Stage-Related Increase in the Proportion of Apoptotic Germ Cells and Altered Frequencies of Stages in the Spermatogenic Cycle Following Gestational, Lactational, and Direct Exposure of Male Rats to *p*-Nonylphenol

LM McClusky[1], C de Jager[2] and MS Bornman[3]

[1] Department of Physiology, Faculty of Health Sciences, University of Pretoria, P.O. Box 2034, Pretoria 0001, SOUTH AFRICA
[2]School of Health Systems and Public Health, Faculty of Health Sciences, University of Pretoria
[3]Department of Urology, Faculty of Health Sciences, University of Pretoria

Abstract

The cumulative effects of environmental toxicants, for example, the alkylphenol, *para*-nonylphenol (*p*-NP) are of concern. Our previous study showed that *p*-NP reduced several testicular morphometric parameters, including sperm counts. The present study reexamined material collected in that study to determine the mechanistic basis of *p*-NP action on spermatogenic development in the offspring. Seven-day pregnant Sprague-Dawley rats were treated with vehicle or 100 or 250 mg/kg *p*-NP through gestation, lactation and afterward directly to all male offspring until 10 weeks of age. Both doses of *p*-NP significantly ($P < 0.02$) increased the number of germ cells with *in situ* end-labeled fragmented DNA (TUNEL positive) by 1.9-fold and 1.7-fold, respectively, and specifically in stages XII–XIV and I–III. TUNEL-labeling was, however, selective, and excluded labeling of basal cells with apoptotic morphology. Cleaved caspase-3 immunohistochemistry strongly labeled basal cells (spermatogonia and early spermatocytes) with condensed marginated chromatin but not degenerate germ cells lacking definitive nuclear material found throughout the epithelium. Only the caspase index (ratio of number of caspase positive to number of degenerate cells) of the 100-mg/kg *p*-NP group was significantly ($p < 0.05$) threefold greater than controls. Whereas both doses and either 250 or 100 mg/kg treatment alone significantly ($p < 0.002$) reduced the frequencies (duration) of stages I–III, VII–VIII, and late VIII–IX (spermiating and recently spermiated tubules), respectively, both doses significantly ($p < 0.002$) increased the frequencies of stages IV–VI and all stages containing late-stage spermatocytes (XII–XIII) and meiotic cell divisions (XIV). Thus, *p*-NP, an environmentally persistent xenoestrogen, insidiously alters the spermatogenic cycle and spermatogenic process in male offspring.

Introduction

The postulated link between estrogenic environmental chemicals and the increased incidence of male reproductive disorders and declining sperm counts in humans has been the subject of much investigation (Auger *et al.*, 1995 ; Sharpe, 2001 ; Sharpe and Skakkebaek, 1993 ; Skakkebaek *et al.*, 1998). A body of evidence from mainly *in vitro* studies, however, has demonstrated the estrogenicity (Soto *et al.*, 1991 ; Vivacqua *et al.*, 2003 ; White *et al.*, 1994), cell proliferative–inducing (Masuno *et al.*, 2003 ; Miura *et al.*, 2005 ; Soto *et al.*, 1991) and caspase-dependent death-inducing (Kudo *et al.*, 2004 ; Yao *et al.*, 2006) effects of weak estrogenic chemicals such as alkylphenolic compounds (e.g., nonylphenol and octylphenol). Nonylphenol production, which in the United States was 147 million pounds in 1980 and grew to over 230 million pounds in 2000 (Blankenship and Coady, 2005), are for diverse uses, ranging from industrial detergents and wetting agents to pesticides, hair dyes, and intravaginal spermicides (Bolt *et*

al., 2001). Treatment of industrial effluent and sewage causes the release of degradative products, that is, nonylphenol and related alkylphenols, which are known to be environmentally persistent and to bioaccumulate, in fish at least (Van den Berg *et al.*, 2003). Assumptions about their possible adverse effects on human reproductive health have been questioned because nonylphenol's potency is 1000 times less than that of 17ß estradiol (Blankenship and Coady, 2005) and its rapid metabolism and excretion out of the body after a single exposure (Doerge *et al.*, 2002 ; Van den Berg *et al.*, 2003 ; Zalko *et al.*, 2003). Some studies, however, showed that repeated exposures of rats to *para*-nonylphenol (*p*-NP) caused a fourfold to fivefold increase in *p*-NP concentration in fat tissue (Green *et al.*, 2003) and tissue accumulation of active NP aglycone and placental transfer into the serum and brains of fetuses (Doerge *et al.*, 2002), albeit at high doses.

All in all, these findings are relevant against the backdrop of concerns about the cumulative effects of environmental (weak) estrogens, which may perturb developmental and/or imprinting processes during critical periods of testicular development, prenatally (Bendsen *et al.*, 2001) or postnatally (Atanassova *et al.*, 2000 ; Chapin *et al.*, 1999 ; Murono *et al.*, 1999 ; and the reported epigenetical transgenerational actions of endocrine disruptors on male reproductive development (Anway *et al.*, 2006 ; Newbold *et al.*, 2006). Indeed, octylphenol treatment of neonatal rats resulted in a small but significant decrease in germ cell apoptosis at day 18 but not at day 25 and paradoxically increased the number of spermatocytes at day 18 but not day 25 (Atanassova *et al.*, 2000). A multigenerational study of the effects of NP in rats did not show any conclusive effects on testicular morphology or other testicular parameters in the F_1 generation but increased sperm morphological abnormalities without a reduction in testis weight in the F_2 generations (Chapin *et al.*, 1999).

In the present report, we reexamined material collected in a previous study, in which it was shown that gestational, lactational, and direct exposure of rats to *p*-NP resulted in small but significant reductions in testicular and epididymal weights, seminiferous tubule diameter, seminiferous epithelium thickness, and epididymal sperm counts, without any gross histopathological changes in the testis (de Jager *et al.*, 1999). By taking advantage of the commercial availability of various tissue-based apoptosis assay kits, such as an antibody to a major executioner of apoptotic morphology, cleaved caspase-3 (Gown and Willingham, 2002), we wished to maximize our research efforts with the limited research material available and despite having only one time point of analysis. Thus, we set out to determine the mechanistic basis of *p*-NP action on spermatogenic development in the F_1 generation.

We report that *p*-NP specifically targets germ cells (spermatogonia and early spermatocytes) in stages XII–XIV and I–III for caspase-3–dependent apoptotic death, as revealed by cleaved caspase-3 and TUNEL immunohistochemical analysis, and these actions were coincident with an increase in the proportion of meiotically active seminiferous tubules. Furthermore, *p*-NP treatment altered the spermatogenic cycle by decreasing the duration of stages I–III and VII–IX and increasing the duration of stages containing late-stage spermatocytes (stages X–XIV).

Materials and Methods

Animals and Treatments

The present study was based on testicular tissues sampled in an earlier study that used the modified Organization for Economic Cooperation and Development 415 one-generation test (de Jager *et al.*, 1999). Briefly, 7-day pregnant Sprague-Dawley rats received 100 or 250 mg/kg *p*-NP or vehicle (cottonseed oil) only intragastrically. Dosing of all groups (n = 20/group) was daily and continued through gestation, lactation, and afterward directly to all male offspring until 10 weeks of age. Animals were housed, according to the Animal Use and Care guidelines of the University of Pretoria, under routine laboratory

conditions (12-h light-dark cycle; 22°C ± 3 room temperature [RT] and 30-70% relative humidity) at the Laboratory Animal Facility of the Faculty of Veterinary Sciences, University of Pretoria and were given access to standard rat chow and tap water *ad libitum*. On the day of sampling, animals were decapitated and the epididymides and both testes were removed. A small slit was made in the testes after which they were immersion fixed in Bouin's solution for 12 h. Before further processing, testes were cut in half and fixed for a further 2.5 days before being transferred to 70% ethanol. Fixed tissues were thoroughly rinsed in 70% ethanol, before they were embedded in Tissue Tek III paraffin wax (Sakura Finetek, Torrance, CA). For positive control, human testicular tissues were obtained from men, aged 57–70 years old, undergoing orchidectomy as treatment for prostate cancer.

Immunohistochemistry

One paraffin-embedded testis from each of the 10 rats in each treatment group was processed for immunohistochemical analysis. Paraffin sections (4-µm thick) were floated in a water bath (45°C), collected on Superfrost Plus slides (Menzel-Glaser, Germany), deparaffinized and rehydrated stepwise through an ethanol series.

TUNEL immunohistochemistry.

Deparaffinized and rehydrated sections were treated with 0.5% Triton X-100 for 10 min at RT before treatment with 3% hydrogen peroxide for 7 min to quench endogenous peroxidase. This was followed by two washes in phosphate-buffered saline (PBS-A: 50mM sodium phosphate, pH 7.4; 200mM NaCl). Subsequent steps for TUNEL staining were carried out using the ApopTag-Peroxidase Kit according to the supplier's instructions (Chemicon, Temecula, CA), except that the terminal transferase enzyme (TdT) was diluted 1:16 with sterile water. Following incubation of the sections with the TUNEL reaction mixture in a humidified chamber at 37°C for 1 h, sections were treated with the antidigoxigenin-peroxidase complex for 30 min at RT. TUNEL-positive cells were finally detected by treating the sections, according to the supplier's instructions (Vectorlabs, Burlingame, CA), for 1–2 min with a 3,3'-diaminobenzidine (DAB) substrate kit that stained positive cells brown. Sections were counterstained with 0.5% crystal violet–containing methyl green in 100mM sodium acetate (pH 4), dehydrated in 100% butanol, cleared in xylene, and mounted with Entellan permanent mounting medium (Merck, Darmstadt, Germany).

Cleaved caspase-3 immunohistochemistry.

Deparaffinized and rehydrated sections were subjected to an antigen retrieval procedure, that is, heating in a microwave oven (800 W) in 10mM sodium citrate buffer (pH 6.0) for 10 min. After cooling for 20 min at RT, sections were rinsed in distilled water and treated in darkness for 10 min with 3% hydrogen peroxide to quench endogenous peroxidase. Following a rinse in distilled water, the sections were placed in 0.1% Tween 20 in PBS-B (137mM NaCl, 29mM $NaH_2PO_4 \cdot H_2O$, 9mM Na_2HPO_4, pH 7.4) for 5 min. Sections were then incubated with blocking solution (5% normal goat serum in 0.1% Tween 20-PBS-B) for 1 h at RT and then overnight at 4°C with a rabbit polyclonal antibody to cleaved caspase-3 (17/19 kDa fragment of activated caspase-3, Cell Signaling Technology, Beverly, MA) diluted 1:100 in blocking solution. After three washes in 0.1% Tween 20-PBS-B, sections were incubated with biotinylated antirabbit IgG for 30 min at RT, washed in PBS-B, and then incubated with the Vectastain avidin-biotin complex (Vectorlabs, Burlingame, CA) for a further 30 min at RT. Following three 5 min PBS-B washes, the antigen was finally detected by treating the sections with DAB, then counterstained, dehydrated, and mounted as described for the TUNEL method. Negative controls were generated by substituting the primary antibody with normal rabbit serum and by serial dilution of the primary antibody. Both weak and strong cytoplasmic as well as entire immunostaining of the cell were considered as caspase-positive labeling.

Microscopy and Data Analysis

Sections were viewed and photographed with a Nikon Optiphot microscope to which was attached a Nikon DXM1200F Digital camera. For each testicular cross section, all cross-sectional tubular profiles (600–800) were counted, stages assigned, and the number of TUNEL- and caspase-positive cells noted for each cross-sectioned tubule. Because of its metachromatic staining of condensed chromatin as is found during spermiogenesis, the methyl green counterstain had utility for the staging of the spermatogenic cycle (Hess, 1990 ; Russell *et al.*, 1990). To investigate a possible link between the basis of *p*-NP's actions to reduce epididymal sperm counts (de Jager *et al.*, 1999) and the highly ordered sequence of germ cell development, criteria for pooling of stages focused primarily on stages containing maturing and mature spermatids and spermiated and postspermiation tubules. Thus, the intensely turquoise-stained heads of the most advanced spermatids in stages IV–VI (readily observed gradual base-to-lumen movement of the heads of the most advanced spermatids) and stages VII–VIII (characteristic arrangement of their spermatid heads and residual bodies on the luminal border of the Sertoli cells) clearly distinguished these stages from the rest of stages in which the short heads of the most advanced spermatids (elongating spermatids) were distinctly blue. The rest of the tubules were pooled as spermiated (stages late VIII–IX), postspermiated (stages X–XI), meiotically active (stage XIV) tubules, and tubules with newly formed spermatids (stages I–III).

Statistics

Data were analyzed by one-way ANOVA using Instat Version 2.03 (GraphPad Software, San Diego, CA). Dunnet multiple comparison test was used to determine which values differed significantly from controls ($p < 0.05$).

RESULTS

TUNEL Immunohistochemistry

To establish whether apoptotic death may underlie the reduced sperm counts in *p*-NP–treated rats, we performed TUNEL immunohistochemistry. In both controls and *p*-NP–treated testes, TUNEL-positive cells were found in the basal and adluminal compartments of the seminiferous epithelium but specifically in stages XII–XIV and I–III (Fig. 1). Apart from an increased number of TUNEL-stained germ cells (Figs. 1B and 1C), *p*-NP–treated testes also showed an increased number of TUNEL-labeled arrested cell divisions (Fig. 1C). TUNEL labeling of basally located germ cells was, however, selective and excluded dying cells with clear apoptotic morphology (cells with a single or several intensely basophilic masses of chromatin, Figs. 1C and 1D). When these observations were analyzed quantitatively, treatment of animals with 100 and 250 mg/kg *p*-NP significantly ($p < 0.02$) increased the number of TUNEL-positive germ cells by 1.9-fold and 1.7-fold, respectively (Fig. 2).

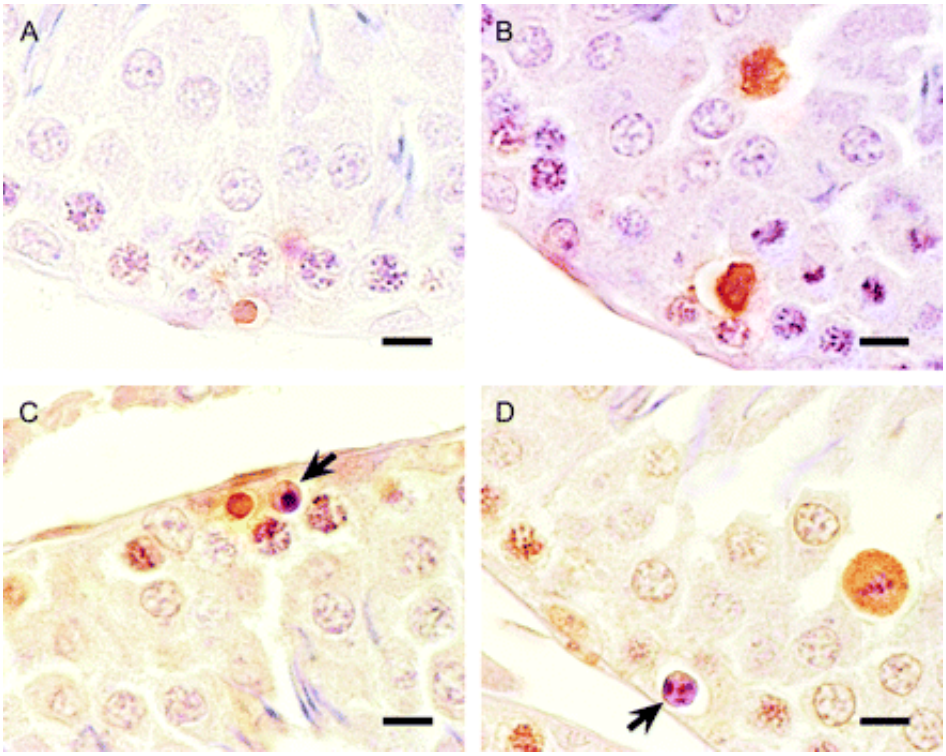

FIG. 1 TUNEL immunostaining in stage XIV tubules in rats treated with (A) vehicle and (B)–(D) 250 mg/kg *p*-NP. (Sections were counterstained with methyl green containing crystal violet). In both controls and *p*-NP treated rats, TUNEL-positive germ cells were specifically found in stages XII–XIV and I–III. Compared to (A) control testes, (B) – (D) *p*-NP–treated testes had an increased number of TUNEL-stained germ cells in both the (C) basal and (B) adluminal compartments, including an increased number of (C) TUNEL-labeled arrested cell divisions. However, TUNEL labeling of basally located dying germ cells was selective, always excluding those cells with (C) a single or (D) several intensely basophilic clumps of chromatin (arrow). Bar = 10 µm.

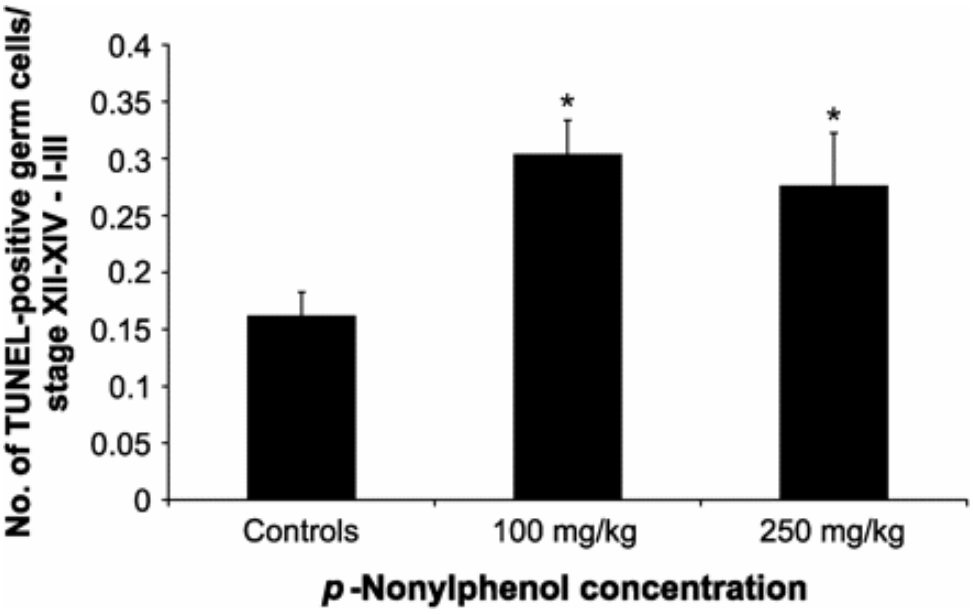

FIG. 2 The effect of long-term *p*-NP treatment on TUNEL immunostaining in testicular cross sections. Values represent the mean ± SEM number of TUNEL-positive germ cells per stage XII–XIV and I–III tubule (TUNEL index). Data are from one testis from each of the 10 animals in each treatment group. Asterisks indicate significant differences from controls ($p < 0.05$).

Cleaved Caspase-3 Immunohistochemistry

Given the observed selectivity of TUNEL labeling, we set out to establish the intracellular apoptotic pathway involved here. Whereas the TUNEL method labels the end stages of apoptotic death (fragmented DNA), immunostaining with a rabbit polyclonal antibody to the cytoplasmically located cleaved caspase-3 labels upstream apoptotic events. As shown in Figure 3, single as well as clusters of caspase-positive germ cells were regularly observed near or along the basement membrane in all tubules either lacking round spermatids or with only early round spermatids (stages XII–XIV and I–III). Caspase immunostaining varied within these basally located germ cells and included intense immunostaining that either (1) filled the entire cell and partly obscuring the nucleus (Figs. 3A and 3B) or (2) was cytoplasmic in cells with (i) a normal-like appearance (not shown), (ii) intensely basophilic clumps of chromatin (Fig. 3B), and (iii) moderate, yet specific immunostaining and with one or two intensely basophilic spherical masses of chromatin (Fig. 3C). Conversely, distinctly degenerate germ cells lying in halos, that lacked any definitive nuclear material and that were located throughout the seminiferous epithelium, were very faintly caspase labeled or caspase negative (Fig. 3D).

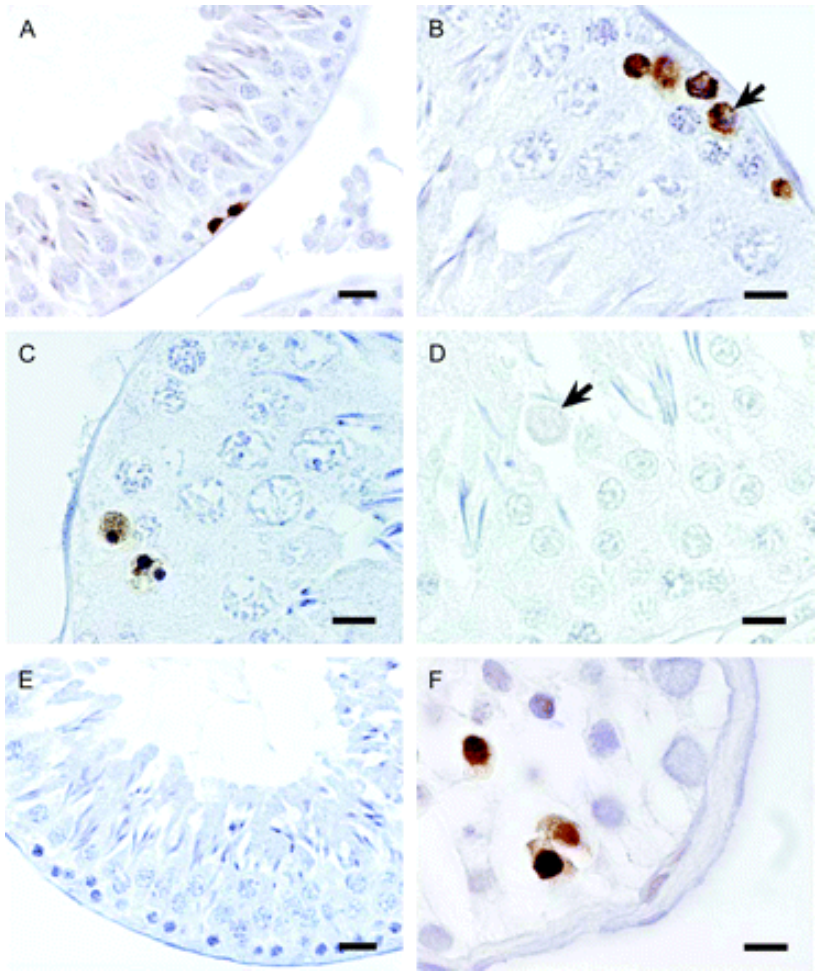

FIG. 3 Immunostaining patterns with the rabbit polyclonal cleaved caspase-3 antibody to detect early-stage apoptotic germ cells in testicular cross sections of *p*-NP–treated ([A] and [D], 100 mg/kg dose; [B] and [C], 250 mg/kg) rats. (Sections were counterstained with methyl green containing crystal violet). Caspase-positive cells were mostly basally located, including (A, stage XII) spermatogonia linked by intercellular bridges and (B, stage XIII) clusters of spermatogonia and zygotene spermatocytes. Depending on the plane of sectioning, intense immunostaining filled the cell but excluded several clumps of intensely basophilic chromatin (arrow). In other caspase-positive cells (C, stage XIII), immunostaining was diminished, coincident with compaction of chromatin into one or more intense basophilic spherical masses. Conversely, immunostaining was faint or absent in (D, stage XIV) distinctly apoptotic cells. Note also the size difference between this dying cell and the surrounding secondary spermatocytes. (E, stage XII) Negative control. (F) Positive control of aging human testis, showing immunostained spermatocytes. Bar: (A) and (E), 20 µm; (B)–(D) and (F), 10 µm.

Given these various manifestations of caspase-3 immunoreactivity, it was argued that the actual rate of *p*-NP–enhanced apoptosis may have been underestimated as the TUNEL method only labels the end stage of the apoptotic death process. When analyzed quantitatively, the number of caspase-3–positive cells in the *p*-NP–treated rats tended to be elevated above that of vehicle-treated animals (Fig. 4A), the shape of the curve resembling that of the TUNEL analysis, though not statistically significant. To understand the contextual relationship of the observed distinctly degenerate but very faintly caspase-labeled and -negative cells, these cells were analyzed separately (Fig. 4B) and as a ratio of the number of caspase-3–positive cells, that is, the caspase index (Fig. 4C). Although not statistically significant, the number of degenerate cells tended to be lower in *p*-NP–treated rats, with the decrease of 1.6-fold in 100-mg/kg-treated animals compared to controls (Fig. 4B). Conversely, differences in the ratio of the number of caspase-3–positive cells to the number of distinctly degenerate cells, among the three treatment groups were significant ($p = 0.05$), with the caspase index of the 100-mg/kg treatment group significantly ($p < 0.05$) threefold greater than controls (Fig. 4C).

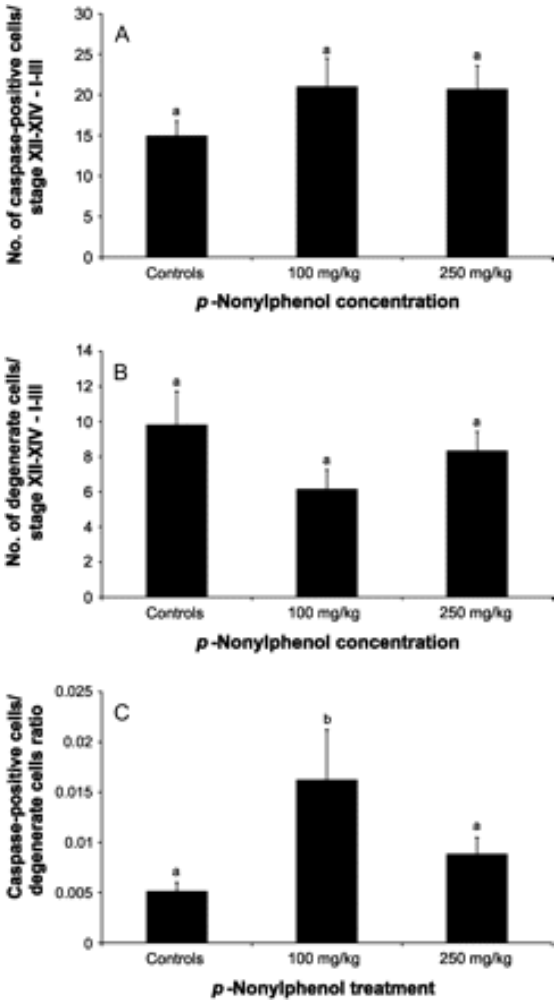

FIG. 4 The effect of long-term *p*-NP treatment on cleaved caspase-3 immunostaining in testicular cross sections. Values represent the mean ± SEM ([A] number of caspase-positive cells, [B] number of degenerate cells, and [C] caspase-positive cells/degenerate cells ratio) per stage XII–XIV and I–III tubule. Data are from one testis from each of the 10 animals in each treatment group. Different letters indicate significant differences ($p < 0.05$).

Changes in the Observed Frequency of Stages of the Spermatogenic Cycle

Since new generations of germ cells commence their development before those that began their development earlier have completed theirs, spermatogenesis is consequently a lengthy, complex process, involving time-dependent development and assembly of intracellular components in germ cells over 14 stages. We wished to establish whether the subtle effects of *p*-NP may be related to dysregulation of the duration of the stages of the spermatogenic cycle. *p*-NP treatment significantly ($p < 0.002$) altered the observed frequencies of six of the seven groups of stages of the spermatogenic cycle (Fig. 5). Whereas both doses and either 250 or 100 mg/kg treatment alone significantly ($p < 0.002$) reduced the frequencies of stages I–III, VII–VIII, and late VIII–IX (spermiating and recently spermiated tubules), respectively, both doses significantly ($p < 0.002$) increased the frequencies of stages IV–VI, XII–XIII, and XIV (meiotic divisions), respectively.

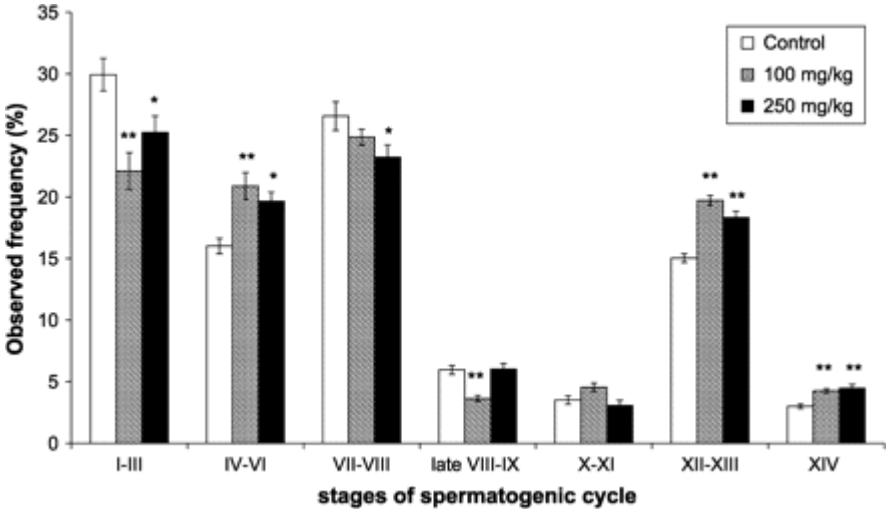

FIG. 5 The effect of long-term *p*-NP treatment on the frequency of the stages in the spermatogenic cycle. Values represent the mean ± SEM observed frequency of stages; $n = 10$ per treatment group. Asterisks indicate significant differences from controls (*$p < 0.05$, **$p < 0.01$).

Discussion

Findings presented here are consistent with the emerging view that environmental (weak) estrogens such as *p*-NP, although crossing the placenta to the fetus in very small amounts (Doerge *et al.*, 2002), may, through their cumulative effects, perturb normal steroid-dependent developmental processes during critical window periods of male reproductive development, all of which may translate into reduced reproductive parameters in the male offspring (Chapin *et al.*, 1999 ; de Jager *et al.*, 1999). Likewise, although the alteration of spermatogenic events are not strikingly overt, the small changes are significant and broadly agrees with the notion that endocrine disruptor-induced small to modest changes in the hormonal milieu of steroid-sensitive tissues in the fetus, often lead to detectable effects later in adulthood (Crews and McLachlan, 2006) and in subsequent generations (Anway *et al.*, 2006 ; Chapin *et al.*, 1999).

Results presented here suggest that inappropriate elevation of germ cell apoptosis may underlie the reduced sperm counts following long-term exposure to *p*-NP. Although not in a dose-related manner, our findings that *p*-NP increased the number of TUNEL-positive germ cells specifically in stages XII–XIV and I–III resembles that reported for another endocrine disruptor (Anway *et al.*, 2006).

Cleaved caspase-3 is the major executioner of nuclear apoptotic morphology, and our findings are consistent with the well-delineated time course of intracellular events during apoptosis, including chromatin condensation and margination. Based on the observed slight decrease and increase in the number of distinctly degenerate cells and caspase-positive cells, respectively, in especially the 100-mg/kg treatment group, our results suggest that the time course of intracellular apoptotic events, which are known to be caspase-3 dependent in the rat testis (Moreno *et al.*, 2006), may have been altered by *p*-NP treatment. Exogenous toxic substances are known to change the time course of intracellular apoptotic events, as was reported for bleomycin (Mekid *et al.*, 2003). In addition, caspase-3 deficiency markedly delays the kinetics of apoptotic events, including DNA fragmentation, in hepatocytes and thymocytes *in vitro* (Zheng *et al.*, 1998).

Other explanations for the discordances between the TUNEL and caspase-3 analyses are also plausible. Firstly, the TUNEL and caspase-3 immunohistochemical methods differ in their specificities, with TUNEL also labeling nonapoptotic death processes, for example, arrested cell divisions. In this regard, inappropriate elevation of meiotic activities (increased frequency of stage XIV tubules) by *p*-NP treatment concomitantly also increased the number of arrested meiotic divisions, and these were TUNEL labeled but not caspase labeled, a further explanation why the TUNEL index was higher than the caspase index. Whether *p*-NP specifically reduces the efficiency of meiotic events remains to be verified (see below). Secondly, considering that the 100 mg/kg dose gave a maximal response for nearly all end points, and was thus at the high end of the dose-response curve for all these end points, another explanation for the lack of a dose-response in particularly the TUNEL analyses in stages XII–XIV and I–III could be that cell death was occurring in stages during which Sertoli cell phagocytic activity is known to be inhibited (Russell *et al.*, 1990). Interestingly, unlike dying preleptotene and mid-pachytene spermatocytes (found in stages VII–VIII), which induce full Sertoli phagocytic activity *in vitro*, dying late-stage spermatocytes, including meiotically dividing cells, that is, spermatocytes present in stages XII–XIV, strongly inhibit Sertoli cell phagocytic activity *in vitro* (Grandjean *et al.*, 1997), which prompts the suggestion that these dying spermatocytes may persist in the epithelium for longer than dying stage VII–VIII spermatocytes. Our observations of dying pachytene/diplotene spermatocytes amid secondary spermatocytes would (Fig. 3D) support this notion. Thus, the mere presence of apoptotic cells is not the signal that triggers full phagocytic activity of the Sertoli cell, but the germ cell type that is apoptotic, is. Lastly, given the importance of critical window periods of susceptibility to hormonal influence (endogenous or exogenous), especially, during early development, it is noteworthy that octylphenol treatment of neonatal rats resulted in reduced germ cell apoptosis at day 18 but not when analyzed on day 25. Thus, given our single end point of analysis (age 10 weeks), it is not known to what extent germ cell death ratios were affected at earlier stages of development.

Spermatogenesis is a long, complex but synchronized process involving time-dependent development of and assembly of intracellular components to germ cells over 14 man-identified stages. *p*-NP treatment decreased the frequencies (i.e., the duration) of some groups of stages (I–III, VII–VIII, late VIII–IX) and increased the duration of others (stages IV–VI, XII–XIII, XIV). Whether these perturbations of the duration of various stages directly or indirectly underlie reduced fertility potential remains to be verified. It is known, however, that *p*-NP administered in drinking water at 50 and 500 µg/l to outbred CD-1 mice for 100 days significantly reduced acrosomal integrity (Kyselova *et al.*, 2003). By extension and assuming the car plant analogy of spermatogenic development as proposed by Sharpe (1994) , it is possible that the lengthy process of acrosome development in Sprague-Dawley rats may well have been similarly adversely affected by *p*-NP. However, the frequency of the stages during which the maturation of elongated spermatids occur and mature spermatids are released (stages VII–VIII) is decreased in a dose-related manner, a finding that is in broad agreement with those found in rats fed with xenoestrogen-contaminated fish (Aravindakshan *et al.*, 2004). Another interesting finding is that the duration of all stages containing late-stage spermatocytes (stages X onward) was gradually increased, culminating in significant increased duration in stages XII,XIII, and XIV (meiotically active tubules). The *p*-NP–induced prolonged passage of

germ cells through the first phase of meiosis may be associated with the increased germ cell apoptosis in these stages. All in all, our findings are consistent with similar observations of increased meiotic divisions at day 18 in rats treated neonatally with octylphenol (Atanassova *et al.*, 2000). Furthermore, our findings may also be interpreted to suggest that testosterone metabolism may have been increased by *p*-NP action, specifically at these stages, because removal of the Leydig cells (through ethane dimethanesulphonate treatment) causes a threefold to fourfold increase in the number of degenerating metaphase spermatocytes in stages XIV-I (Sharpe *et al.*, 1988). Thus, the notion of increased testosterone metabolism is in broad agreement with our apoptotic analyses which showed no apoptotic spermatocytes in the exquisitely androgen-sensitive stages (VII–VIII). Indeed, octylphenol stimulates testosterone secretion in isolated neonatal Leydig cells (Murono *et al.*, 1999) and in cultured fetal rat testes (Haavisto *et al.*, 2003), which may explain increased spermatogonial cell divisions in organ cultured Japanese eel testis upon exposure to *p*-NP *in vitro* (Miura *et al.*, 2005).

Taken together, these and other findings are consistent with other *in vitro* studies which show that nonylphenol and other environmental toxicants exert their dysregulatory effects via Sertoli cells (Aravindakshan and Cyr, 2005 ; Fiorini *et al.*, 2004). A characteristic feature of *p*-NP–treated testes was tubules that partially resembled stage VII in one area of the tubule, but in other areas clearly containing bundles of elongating spermatids that were still deeply embedded in the Sertoli cell cytoplasm and that have not yet completed the base-to-lumen adjustment of the spermatid heads, which necessitated their classification as stages IV–VI. Thus, a reduced ability of Sertoli cells to facilitate this base-to-lumen adjustment may have resulted in reduced frequencies of stages VII–VIII, that is, reduced proportions of mature and spermiating tubules.

Given that Sprague-Dawley rats are known to be more resistant to estrogenic substances than other rodent strains, the actual disruptive effects of *p*-NP on testicular development may be underestimated (Spearow and Barkley, 2001) or may be manifested only in subsequent generations (Chapin *et al.*, 1999). Admittedly, the doses administered were high, but some studies showed that repeated exposures of rats to *p*-NP, caused, though at very low levels, a fourfold to fivefold increase in *p*-NP concentration in fat tissue (Green *et al.*, 2003), tissue accumulation of active NP aglycone in lipophilic endocrine-responsive tissues, and placental transfer into the serum and brains of fetuses (Doerge *et al.*, 2002). Whether this accumulated *p*-NP might be responsible for the insidiously altered spermatogenesis and cell death ratios described here remains to be established. Nevertheless, it is concluded that the *p*-NP–enhanced germ cell apoptosis underlies the reduced epididymal sperm counts. What is clear is that alkylphenols may influence germ cell kinetics in various ways, but each effect is cell context and developmental stage dependent and, probably, mediated *via* the Sertoli cells, which also undergo spatiotemporal changes at defined stages of testicular development.

References

Anway MD, Memon MA, Uzumcu M, Skinner MK. (2006) Transgenerational effect of the endocrine disruptor vinclozolin on male spermatogenesis. *J. Androl.* 27:868–879.

Aravindakshan J and Cyr DG. (2005) Nonylphenol alters connexin 43 levels and connexin 43 phosphorylation via an inhibition of the p38-mitogen-activated protein kinase pathway. *Biol. Reprod.* 72:1232–1240.

Aravindakshan J, Gregory M, Marcogliese DJ, Fournier M, Cyr DG. (2004) Consumption of xenoestrogen-contaminated fish during lactation alters adult male reproductive function. *Toxicol. Sci.* 81:179–189.

Atanassova N, McKinnell C, Turner KJ, Walker M, Fisher JS, Morley M, Millar MR, Groome NP, Sharpe RM. (2000) Comparative effects of neonatal exposure of male rats to potent and weak (environmental) estrogens on spermatogenesis at puberty and the relationship to adult testis size and fertility: Evidence for stimulatory effects of low estrogen levels. *Endocrinology* 141:3898–3907.

Auger J, Kunstman JM, Czyglik F, Jounnet P. (1995) Decline in semen quality among fertile men in Paris during the past 20 years. *N. Engl. J. Med.* 332:281–285.

Bendsen E, Laursen SB, Olesen C, Westergaard LG, Andersen CY, Byskov AG. (2001) Effect of 4-octylphenol on germ cell number in cultured human fetal gonads. *Hum. Reprod.* 16:236–243.

Blankenship AL and Coady K. (2005) Nonylphenol. In Wexler P (Ed.). Encyclopedia of Toxicology 2nd ed. (Elsevier Academic Press, Oxford) Vol. 3: pp. 260–263.

Bolt HM, Janning P, Michna H. (2001) Comparative assessment of endocrine modulators with oestrogenic activity: I. Definition of a hygiene-based margin of safety (HBMOS) for xeno-oestrogens against the background of European developments. *Arch. Toxicol.* 74:649–662.

Chapin RE, Delaney J, Wang Y, Lanning L, Davis B, Collins B, Mintz N, Wolfe G. (1999) The effects of 4-nonylphenol in rats: A multigenerational reproduction study. *Toxicol. Sci.* 52:80–91.

Crews D and McLachlan JA. (2006) Epigenetics, evolution, endocrine disruption, health, and disease. *Endocrinology* 147:Suppl., S4–S10.

de Jager C, Bornman MS, Oosthuizen JMC. (1999) II. The effect of *p*-nonylphenol on the fertility potential of male rats after gestational, lactational and direct exposure. *Andrologia* 31:107–113.

Doerge DR, Twaddle NC, Churchwell MI, Chang HC, Newbold RR, Delclos KB. (2002) Mass spectrometric determination of *p*-nonylphenol metabolism and disposition following oral administration to Sprague-Dawley rats. *Reprod. Toxicol.* 16:45–56.

Fiorini C, Tilloy-Ellul A, Chevalier S, Charuel C, Pointis G. (2004) Sertoli cell junctional proteins as early targets for different classes of reproductive toxicants. *Reprod. Toxicol.* 18:413–421.

Gown AM and Willingham MC. (2002) Improved detection of apoptotic cells in archival paraffin sections: Immunohistochemistry using antibodies to cleaved caspase 3. *J. Histochem. Cytochem.* 50:449–454.

Grandjean V, Sage J, Ranc F, Cuzin F, Rassoulzadegan M. (1997) Stage-specific signals in germ line differentiation: Control of Sertoli cell phagocytic activity by spermatogenic cells. *Dev. Biol.* 184:165–174.

Green T, Swain C, Van Miller JP, Joiner RL. (2003) Absorption, bioavailability, and metabolism of *para*-nonylphenol in the rat. *Regul. Toxicol. Pharmacol.* 38:43–51.

Haavisto TE, Adamsson NA, Myllymäki SA, Toppari J, Paranko J. (2003) Effects of 4-*tert*-octylphenol, 4-*tert*-butylphenol, and diethylstilbestrol on prenatal testosterone surge in the rat. *Reprod. Toxicol.* 17:593–605.

Hess RA. (1990) Quantitative and qualitative characteristics of the stage and transitions in the cycle of the rat seminiferous epithelium: Light microscopic observations of perfusion-fixed and plastic-embedded testes. *Biol. Reprod.* 43:525–542.

Kudo C, Wada K, Masuda T, Yonemura T, Shibuya A, Fujimoto Y, Nakajima A, Niwa H, Kamisaki Y. (2004) Nonylphenol induces the death of neural stem cells due to activation of the caspase cascade and regulation of the cell cycle. *J. Neurochem.* 88:1416–1423.

Kyselova V, Peknicova J, Buckiova D, Boubelik M. (2003) Effects of *p*-nonylphenol and resveratrol on body and organ weight and in vivo fertility of outbred CD-1 mice. *Reprod. Biol. Endocrinol.* 1:30–40.

Masuno H, Okamoto S, Iwanami J, Honda K, Shiosaka T, Kidani T, Sakayama K, Yamamoto H. (2003) Effect of 4-nonylphenol on cell proliferation and adipocyte formation in cultures of fully differentiated 3T3-L1 cells. *Toxicol. Sci.* 75:314–320.

Mekid H, Tounekti O, Spatz A, Cemazar M, El Kebir FZ, Mir LM. (2003) *In vivo* evolution of tumour cells after the generation of double-strand DNA breaks. *Br. J. Cancer* 88:1763–1771.

Miura C, Takahashi N, Michino F, Miura T. (2005) The effect of *para*-nonylphenol on Japanese eel (*Anguilla japonica*) spermatogenesis *in vitro*. *Aquat. Toxicol.* 71:133–141.

Moreno RD, Lizama C, Urzúa N, Vergara SP, Reyes JG. (2006) Caspase activation throughout the first wave of spermatogenesis in the rat. *Cell Tissue Res.* 325:533–540.

Murono E, Derk RC, de León JH. (1999) Biphasic effects of octylphenol on testosterone biosynthesis by cultured Leydig cells from neonatal rats. *Reprod. Toxicol.* 13:451–462.

Newbold RR, Padilla-Banks E, Jefferson WN. (2006) Adverse effects of the model environmental estrogen diethylstilbestrol are transmitted to subsequent generations. *Endocrinology* 147:(Suppl.), S11–S17.

Russell LD, Ettlin RA, Sinha Hikim AM, Clegg ED. (1990) Histological and Histopathological Evaluation of the Testis(Cache River Press, Clearwater, FL).

Sharpe RM. (1994) Regulation of spermatogenesis. In Knobil E and Neill JD (Eds.). The Physiology of Reproduction 2nd ed. (Raven Press, New York) pp. 1363–1434.

Sharpe RM. (2001) Hormones and testis development and the possible adverse effects of environmental chemicals. *Toxicol. Lett.* 120:221–232.

Sharpe RM, Fraser HM, Ratnasooriya WD. (1988) Assessment of the role of Leydig cell products other than testosterone in spermatogenesis and fertility in adult rats. *Int. J. Androl.* 11:507–523.

Sharpe RM and Skakkebaek NE. (1993) Are oestrogens involved in falling sperm counts and disorders of the male reproductive tract? *Lancet* 1:1392–1395.

Skakkebaek NE, Rajpert-De Meyts E, Jørgensen N, Carlsen E, Petersen PM, Giwercman A, Andersen A-G, Jensen TK, Andersson A-M, Müller J. (1998) Germ cell cancer and disorders of spermatogenesis: An environmental connection? *APMIS* 106:3–12.

Soto AM, Justica H, Wray J, Sonnenschein C. (1991) *p*-Nonyl-Phenol: An estrogenic xenobiotic released from "modified" polystyrene. *Environ. Health Perspect.* 92:167–173.

Spearow JL and Barkley M. (2001) Reassessment of models used to test xenobiotics for oestrogenic potency is overdue. *Hum. Reprod.* 16:1027–1029.

Van den Berg M, Sanderson T, Kurihara N, Katayama A. (2003) Role of metabolism in the endocrine-disrupting effects of chemicals in aquatic and terrestrial systems. *Pure Appl. Chem.* 75:1917–1932.

Vivacqua A, Recchia AG, Fasanella G, Gabriele S, Carpino A, Rago V, Di Gioia ML, Leggio A, Bonofiglio D, Liguori A, et al. (2003) The food contaminants bisphenol A and 4-nonylphenol act as agonists for estrogen receptor alpha in MCF7 breast cancer cells. *Endocrine* 22:275–284.

White R, Jobling S, Hoare SA, Sumpter JP, Parker MG. (1994) Environmentally persistent alkylphenolic compounds are estrogenic. *Endocrinology* 135:175–182.

Yao G, Yang L, Hu Y, Liang J, Liang J, Hou Y. (2006) Nonylphenol-induced thymocyte apoptosis involved caspase-3 activation and mitochondrial depolarization. *Mol. Immunol.* 43:915–926.

Zalko D, Costagliola R, Dorio C, Rathahao E, Cravedi J-P. (2003) *In vivo* metabolic fate of the xeno-estrogen 4-n-nonylphenol in Wistar rats. *Drug Metab. Dispos.* 31:168–178.

Zheng TS, Schlosser SF, Dao T, Hingorani R, Crispe IN, Boyer JL, Flavell RA. (1998) Caspase-3 controls both cytoplasmic and nuclear events associated with Fas-mediated apoptosis *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.* 95:13618–13623.