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Disruption of Male Reproductive Tract Development by Administration of the Xenoestrogen, Nonylphenol, to Male Newborn Rats

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Nonylphenol (NP) treatment of neonatal male rat pups decreased the size of their testes, epididymis, seminal vesicle, and ventral prostate, and increased the frequency of cryptorchidism (60.7%, *n* = 56 vs 0% in vehicle-treated control, *n* = 58) when examined at 31 d of age. NP effects are dose-dependent. These effects were only seen when NP was given at ≥0.8 mg/kg daily for 15 d. There is a critical period of vulnerability to NP during male reproductive development in the neonatal stage. Changes were found when NPs were given to male pups before 13 d of age, but not when given at ≥13 d of age. NP acts on the male reproductive tissues through the estrogen receptor (ER), since concomitant treatment with ICI 182,780, a specific ER antagonist, blocked NP's effects on the testis and male accessory organs. NP-treated males in the neonatal period had greatly reduced their subsequent capacity to impregnate young fertile females. Our results suggest that exposure of neonatal male rats to NP is potentially deleterious to their reproductive development and affects their reproductive performance.

Key Words: Xenoestrogen; testis; fertility; cryptorchidism; rats.

Introduction

Differentiation and morphogenesis of the reproductive tract, as in many other tissues and organs, are sensitive to various stimuli, particularly during the perinatal period. Development of the male reproductive tract is precisely regulated by sex and other hormones. Upsetting this hormonal balance is likely to affect the normal path of development and results in abnormalities in the various organs of the reproductive tract. Long-lasting suppression of spermatogenesis and atrophy of the testis and male accessory organs in rodents have been shown by many investigators following subchronic (5–15 d) treatment of newborn male pups with estrogen *(1–3)*. A single injection of estradiol at day 4 after birth to rat pups led to a marked delay in the onset of increase in testicular and accessory gland weights and spermatogenesis at puberty *(4)*. Neonatal estrogen treatment often interferes with the descent of the testis. Both unilateral and bilateral cryptorchidism were found with high frequency in male rats and mice treated with estrogen early in postnatal life *(5,6)*. Perinatal treatment with diethylstilbestrol (DES), a potent synthetic estrogen analog, not only led to cryptorchidism, but also caused sterility of the treated male mice *(5)*.

Alkylphenol polyethoxylates (APEs) are produced in large quantities, estimated to be close to half a billion pounds in 1990 in the US alone *(7)*. They are widely used as components of detergents, paint, herbicides, pesticides, and other formulated products. Approximately 80% of APEs are nonylphenol (NP) polyethoxylates with the remaining 20% being octylphenol polyethoxylates. It is estimated that 60% of APEs found their way into the aquatic environment as the major degradative products, NP and octylphenol. NPs have been documented to appear in the aquatic environment, particularly in sediment, and can reach up to 3000 ppb in rivers and lakes *(8)*. More important, water treatment stabilizes and renders NPs water-soluble *(9)*. Various reports even indicated the presence of NPs in drinking water *(9–11)*.

NP was first reported to exert estrogenic activity based on its induction of proliferation and upregulation of the progesterone receptor in human estrogen-sensitive breast tumor cells and its ability to increase the mitotic index in endometrial epithelium of ovariectomized female rats *(12)*. Octylphenol also has been shown to be mitogenic on breast cancer cells and to stimulate transcriptional activity of the target tissue *(13)*. Commercial preparation of NPs has been shown to stimulate the production of vitellogenin, an estrogen-responsive protein, in rainbow trout in vivo *(14)*. A recent report indicated the NP at a low dose (1 mg/kg) could elicit the synthesis of another estrogen-responsive protein,

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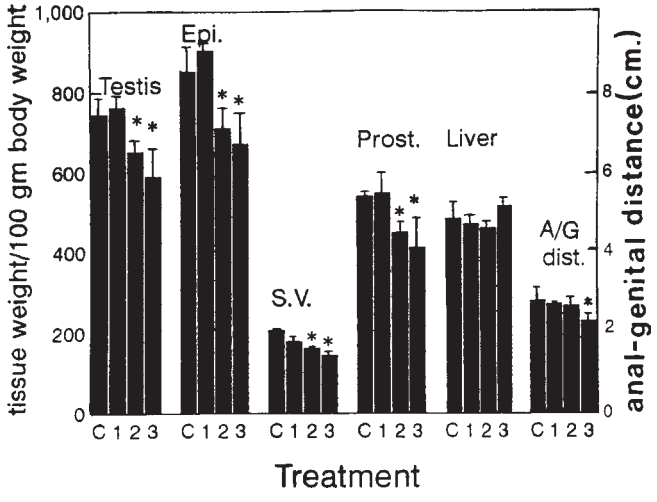

Fig. 1. Dose–response of male reproductive tissues and liver to NP administration. NPs at (1) 0.08 mg, (2) 0.8 mg, and (3) 8.0 mg/kg body wt were given to male pups daily from days 1 to d 15 after birth. Controls were given identical volume of vehicle (DMSO) for the same duration. Animals were sacrificed at 31 d of age. Values represent mean ±SD from at least 3 animals. *Denotes values significantly different from corresponding control value. Tissues weights are as follows: Testis = mg, Epi (epididymis), S.V. (seminal vesicle), and Prost. (ventral prostate) = mg X 1/10, liver = mg × 10. All are normalized to 100 g body wt. A/G dist. = anal-genital distance.

the *zona radiata* protein, in salmon *(15)*. We have shown that NPs induce uterine growth in immature female rats in a manner similar to that of estrogen *(16)*. Relatively few studies have looked at the effects of alkylphenols in the males. In adult male rats, chronic administration of octylphenol caused shrinkage of testes and male accessory organs. Spermatogenesis was disrupted, sperm deformities were seen *(17)*, and luteinizing hormone (LH), follicle stimulating hormone (FSH), prolactin, and testosterone secretion was altered in these animals *(18)*. Alkylphenols thus can disrupt the endocrine balance and may interfere with the normal reproductive processes in wildlife and humans.

The present study was performed to determine the effect of neonatal exposure to NP on subsequent male reproductive tract development using rats as the model. In addition, we evaluated if NP acts through the estrogen receptor (ER) using the ER-specific antagonist ICI 182,780 to see if it could block the NP effects.

Results

Dose Dependence

NPs administered to male rat pups starting from d 1 after birth for 15 d resulted in smaller reproductive organ weight at 31 d of age. The NP effects on the male reproductive tract were dose-dependent. Treatment with 0.08 mg/kg body wt had no significant effect on reproductive organ weight/body wt basis (Fig. 1). On the other hand, rat pups treated with 0.8 mg/kg body wt resulted in decreases in testis, epididymis, seminal vesicle, and ventral prostate gland weight/body wt basis compared to those from control-treated pups. Further reductions in these parameters were evident when the NP dose was increased to 8.0 mg/kg body wt. Only at this high dose of NP (8.0 mg/kg body wt) was there a decrease in the anal-genital distance. By contrast, the liver weight/body wt basis of the NP-treated animals did not differ from those of control-treated animals. Body weights of pups were slightly lower following the treatment with NPs at the high dose of 8.0 mg/kg body wt, but not at the lower two doses of NP.

Period of Vulnerability

Since a determining phase of development of the male reproductive tract occurs in the perinatal period, certain stages during the early postnatal life should be more susceptible to exogenous influences. In experiments designed to determine if there was a developmental period in which the male reproductive system was more sensitive to NP treatment, three series of pups were studied to cover the age period from immediate postnatal to weaning stages. NPs were administered for a similar duration (18 d total), but starting at the ages of 1, 6, or 13 d after birth. All were sacrificed at 31 d of age. Table 1 summarizes the results. When NPs were given from days 1 to d 18, decreases in testes, epididymis, seminal vesicle, and ventral prostate glands weight/body wt basis were evident. Similar decreases were also seen when NPs were given from age 6 d to 24 d. However, when the start of NP-treatment was delayed until age 13 days, no effect occurred. There is therefore a window of time during the immediate postnatal period that is critical for the normal development of the male reproductive tract. This is the period when the neonates are most sensitive to NP treatment.

Descent of Testis

NP treatment in newborn male rat pups resulted in frequent failure of the testis to descend properly. In affected animals, abnormal descent of the testis resulted in cryptorchidism and occurred predominantly unilaterally on the left side. In these cases, the left testis and epididymis not only remained in the abdomen, but usually also became atrophic. Bilateral cryptorchidism was less common. Even in this case, the left testis showed a higher degree of atrophy than the right testis. Unilateral cryptorchidism of the right testis was rare (Table 2). The duration of NP treatment affects the type and frequency of cryptorchidism. Treatment of newborn male pups with NP for 1 d did not result in cryptorchidism. NP administration from ages 1 to 5 d resulted in cryptorchidism in 33% of the treated animals. When NP administration was extended to 10 d, the frequency of cryptorchidism increased to 55%. NP treatment for longer than 10 d (15 and 18 d) resulted in a further increase in the frequency of cryptorchidism (to about 62%) (Table 2). In addition, the longer duration of NP treatment increased the involvement of the right testis as evidenced

Table 1
Treatment Age and Effect of NPs on Male Reproductive Organ Development[a]

Durations[b]	Treatments	Body wt, g	Tissue weight, mg/100 g body wt			
			Testis	Epididymis	Seminal vesicle	Prostate
1–18	(I) Control	116.8 ± 5.5	784.8 ± 39.3	91.8 ± 3.2	28.0 ± 2.5	54.9 ± 4.2
	NPs	106.1 ± 9.1	651.2 ± 81.4[c]	75.9 ± 6.2[c]	23.3 ± 0.9[c]	40.1 ± 4.9[c]
	(II) Control	97.2 ± 8.3	705.7 ± 20.0	94.8 ± 8.0	24.8 ± 3.8	54.5 ± 2.1
	NPs	88.2 ± 4.4[c]	355.7 ± 150.5[c]	71.3 ± 6.6[c]	19.8 ± 3.3	39.7 ± 8.6[c]
6–24	Control	93.9 ± 3.6	739.4 ± 21.4	97.4 ± 8.0	32.2 ± 1.7	63.2 ± 5.4
	NPs	80.4 ± 12.6	591.0 ± 64.6[c]	81.2 ± 7.9[c]	22.4 ± 4.3[c]	41.3 ± 6.3[c]
13–30	Control	102.4 ± 3.9	697.3 ± 61.0	78.7 ± 2.7	21.2 ± 1.6	40.9 ± 2.9
	NPs	93.0 ± 6.4	720.6 ± 88.3	86.5 ± 3.0	21.4 ± 3.3	40.1 ± 2.4

[a]Each value represents the mean ±SD of 3–4 animals.
[b]First number denotes age in days of pups when injection was started. The second number denotes the age in days when injection was stopped. Pups were given NPs (8 mg/kg, daily). Controls were injected with vehicle at the same schedule as NP-treated groups. All pups were sacrificed at 31 d of age.
[c]Values significantly differ from corresponding values in control group ($p \leq 0.05$).

Table 2
Treatment Duration and Frequency of Cryptorchidism

Treatment duration[a] *(n)*	Normal descent	Abnormal descent		
		Left only	Right only	Both sides
1 d only (6)	6 (100%)[b]	0	0	0
1–5 d (10)	6 (66.7%)	2 (22.2%)	0	1 (11.1%)
1–10 d (11)	5 (45.4%)	3 (27.3%)	1 (9.0%)	2 (18.1%)
1–10 d (34)	13 (38.2%)	14 (41.2%)	2 (5.9%)	5 (14.7%)
NP + ICI (13) (1–5 and 10 d)	13 (100%)	0	0	0

[a]First number denotes age in days of pups when NP treatment was started; the second number denotes the age in days when treatment was stopped. Pups were given NPs (8.0 mg/kg) daily for the duration specified. Control age-matched pups were given vehicle only. NP + ICI groups were given ICI 182,780 (0.5 mg/kg) just before NP. All control pups showed normal descent of the testes. All animals were sacrificed at 31 d of age.
[b]Values calculated as percent of total.

by the increase in frequency of undescended right testis (both unilateral on the right side and bilateral) (Table 2). NP treatment for 1 d resulted in no abnormal descent of the testis, but caused smaller testis weight/body wt basis in two of the six NP-treated animals, suggesting some subtle changes might have already resulted from the single dose of NP (data not shown).

Effect of ER Antagonist

NP is known to have estrogenic properties both in vitro and in vivo. Therefore, it is necessary to investigate if the effects of NP on the male reproductive tract development during the neonatal stage were mediated by the ER. Day-old male pups were given NP alone or together with ICI 182,780, a specific ER receptor antagonist. Age-matched control male pups were given vehicle only or ICI 182,780 only at the same volume and dose as the treatment groups for comparison. Figure 2 shows that ICI 182,780 by itself has no effect on the testis, epididymis, seminal vesicle, and ventral prostate gland weight/body wt basis. NP caused the typical decrease in these weight parameters of the male reproductive tract. The same dose of ICI 182,780 when given together with NP blocked the atrophic effect on all the organs measured. Additionally, ICI 182,780 also prevented the NP-induced cryptorchidism when given simultaneously with NP (Table 2, last line).

Reproductive Effects

To evaluate the long-term reproductive effects of NP treatment of newborn male pups, two series of experiments were performed. Each started with eight newborn males from the same litter. Four of the newborns were given NPs at 8.0 mg/kg body wt daily from days 1–15 after birth. The remaining four were given the vehicle in an identical manner. They were raised to adults and individually pair-mated with young adult fertile females repeatedly. Fertility was scored as the number of fertile females rendered pregnant by the test males from both groups. Table 3 summarizes the

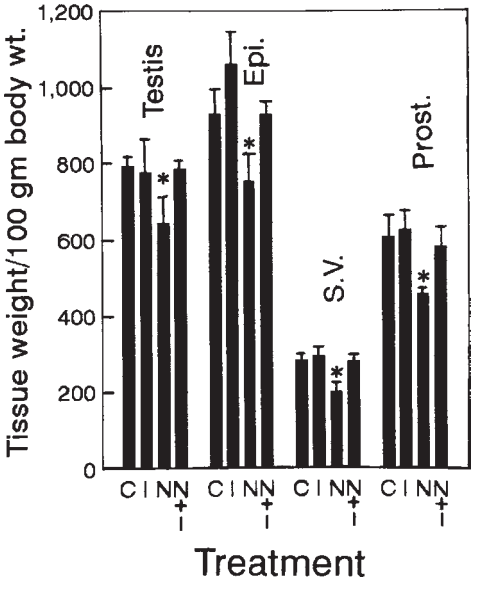

Fig. 2. Effect of the ER antagonist, ICI 182, 780, on NP-induced impairment of male sex organ development. NP was given to day-old male pups at 8.0 mg/kg body wt daily for 5 d in the absence (*N*; $n = 7$) and presence of ICI (0.5 mg/kg) (*N* + I; $n = 7$). Control (C; $n = 6$) age-matched pups were given vehicle only. Some pups were give ICI only (I; $n = 3$). Pups were sacrificed at 31 d of age. Values represent mean + SD from all animals in the treatment group. *Denotes values significantly different from corresponding control values ($p \leq 0.05$). Tissue weights are as follows: Testis = mg; Epi. (epididymis), S.V. (seminal vesicle), and prost. (ventral prostate) = mg × 1/10.

results from two separate experiments. Each animal (NP-treated or control) was scored separately. In experiment #1, neonatal NP-treatment had no effect on one (I) and affected only slightly another (II) male. One was infertile (III) in that it failed to sire any of the 10 different females during 10 separate matings. One (IV) was initially fertile, but then did not sire any more litters after the 7th mating. In contrast, three of four control males were fertile up to the 20th mating and the remaining one was fertile up to the 19th mating. There were no observed difference in the mean litter size from the dams sired by either control or NP-treated males. The sex distribution of the litters was also similar. At necropsy, rat I showed cryptorchidism and slight atrophy of the left testis (~80% of control). Rat II showed no cryptorchidism, but slight atrophy of both testes (>80% of control). Rat III had no cryptorchidism, but severe atrophy of both testes (<30% of control). Rat IV showed cryptorchidism of the left testis and atrophy of both testes. The results of experiment #2 essentially confirmed the findings from experiment #1 in that all controls were fertile. One NP-treated males (I) could only be tested once, since it died after one mating and failed to sire a fertile female. At necropsy, it showed cryptorchidism of the left testes and atrophy in both testis. Another NP-treated male (II) sired two females during the first two mating, did not sire a third female, but sired another female the fourth time and became sterile for two subsequent matings. One (III) was sterile and failed to sire fertile females in seven tries. One (IV) was fertile through the fifth mating and did not sire the females during two additional matings.

Discussion

The present results clearly document that exposure of newborn male rat pups to NPs can adversely alter the development of the male reproductive tract. The most significant changes that occurred were restricted development of the testis, epididymis, seminal vesicles, and ventral prostate gland, thus resulting in significant reduction in the size of each organ compared to age-matched, vehicle-treated controls. These effects of NPs on the development of the male reproductive tract were dose-dependent. In a standard treatment protocol of from 1 to 15 d of age, the threshold dose of NPs was between 0.08 and 0.8 mg/kg body wt. These doses were within the range of tissue NP concentrations (1.0 to 6.0 mg/kg) present both in laboratory settings and in the wild *(19,20)*. Our findings of NPs having effects at these equivalent concentrations therefore are of physiological importance, since it reflects the concentrations achievable through environmental exposure. It is of further significance in that these dosages are not otherwise toxic and had minimal effects on other growth parameters, since there are no detectable changes in the liver weight and only a slight decrease in body weight observed only after treatment with high doses of NPs. Of a more serious nature is the observed big increase in cryptorchidism, i.e., failure of the testis to descend from the abdomen. An overall incidence of >60% of the newborn pups treated with 8.0 mg/kg body wt for five or more days showed unilateral or bilateral cryptorchidism as compared to 0% in vehicle-treated control animals. The frequency of cryptorchidism increased with increases of duration of NP treatment. Cryptorchidism was evident after a 5-d treatment with the NP dose used. An additional increase in frequency was seen when the treatment was prolonged to 10 d. Further prolongation of the treatment duration appeared to reach a plateau, suggesting the period between 1 and 10 d of age represented an important time for the development and normal descent of the testis. Cryptorchidism has been documented in mice after prenatal treatment with estrogen or the synthetic analog, diethylstilbestrol (DES) *(21,22)*. More important, nondescended testis have a higher incidence of hyperplasia and neoplastic changes *(23)*. The increase in cryptorchidism found following neonatal treatment with NP in the present study suggests a possibility of increased metaplastic changes in later life of these affected males. The predominance of unilateral effect on the testis seems to be a general phenomenon. Treatment of neonatal mice with a high dose of estrogen has been reported to affect primarily the descent of one side of the testes *(6)*. The differential sensitivity of the two testes might reflect the differential timing of the events associated with the migration of the testis of each side from

Table 3
Comparison of Fertility Results of Control and NP-Treated Males in Their Ability to Father Offsprings with Fertile Females

Treatments	Rats #	# of females pregnant/total (%)	Mean litter size
Exp. #1	1	20/20 (100)	13.5
Control	2	20/20 (100)	13.0
(Vehicle)	3	20/20 (100)	14.0
	4	19/20 (95)	13.5
NPs	I	20/20 (100)	14.0
(8.0 mg/kg from	II	19/20 (95)	13.5
days 1 to 15)	III	0/10 (0)[a]	—
	IV	7/20 (35)[a]	13.0
Exp. #2	1	6/6 (100)	—
Control	2	6/6 (100)	—
(Vehicle)	3	6/6 (100)	—
	4	7/7 (100)	—
NPs	I	0/1 (0)[b]	—
(8.0 mg/kg from	II	3/6 (50)[a]	—
days 1 to 15)	III	0/7 (0)[a]	—
	IV	5/7 (71)[a]	—

[a]All females for these matings were rechecked by mating with control males and proved to be fertile. Males and females were allowed to stay together for at least 15 d.
[b]NPI from Exp. 2 died after one mating.

the abdomen to the scrotal sac. These events have been shown to involve complicated interactions of hormonal and developmental processes that are at present more speculative than known *(24)*. NP can interfere with one or more of these processes.

The adverse effect of NP on the pubertal increase in the testis and male accessory organs was age-dependent, since the effect was observed when NP treatment was given before, but not after 13 d of age. The present results indicate that there is a critical period, most likely during the first 2 wk of life, during which the determinants for the male reproductive tract were laid down. This period would be vulnerable to any interference that perturbs the precisely controlled sequence of events leading to pubertal increases in male accessory organ sizes and proper descent of the testes. A similar observation of a critical time for treatment to be effective was also reported for neonatal estrogen exposure in male mice. Only those mice treated from ages 1 to 15 d, but not those from ages 10 to 25 d showed estrogen effects, including cryptorchidism and abnormal spermatogenesis *(6)*.

Adult rats treated with another alkylphenol, octylphenol, had been reported to lead to shrinkage of their testes and male accessory organs *(16)*. The dosage of octylphenol used was, however, 10 times higher (80.0 mg/kg body wt) and the treatment duration (1–2 mo) was 2 to 4 times longer than those used in our study. Further, octylphenol has been shown to be more potent than NP in its estrogenic actions *(25)*. In relation to this, feeding of octylphenol to pregnant rat dams has been shown to result in male progenies with smaller testis and a reduction in the daily sperm production as these affected males reached adulthood *(26)*. Our results showed that the early neonatal period is also sensitive to alkylphenols.

Although the exact mechanism by which NP interferes with male reproductive development is at present unknown, our results suggest that NPs most likely mimic the action of estrogen and act directly through the ER. First, NP treatment resulted in male developmental defects, such as delayed onset of pubertal increase in testis and male accessory organs and cryptorchidism, which are very similar to those found after neonatal treatment with estrogen in both mice and rats *(1,4)*, suggesting a similarity to estrogen actions. Second, both organ atrophy and cryptorchidism were completely blocked by the ER-specific antagonist, ICI 182, 780, suggesting the involvement of ER in NP's action. Our results further showed that blocking endogenous estrogen action by ICI 182, 780 during the neonatal period had no effect on the pubertal developmental increase in the testis and male accessory organ weights, suggesting that this stage of male reproductive tract development is independent of estrogen. Functional ER, however, is essential for the complete development of the male reproductive system. It has been shown that although ER knockout male mice develop normally, they have distinctly smaller testes *(27)*. Further, these ER knockout mice were basically infertile. We have not yet followed through with the ER inhibitor-treated males to maturity. It is likely that a functional ER is required at a stage later than 15 d of age.

Alternatively, NP may act indirectly through modulation of growth factors and/or their receptors. Estrogen is known to upregulate *(28,29)* and to interact with epidermal

growth factor (EGF) and insulin-like growth factor (IGF) *(30)*. Though by analogy, xenoestrogen should interact similarly with these growth factors, few studies have been reported. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) was shown to increase the expression of tumor necrosis factor-α in human endometrial adenocarcinoma cells *(31)*. However, TCDD was also demonstrated to downregulate EGF binding in human keraotocytes *(32)* and to inhibit estrogen-stimulated increases in rat uterine EGF receptors *(33)*. NPs might behave similarly to TCDD in the inhibition of the actions of EGF or other growth factors that are required for the normal development of the male reproductive tract. This remains to be established.

Last but not least, our results showed that the reduction in testis and male accessory organ sizes have serious reproductive consequence. The neonatal treatment of male pups with NPs significantly reduced their reproductive capacity in that more than half of the treated males were either infertile or less fertile than control vehicle-treated males. The exact cause of sterility in these NP-treated males is unknown at present. Testicular atrophy and cryptorchidism were found in most, but not all NP-treated sterile males on necropsy. Microscopic examination of epididymal sperms from these males showed a reduction in motility compared to those from age-matched, vehicle-treated control males. Further studies of these and other subtle changes both biochemically and physiologically are necessary to delineate the exact causes.

Materials and Methods

Chemicals

Except where otherwise stated, all chemicals were from Sigma Chemical Company (St. Louis, MO.). NP was from American Cyanamid Co. (Wayne, NJ). ICI 182, 780, a specific ER antagonist, was a gift from A. Wakeling of Zeneca Pharmaceuticals (Cheshire, England).

Animals

Pregnant Sprague-Dawley rats from an inbred colony maintained at the Medical College of Wisconsin were housed in individual cages and maintained on a 12-h alternate light–dark cycle. On the expected date of delivery, cages were inspected every 6 h for birth. The day of birth was regarded as day 0. Pups were allowed to suckle freely until 20–21 d of age. The NIH guidelines for the care and use of laboratory animals were followed to ensure that animals were not subjected to pain and discomfort.

The following experiments were performed:

1. Dosage effect: To see if the NP effects were dose-dependent, newborn male pups were given NPs at varying doses by ip injections: 0 (control), 0.08, 0.8, and 8.0 mg/kg body wt daily for 15 d. Animals were sacrificed at 31 d of age.
2. Age dependence: To see if there was a critical window of vulnerability during the neonatal phase of development, male pups were given NPs (8.0 mg/kg body wt, ip daily) for the same duration, but started at different ages as follows: group A from days 1 to 18; group B, from days 6 to 24, and group C from days 13 to 30. Control age-matched male pups were given the same volume of vehicle only in an identical manner. All animals were sacrificed at 31 d of age.
3. Duration of treatment: To see if the NP effects were dependent on the length of treatment duration, newborn male pups were given NPs (8.0 mg/kg body wt, ip) daily for 1, 5, 10, and 15–18 d and stopped. Control age-matched pups were given the same volume of vehicle only in an identical manner. All animals were sacrificed at 31 d of age.
4. Effect of ER antagonist: To see if the NP effects on male reproductive tract development were dependent on ER function, newborn male pups were given NPs (8.0 mg/kg body wt, ip) alone, ICI 182,780 (0.5 mg/kg, ip) alone, or NPs (8.0 mg/kg) + ICI (0.5 mg/kg) together daily for 5 d. Control age-matched male pups were given the same volume of vehicle only. All animals were sacrificed at 31 d of age.
5. Reproductive effect: To see if NP's effect on male reproductive tract development might also affected its reproductive function, newborn pups were divided into two groups. One group was given NPs at 8.0 mg/kg body wt, ip, daily for 15 d and the other groups (control) was given vehicle only also daily for 15 d. They were allowed to grow to maturity (8–10 wk of age) and then used to test for fertility by repeated mating with fertile young females. Mating pairs were allowed to stay together for at least 15 d before transferring the test male to another cage with a different female. For females that failed to get pregnant after mating with NP males, they were rechecked with control males to assure that these females were capable of bearing young.

At the time of sacrifice, the external appearance of the genital area was examined and the anal-genital distance was measured before dissection. Before tissue harvesting, the abdominal cavity was examined for nondescended testis. Testis, epididymis, seminal vesicle, and ventral prostate were removed, trimmed of fats, and their weights determined. The weight of the liver was also determined to serve as a control.

Statistics

Results are reported as means ±SD. ANOVA was used to evaluate the difference between multiple groups. If significance was observed between groups, then a post-hoc *t*-test was used to compare the means of two specific groups, with $p < 0.05$ considered as significant.

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