH) was coinjected with testosterone propionate, more advanced spermatids were observed as compared to spermatids from control rats on PND 35. The results (35) indicated that an early increase in serum FSH concentration accelerated the onset of spermatogenesis. This supports our conclusion.

In the analysis of body fat from female offspring and dams, we detected DDE and PCBs in both exposed and control animals, and concentrations of these compounds did not differ between the two groups. The feed used in this study contained approximately 200 ppt DDE and PCBs; these compounds found in the fat probably originated in the feed. Commercial rodent diets often contain high amounts of phytoestrogens (36,37). Therefore, the results of this study might not apply to a situation in which animals are exposed only to 1,2,3,4,6,7-HxCN.

In the 1,2,3,4,6,7-HxCN group, the newborn female rats contained 1.48 ± 1.64 ng 1,2,3,4,6,7-HxCN, and the female offspring at weaning were estimated to contain 13.1–26.2 ng of this compound in fat. These
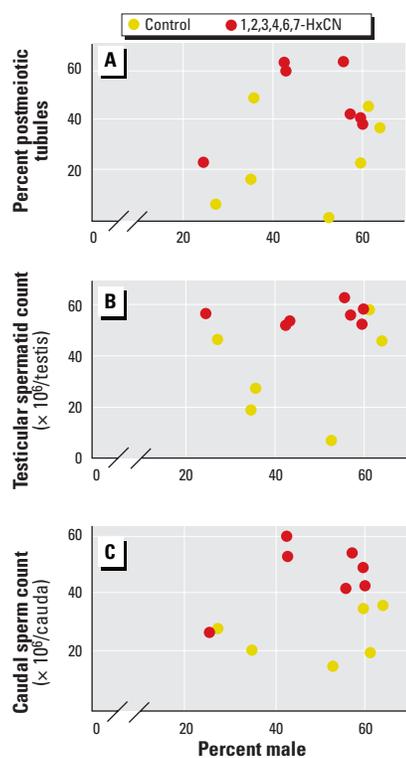
Figure 5. Relationship between the percentage of males at birth and (*A*) the percentage of postmeiotic tubules on PND 31, (*B*) the homogenization-resistant testicular spermatid count on PND 48, and (*C*) the caudal sperm count on PND 62 in control and 1,2,3,4,6,7-HxCN-treated rats.

Table 4. Effects of administration of 1.0 µg/kg bw/day 1,2,3,4,6,7-HxCN on days 14–16 of gestation on serum concentrations of LH and FSH.

	PND 31	PND 48	PND 62	PND 89
LH (ng/ml)[a]				
Control	1.9 ± 0.4	3.7 ± 1.3	3.6 ± 1.1	4.2 ± 1.2
1,2,3,4,6,7-HxCN	$3.3 \pm 1.1^*$	3.4 ± 1.4	3.7 ± 1.2	3.6 ± 0.8
FSH (ng/ml)[a]				
Control	13.7 ± 5.3	17.3 ± 5.9	13.9 ± 4.7	13.0 ± 3.7
1,2,3,4,6,7-HxCN	$19.1 \pm 1.7^*$	18.8 ± 8.2	14.9 ± 7.8	11.3 ± 2.1

Statistical significance was analyzed with Student's *t*-test.
[a]Results are expressed as mean ± SD; $n = 7$ except on PND 62 ($n = 6$). $^*p < 0.05$.

Table 5. Concentrations of 1,2,3,4,6,7-HxCN, DDE, and PCBs in fat from female offspring and dams (ppb).

Females	Group	1,2,3,4,6,7-HxCN	DDE	PCBs
Offspring				
PND 0	Control	ND	10.52 ± 4.89	6.36 ± 1.00
	1,2,3,4,6,7-HxCN	22.18 ± 6.59	13.49 ± 6.37	6.73 ± 3.17
PND 21	Control	ND	35.56 ± 12.86	13.59 ± 4.81
	1,2,3,4,6,7-HxCN	9.78 ± 2.86	27.98 ± 9.39	9.28 ± 3.89
PND 89	Control	ND	16.44 ± 2.88	9.01 ± 1.07
	1,2,3,4,6,7-HxCN	0.45 ± 0.08	15.86 ± 3.40	9.70 ± 1.01
Dams	Control	ND	15.78 ± 2.98	14.06 ± 5.17
	1,2,3,4,6,7-HxCN	5.75 ± 2.81	15.43 ± 3.11	14.57 ± 3.95

ND, not detected. Results are expressed as mean ± SD ($n = 7$). The detection limits of 1,2,3,4,6,7-HxCN in offspring were 5 ppb for PND 0, 2 ppb for PND 21, 0.05 ppb for PND 89, and 0.1 ppb for dams.

results indicated that 1,2,3,4,6,7-HxCN was transferred from dam to offspring via both the placental and lactational routes and that the amount of this compound transferred via the latter route was one order of magnitude greater than that transferred via the former route. This transfer pattern was the same as that of TCDD, PCBs, and polychlorinated dibenzofurans (*38–41*).

In this study, we detected 5.75 ± 2.81 ppb 1,2,3,4,6,7-HxCN in the fat of dams treated with this compound during gestation. Three studies have reported the concentration of PCNs in human adipose tissue:

Takeshita and Yoshida (*7*) examined the adipose tissue obtained during forensic autopsies and detected 6.5 ± 4.5 ppb PCNs (2.8–16.9 ppb; *n* = 10).

Williams et al. (*6*) examined the adipose tissue obtained from autopsies in seven Canadian municipalities. The lowest and highest mean values in seven municipalities, respectively, were 0.56 ppb and 4.89 ppb pentachlorinated naphthalenes, 0.43 ppb and 1.04 ppb 1,2,3,4,6,7/1,2,3,5,6,7-HxCNs (mixture of the two hexachlorinated naphthalenes), and 0.02 ppb and 0.48 ppb HxCN except for 1,2,3,4,6,7/1,2,3,5,6,7-HxCNs.

Haglund et al. (*5*) examined the adipose tissue of a person intoxicated by consumption of a commercial rice oil contaminated with technical grade PCBs (Yusho rice oil). They detected 1.401 ppb PCNs, and > 40% of PCNs (0.605 ppb) was 1,2,3,4,6,7/1,2,3,5,6,7-HxCNs. Considering these concentrations, the concentration of 1,2,3,4,6,7-HxCN in the fat of dams treated with this compound was 5–10 times higher than that found in human adipose tissue.

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