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Testicular Abnormalities in Male Rats After Lactational Exposure to Nonylphenols

Ping C. Lee,[1,2] Paul Arndt,[1] and Katherine C. Nickels[1]

[1]Department of Pediatrics and [2]Department of Pharmacology and Toxicology, Medical College of Wisconsin, Milwaukee, WI.

Lactational exposure of male rat pups to nonylphenols (NPs) decreased the size of their testes and male accessory glands. At 31 d of age, NP-treatment of male rats resulted in less cellular differentiation of the seminiferous tubules (STs) and increased intertubular space compared to controls. At maturity, NP-treated males showed varying degrees of abnormalities in the affected testes. In the moderately affected ones, about 20–30% of their STs had poorly differentiated germinal elements. Cell lineage was less organized. In extreme cases, all STs of the affected testis failed to differentiate into germinal elements. These abnormalities in germinal element differentiation might be the primary cause for a number of the NP-treated males having a lower epididymal sperm count and a lower percentage of motile sperm compared to age-matched control males. Zymogram analysis of testis homogenates by sodium dodecyl sulfate gelatin gels revealed two major forms (64–66 kDa and 50–52 kDa) of gelatinases. Only the 50–52-kDa form was greatly reduced or absent in the affected testis. Lactational exposure of male pups to NPs thus leads to various testicular abnormalities including lack of differentiation of STs, lowering of sperm count, and reduction in the percentage of motile sperm and modulation of a specific form of testicular proteinases.

Key Words: Xenoestrogen; testis; sperm counts; sperm motility; proteinases; rats.

Introduction

Alkylphenol ethoxylates (APEs), widely used as components of detergents, paint, herbicides, pesticides, and other formulated products, are produced in large quantities, estimated to be close to half a billion pounds in 1990 in the United States alone *(1,2)*. About 80% of APEs are derived from nonylphenol (NP) and the remaining 20% from octylphenol. It is estimated that 60% of APEs find their way into the environment as the major degradative products: NP and octylphenol. NPs are found in the aquatic environment, particularly in sediment, and in some rivers and lakes they have been documented to reach up to 3000 ppb in sediments *(3)*. Water treatment stabilizes and renders NPs water soluble *(4)*, and NPs have been reported to be present in drinking water *(4–6)*. Soto et al. *(7)* first showed that NPs exerted estrogenic activity by inducing the proliferation and upregulation of the progesterone receptor in human estrogen-sensitive breast tumor cells and increasing the mitotic index in endometrial epithelium of ovariectomized female rats. Likewise, octylphenol is mitogenic on breast cancer cells and stimulates transcriptional activity of the target tissue *(8)*. Commercial preparation of NPs stimulates the production of vitellogenin in rainbow trout in vivo *(9)* and, at a relatively low dose (1 mg/kg), could elicit the synthesis of another estrogen-responsive protein, the zona radiata protein, in salmon *(10)*. We have shown that NPs induce uterine growth in immature female rats in a manner similar to that of estrogen *(11)*. Few studies have examined the effects of alkylphenols in males. In adult male rats, the administration of octylphenol for 2 mo caused shrinkage of testes and male accessory organs. Spermatogenesis was disrupted; sperm deformities were seen *(12);* and luteinizing hormone, follicle-stimulating hormone, prolactin, and testosterone secretion were altered in these animals *(13)*. NPs thus have the potential to disrupt the endocrine balance and interfere with normal reproductive processes.

Various reports have suggested an increase in the incidence of male reproductive anomalies in humans and wildlife *(14)*. Environmental chemicals can cause endocrine disruption and have been implicated as a possible cause *(15,16)*. Development of the reproductive tract, particularly during the perinatal period, is sensitive to various stimuli. Differentiation of the male reproductive tissue is precisely regulated by sex and other hormones. Hormonal imbalance is likely to alter the path of development and

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Author to whom all correspondence and reprint requests should be addressed: Ping C. Lee, Department of Pediatrics, Medical College of Wisconsin, MACC Fund Research Center, 8701 Watertown Plank Road, Milwaukee, WI. E-mail: pclee@mcw.edu

results in abnormalities in various organs of the male reproductive tract. Subchronic (5–15 d) treatment of newborn male pups with estrogen led to long-lasting suppression of spermatogenesis and atrophy of the testis and male accessory organs in rodents *(17–19)*. A single injection of estradiol to male rat pups at d 4 after birth resulted in a marked delay in the onset of increase in testicular and accessory gland weights and spermatogenesis at puberty *(20)*. 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 *(21,22)*. Perinatal treatment with diethylstilbestrol, a potent synthetic estrogen analog, not only led to cryptorchidism but caused sterility of the treated male mice *(21)*. We have recently shown that male rat pups exposed to NPs immediately after birth resulted in decreased size of testes and associated male reproductive organs and increased incidences of cryptorchidism and infertility *(23)*.

The present study was performed to examine the possible cause(s) of infertility by determining the effect of lactational exposure of rats to NPs on testicular differentiation and the subsequent influences on sperm counts and sperm motility. In addition, we evaluated whether NP treatment would lead to specific changes in matrix metalloproteinases (MMPs) that may alter the differentiation process.

Results

General Histology

Treatment of neonatal male pups with 8.0 mg/kg body wt of NPs for 15 d from birth caused marked changes in the histology of the testes when examined at 31 d of age and at adulthood (~8 mo of age). Figure 1 shows a comparison of the control vs NP-treated animals at 31 d of age. The most striking difference is the increased intertubular spaces. The seminiferous tubules (Sts) in the NP-treated animals appear only a bit smaller, but the cellular architecture is different. The control tubules appear to be more differentiated in that the column of cells are six to seven cells thick whereas the NP-treated ones are only four to five cells thick. Simultaneous treatment with ICI 182,780 countered some of the NP effects. The intertubular spaces in the ICI- and NP-treated testis are similar to the controls, and cellular differentiation shows improvement over those from NP treatment alone (cf. Fig. 1C to Fig. 1B).

In adult males that have undergone lactational treatment with NPs, distinct histological changes are also observed. In the moderately affected ones, an increase in intertubular spaces is evident (Fig. 2B). Many tubules are not fully differentiated. Of those tubules that differentiate and produce mature sperm, many more sperm heads are found close to the basal membrane compared to those from controls. In the most severe cases, all tubules remain undifferentiated (Fig. 2C). They are much smaller in diameter, and no germinal tissue can be discerned. Cellular architecture is completely changed and the center of the tubule is occupied by large vacuoles.

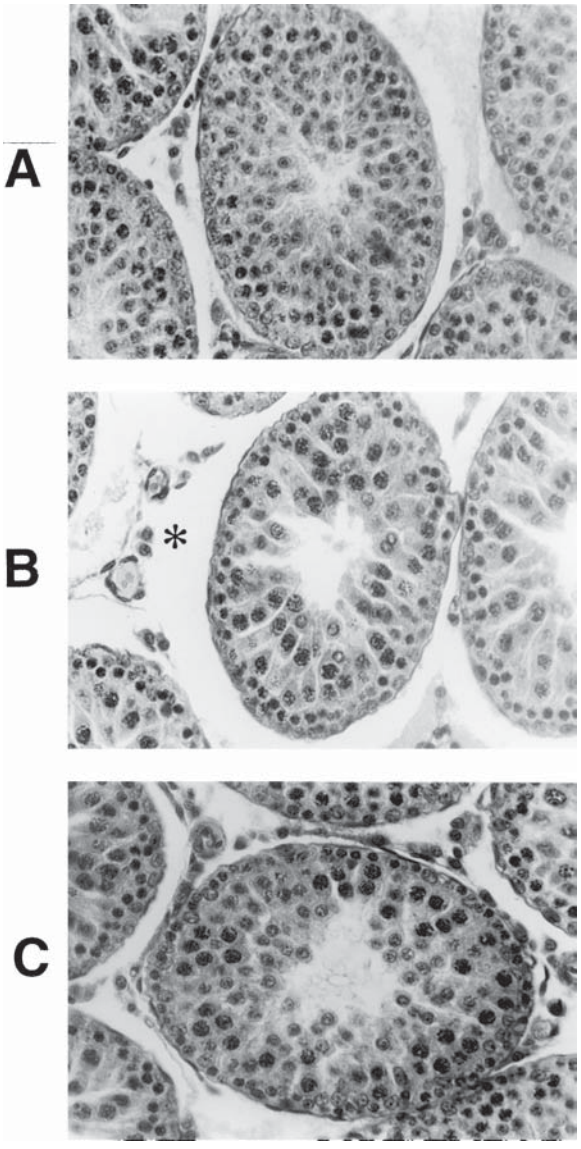

Fig. 1. Representative photomicrograph (×240) of testis sections from 31-d-old rats treated with **(A)** vehicle; **(B)** NPs (8.0 mg/kg of body weight); **(C)** NPs (8.0 mg/kg of body weight) and ICI 182,780 (0.5 mg/kg of body weight) daily from age 1 to 15 d. Asterisk denotes increase in intertubular spaces in NP treated animals.

Epididymal Sperm Counts and Sperm Motility

In adult rats, exposure to NPs during their lactation period resulted in a decrease in testes sizes in many but not all individuals in this set of experiments. They were therefore arbitrarily divided into two subgroups (NP I and NP II) for comparison. Of the nine NP-treated rats evaluated, three showed atrophy in their left testis only (NP I) whereas six showed no decrease in size in either their right or left testis (NP II) compared to controls (Table 1). In the subgroup NP I, the epididymis from the side of the atrophic testis (left in

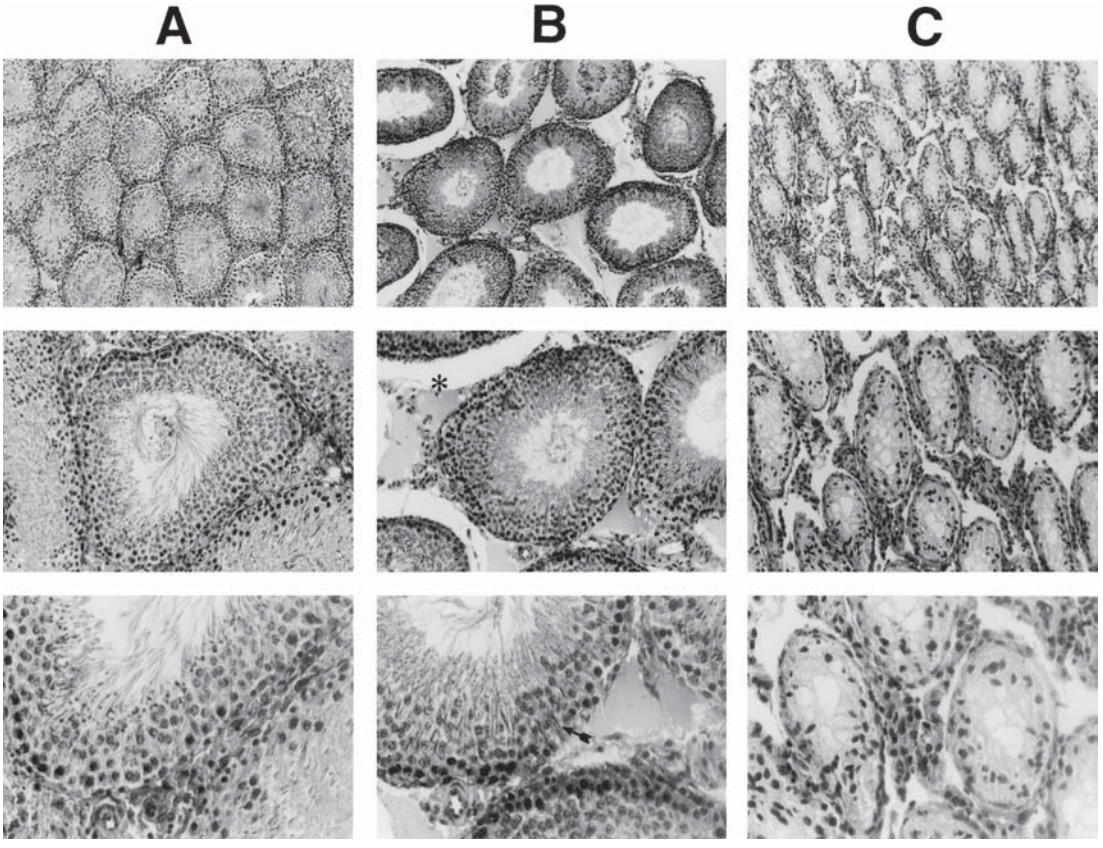

Fig. 2. Representative photomicrographs of testis sections from adult (~8 mo old) rats treated with **(A)** vehicle; **(B,C)** NPs (8.0 mg/kg of body weight) daily from age 1 to 15 d. Magnifications: top row (×80); middle row (×160); bottom row (×240). Asterisk denotes increase in intertubular spaces compared to control sections. Arrow indicates mature sperm head near the basal membrane.

these animals) contained no recognizable sperm whereas the corresponding epididymis from the opposite side (right in these animals), although having a normal-sized testis, contained a lower number of sperm compared to epididymis from either side of the control animals. The motility of epididymal sperm from both sides was invariably decreased. On the other hand, the epididymis from the subgroup NP II, which showed no observable difference in the weight of their testis, had a similar number of sperm to that of the epididymis from the control group. However, their sperm motility (as percentage of total sperm from the epididymis) was lower.

As a comparison, male rat pups were treated with estradiol for the same duration as with NPs. Estradiol led to uniform decreases in their testicular weights with corresponding decreases in their epididymis sperm counts and motility (Table 2).

Zymogram Analysis

Because of the observed histological changes, specifically the increase in intertubular spaces and the vacuolization of the tubular core, in NP-treated testis, we examined where proteinases, particularly MMPs, are involved. A zymogram using gelatin gels revealed the presence of gelatinases in testicular extracts (Fig. 3). The testis extract contains two major molecular forms; a 64 to 66- and a 50 to 52-kDa gelatinase. Comparison of control and NP-treated testis indicated that only the 50 to 52-kDa gelatinase was reduced or absent in the affected testis. In Fig. 3, testes from six animals (three from NPs and three from age-matched control littermates) were presented to illustrate the range of effects. For reference, the testis weights of these same animals are listed in Table 3 to indicate the degree of atrophy of the affected testis. In pair I, the left testis from the NP-treated animal had a severe reduction in size (Table 3). The corresponding tissue extract showed that the 50 to 52-kDa gelatinase was almost completely absent. The right testis from the same animal showed no atrophy, and the corresponding tissue extract showed a similar gelatinase profile as that of the control. In pair II, the right testis of the NP-treated male was moderately affected. The corresponding tissue extract showed a reduction in the 50 to 52-kDa gelatinase whereas the unaffected left testis extract had a similar gelatinase profile as that of the control. In pair III, the left testis was slightly affected. The corresponding tissue extract showed a reduction in the 50 to 52-kDa gelatinase. The right testis was unaffected as was its 50 to 52-kDa gelatinase. To determine whether these gelatinases are indeed MMPs, two identical gels were run: One was incubated in the presence of calcium and the other in

Table 1
Effects of NPs on Testis Weights, Epididymal Sperm Counts, and Sperm Motility[a]

Treatment	Testis: Right	Testis: Left	Testis: Right + left
Weight (mg/100 g body weight)			
Control (6)	460 ± 30	460 ± 30	906 ± 48
NP I (3)	530 ± 20	150 ± 70[b]	683 ± 94[b]
NP II (6)	450 ± 40	450 ± 40	909 ± 87
Sperm count (10^8)			
Control (6)	1.08 ± 0.1	1.08 ± 0.1	2.17 ± 0.15
NP I (3)	0.83 ± 0.3[b]	0[b]	0.83 ± 0.34[b]
NP II (6)	1.09 ± 0.3	1.09 ± 0.2	2.20 ± 0.49
Sperm motility (% of total sperm)			
Control (6)	78.4 ± 7.2	73.2 ± 12.4	77.6 ± 11.4
NP I (3)	48.8 ± 0.8[b]	0[b]	
NP II (6)	55.1 ± 5.5[b]	54.0 ± 10[b]	54.3 ± 8.6[b]

[a]NP I and II represent arbitrary groups based on their testicular sizes in response to NP treatment. Values are means ± SD. NPs were given at a dose of 8.0 mg/kg of body weight daily from ages 1 to 15 d. Animals were sacrificed at age 60–68 days. Numbers in parentheses represent the number of animals used. Litter-mates from three separate litters were used with each litter providing two-control and three NP-treated animals.

[b]Significantly different from corresponding values from control group with $p < 0.05$.

Table 2
Effects of Estradiol on Testis Weights, Epididymal Sperm Counts and Sperm Motility[a]

Treatment	Testis: Right	Testis: Left	Testis: Right + left
Weight (mg/100 gm body weight)			
Control (3)	460 ± 20	460 ± 50	926 ± 26
E2 (3)	340 ± 50[b]	340 ± 50[b]	679 ± 101*
Sperm count (108)			
Control (3)	1.47 ± 0.40	1.31 ± 0.1	2.46 ± 0.30
E2 (3)	0.68 ± 0.12[b]	0.76 ± 0.2[b]	1.43 ± 0.15[b]
Sperm motility (% of total sperms)			
Control (3)	72.5 ± 6.9	73.2 ± 9.9	72.9 ± 4.9
E2 (3)	57.7 ± 5.5[b]	49.8 ± 11.1[b]	53.9 ± 3.8[b]

[a]Values are mean ± SD. E2 was given at 5 μg/kg of body weight daily to pups from age 1 to 15 d. Animals were sacrificed at about 65 d of age.

[b]Significantly different from corresponding values from control group with $p < 0.05$.

the absence of calcium but with added EDTA. Figure 4 shows the results. In the absence of calcium, only the 50 to 52-kDa gelatinase was seen. It is evident that the 64 to 66-kDa gelatinase is calcium dependent, but not the 50 to 52-kDa form. A comparison of testicular extract and sperm extract (Fig. 5) revealed that the sperm extract also contained a 50 to 52-kDa gelatinase that possibly matched the same molecular form from the testis.

Discussion

We have reported previously that exposure of neonatal male rat pups to NPs led to cryptorchidism, testicular atrophy, and infertility *(23)*. Our present results clearly demonstrate that lactational exposure of rat pups to NPs seriously alters testicular development. The most significant changes in pups exposed to NPs are the loss of architecture of the STs. These changes are detectable as early as 31 d of age by an increase in intertubular space and a decrease in cellular differentiation of the ST. Interestingly, these changes were blocked by simultaneous administration of the estrogen receptor (ER)–specific antagonist, ICI 182,780. The blockage was not complete, since the histological appearance of the ICI + NP–treated animals was in between that of the control and NP-treated ones. The results suggested that, at least in part, NP acts through the ER. It also hints to other possible NP effects that might be direct and toxic to these cells. The adult testis from the NP-treated animals showed an even more marked difference from the control age-matched littermates. Not only the wide intertubular spaces persisted, but many tubules failed to mature completely and produced little or no sperm in the lumen. Even in those tubules that appeared fully differentiated, there were many more sperm heads closer to the basal membranes compared to controls, suggesting an abnormality in the sperm maturation process. In extreme cases, none of the tubules showed differentiation and the central luminal spaces were occluded by highly vacuolated cells. This complete lack of differentiation and spermatogenesis was reflected in the epididymal sperm content. Our results showed that epididymal sperm counts from NP-treated males were lower than in controls in moderately affected individuals and totally absent in severely affected ones. Additionally, we found that the percentage of motile sperm was in general lower when they were isolated from epididymis of NP-treated animals as compared to those isolated from controls. We also noticed that some of the NP-treated males had "normal"-sized testis and yielded similar sperm counts as that of the control testis. However, the percentage of motile sperm from the epididymis of these NP-treated animals with "normal"-sized testis was still significantly lower than that of the control, suggesting the existence of abnormality even with apparent functional spermatogenesis in these animals.

The results from our estradiol experiments indicate some similarities but, at the same time, some differences to the response of lactational NP treatment. The major similarities are that both estradiol and NP treatment reduce the size of the testes, their epididymal sperm counts, and the percentage of motile sperms. The chief difference is that estradiol affects all whereas NP treatment affects some but not

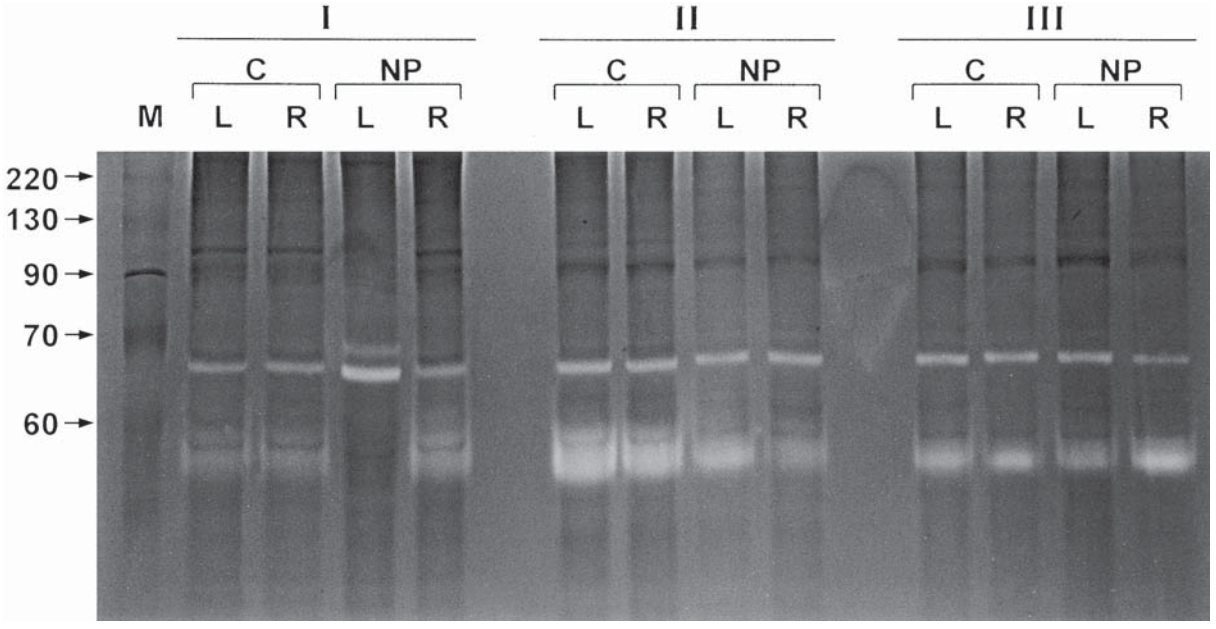

Fig. 3. Zymogram comparing gelatinase activity of Tx-100 extracts of testis homogenates (60 μg protein/lane) from paired adult (~8 mo old) control and NP-treated rats. Control (C) vehicle and nonylphenol (NP) NPs administered at 8.0 mg/kg of body weight daily from age 1 to 15 d. Each pair represents animals that were littermates. Pairs I, II, and III were from different litters. M, = molecular weight markers; L and R, left and right testis from the same animal, respectively. Individual body and testis weights are listed in Table 3 for reference purpose.

Table 3
Comparison of Individual Testicular Weights
for Rat Pairs Used for Zymogram Analysis in Fig. 3

	Rat pair I		Rat pair II		Rat pair III	
	Control	NPs	Control	NPs	Control	NPs
Body weight (g)	560	600	575	525	600	602
Testis weight (g)						
Left	1.90	0.30[a]	2.19	2.10	1.99	1.29[a]
Right	1.80	2.10	2.09	1.10[a]	1.87	2.07

[a]indicates testis smaller than corresponding controls.

all males. These results suggest that NP acts only partly like estradiol, as is also indicated by the behavior toward ICI 182,780 (see Results).

Our results are in general agreement with those reported by other laboratories for rats treated with alkylphenols at other stages of development. Boockfor and Blake *(12)* reported that long-term (up to 2 mo) administration of octylphenols to young adult males led to a reduction in sperm count and disruption of spermatogenesis. Sharpe et al. *(24)* also showed that feeding octylphenol to pregnant rats resulted in male progenies with smaller testes and a reduction in daily sperm production. The present results indicate that a shorter-term exposure during the lactational period is also effective in bringing about these impairments, suggesting that alkylphenols perhaps can act at all ages. Recent reports show that alkylphenols also affect the male reproductive organs of fish. When given to male eelpout *Zoarcs vivparus* during active and late spermatogenesis, NPs caused testicular abnormalities including seminiferous lobules containing spermatogenic cysts with few spermatozoa to general degeneration of seminiferous lobule *(25)*. In male Japanese Medaka, exposure to 4-*tert*-octylphenol resulted in inhibited spermatogenesis and formation of oocytes in their testes *(26)*. The effects of NP are not restricted to rodents; male of other species appear to be affected as well.

The MMPs are a special family of enzymes that have evolved to aid in the remodeling of specific extracellular matrix components *(27)*. As such, they play important roles in development and tissue differentiation. Of particular relevance to the present study, MMP activity has been linked to reproductive processes. MMPs have been impli-

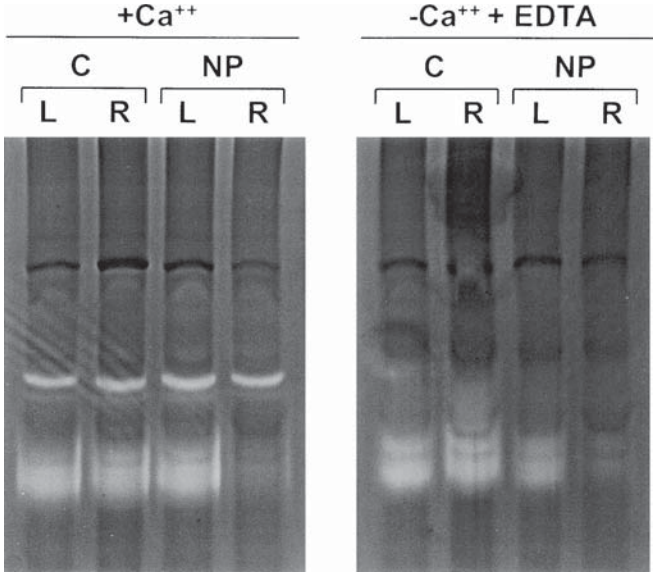

Fig. 4. Zymogram comparing gelatinase activity of Tx-100 extracts (60 μg of protein/lane) of testis from control (C) vehicle and nonylphenol (NPs) NPs administered at 8.0 mg/kg of body weight daily from age 1 to 15 d. **(Left)** A gel incubated in the presence of $Ca^{++,}$ **(right)** a duplicate gel incubated in the absence of Ca^{++} with the presence of EDTA.

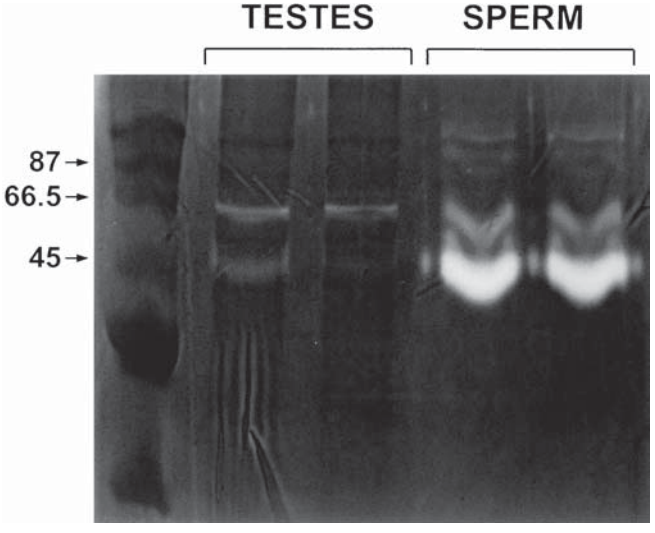

Fig. 5. Zymogram comparing gelatinase activity of Tx-100 extracts from testis (60 μg of protein/lane and mature epididymal sperms [20 μg of protein/lane]). Protein molecular weight markers are shown on the left.

cated to be involved in ovulation *(28)* and mammary gland and uterine involution *(29,30)*. All these processes are well ordered events that are tightly controlled by hormones. Testicular development is also a precisely controlled event subject to hormonal influences. The histological changes, particularly the increase in intertubular spaces and occulting of the lumens of the STs with highly vacuolated cells, observed following NP treatment might possibly be related to changes in MMPs during a critical phase of testicular differentiation.

Because most MMPs are also gelatinases, we started by examining whether any change in gelatinase activity could be found in NP-treated testis. Our results showed that the testis has two major forms of gelatinase: a 64 to 66 and a 50–52-kDa form. In NP-treated testis, the 50 to 52-kDa form was affected compared to control and ranged from a slight decrease to almost complete absence. The 64 to 66-kDa form was not affected. Further studies indicated that the 62 to 64-kDa form but not the 50 to 52-kDa form was Ca^{++} dependent. The 50 to 52-kDa form therefore is unlikely to be a MMP. Moreover, the 50 to 52-kDa form appeared to be derived from sperm, since a comparison of zymogram profiles from sperm and testis extract showed similar 50 to 52-kDa bands with the sperm extract having a much higher specific activity per protein basis. It seems that the graded loss of the 50-52 kDa form from the testis of NP-treated animals is a mere reflection of the result of failure in spermatogenesis rather than a cause for the developmental derangement.

In summary, lactational treatment of male rats with NPs leads to abnormal development and differentiation of their testes. This results in the impairment of spermatogenesis and affects adversely the production of normal sperm (decrease in number and decrease in motility). These impairments would at least partly account for the decrease in fertility of the NP-treated males.

In view of the environmental presence of NPs and the recent report implicating NPs as the causal agent for disrupting the breeding of Atlantic Salmon *(31)*, it is tempting to extrapolate the present findings to that of wildlife and even human health. Caution is required, however, in such extrapolation because the tissue doses and route of exposure reached in experimental animals and those encountered by wildlife and humans in the environment are substantially different. Further laboratory and field studies are required.

Materials and Methods

Chemicals

Unless otherwise stated, all chemicals were from Sigma (St. Louis, MO). NP was from American Cyanamid (Wayne, NJ). ICI 182,780, a specific ER antagonist, was a gift from Dr. 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 to 21 d of age. The National Institutes of Health guidelines for the care and use of laboratory animals were followed to ensure that animals were not subjected to pain and discomfort.

Experiments

The following experiments were performed:

Histology

To determine whether NPs affect testicular development, newborn male pups were given by ip injection NPs at a dose of 8.0 mg/kg of body weight daily for 15 d. Control male littermates were given vehicle (dimethysulfoxide alone). Animals were sacrificed at 31 d and ~8 mo of age. As an extra control, one group of male pups was given NPs and ICI 182,780 simultaneously for the same duration. These pups were sacrificed at 31 d of age. At sacrifice, the left and right testis were removed and immediately placed in Bouin's fixative for ~1 wk. Fixed testes were dehydrated in graded ethanols and stored in cedar wood oil, absolute ethanol (7:3), for another week. Tissues were then soaked in xylene and embedded in wax. Five-micrometer thick sections were cut, mounted on slides, and stained with hematoxylin and eosin.

Sperm Counts and Motility

All glasswares used including hemocytometer and cover slips were siliconized to prevent sperm from adhering to the glass surface. Newborn male pups were given ip injections of NPs at 8.0 mg/kg of body weight daily for 15 d. Control littermates were given vehicle only for the same duration. For comparison, one group of male pups was given ip injections of estradiol at 5.0 μg/kg of body weight daily for 15 d. All animals were sacrificed at about 60–68 d of age. The left and right epididymis were removed immediately following sacrifice. Tissue from each side was separately minced with sharp scissors in 10 mL of warm (~35°C) 0.9% saline. To determine motility, the hemocytometer, its cover slips, and an epididymal cell-free fraction (prepared by centrifugation, 2000*g* for 20 min, to remove tissues and cell debris) prepared previously from control adult males were prewarmed to 37°C. The epididymal suspension was vortexed and a 20 μL aliquot was removed, after settling for 5 s, from the top of the suspension to 980 μL of the prewarmed epididymal cell-free fraction, and then mixed. Motile sperm was counted on the hemocytometer. Preliminary studies indicated that dilution in saline renders the sperm less motile and the motility lasts shorter as compared to dilution with the epididymal cell-free fraction. A proper dilution normally yields 20–50 motile sperm in each of the 10 squares within the grid. For each epididymal preparation, 10 separate aliquots were sampled and counted. Total sperm count was performed at room temperature using saline as the diluent (degree of dilution depends on the total number of sperm obtained). Sperm counts were also performed 10 times with 10 separate samplings from each side of the epididymis from each animal. The averages of the 10 counts for motile sperm and total sperm were taken. Sperm motility was calculated as the number of motile sperm/total number of sperm in a given volume ×100.

Zymography

Newborn male pups were given ip injections of NPs at 8.0 mg/kg of body weight daily for 15 d. Controls were littermates given vehicle only for the same duration. All animals were sacrificed at about 8 mo of age. The left and right testis were removed and their weights determined before snap-freezing and stored separately at –80°C until use. Thawed samples of testis were used for homogenization. Tissue was homogenized in Tris-HCl (50 m*M*, pH 7.5) containing NaCl (200 m*M*), $CaCl_2$ (10 m*M*), aprotinin (10 μg/mL), and Triton X-100 (1%) and extracted in ice buffer for 20 min with occasional stirring. The homogenate was centrifuged for 10 min in the cold (~13,000*g*) in a microfuge. The supernatant fraction was collected and protein concentration was determined by the Bradford method *(32)* using the Bio-Rad reagent (Bio-Rad, Hercules, CA) with bovine serum albumin as the standard. Zymography was performed in a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel containing gelatin (1.5 mg/mL) according to Kleiner and Stetler-Stevenson *(33)*. Briefly, testis samples (60 μg of protein) were mixed with Laemmli loading buffer (minus reducing agent and no boiling) and subjected to electrophoresis. The gel was removed and washed in 2.5% Triton X-100 solution four times, 15 min each and then in distilled water four times, 15 min each. Gels were incubated in Tris-HCl (50 m*M*, pH 7.5) containing NaCl (200 m*M*) and $CaCl_2$ (10 m*M*) for 48 h at 37°C. To confirm whether proteinases detected were Ca^{2+} dependent, in some cases, a duplicate gel was incubated in the same buffer but without Ca^{++} and in the presence of EDTA (1 m*M*). Gels were then stained with Coomassie brilliant blue.

Statistics

Results are reported as means ± SD. Analysis of variance 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 the two specific groups, with $p < 0.05$ considered as significant.

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