2,5-Hexanedione (2,5-HD), a taxol-like promoter of microtubule assembly, and carbendazim (CBZ), a colchicine-like inhibitor of microtubule assembly, are two environmental testicular toxicants that target and disrupt microtubule function in Sertoli cells. At the molecular level, these two toxicants have opposite effects on inhibiting microtubule-dependent functions of Sertoli cells. By studying a combined exposure to 2,5-HD and CBZ, we sought to determine whether CBZ would antagonize or exacerbate the effects of an initial 2,5-HD exposure. In vitro, 2,5-HD-treated tubulin had a decreased lag time and an increased maximal velocity of microtubule assembly. These 2,5-HD-induced in vitro alterations in microtubule assembly were normalized by CBZ exposure. In vivo, adult male rats were exposed to a 1% solution of 2,5-HD in the drinking water for 2.5 weeks. CBZ was administered by gavage (200 mg/kg body weight) at the same time as unilateral surgical ligation of the efferent ducts, 24 h before evaluation of the testis. Measures of testicular effect (testis weight, histopathologic changes [sloughing and vacuolization], and seminiferous tubule diameters) were all significantly altered with combined exposure. The testicular effects in the combined exposure group were either different (seminiferous tubule diameters), additive (% vacuolization), or greater than additive (% sloughing) compared to the effects of the individual toxicant exposure groups referenced to the controls. Therefore, CBZ coexposure does not antagonize the effects of an initial 2,5-HD exposure, as might be expected if their molecular effects on microtubule assembly were solely responsible for their combined toxicity; instead, 2,5-HD and CBZ act together to exacerbate the testicular injury.

Key Words: 2,5-hexanidine; carbendazim; microtubule; Sertoli; testis.

Real-life exposures to testicular toxicants most often involve complex mixtures of hazardous chemicals. Because of the multiplicity of testicular targets and the complexity of the exposures, few mechanistic data are available concerning the effects of coexposures to testicular toxicants. These experiments begin to address this deficiency by comparing single exposures with coexposure to two well-described testicular toxicants.

The environmental toxicants 2,5-hexanedione (2,5-HD; acetonyl acetone) and carbendazim (CBZ; methyl-2-benzimidazole carbamate) are suspected of targeting and disrupting microtubule function in Sertoli cells leading to testicular injury (Boekelheide et al., 2003; Carter et al., 1987; Nakai et al., 1992). These toxicants have opposing actions on microtubules and different molecular mechanisms of toxicity. However, disruption of Sertoli cell microtubules by both these toxicants results in broadly similar physiological consequences including inhibiting seminiferous tubule fluid formation, inducing sloughing of seminiferous epithelium, and germ cell death (Boekelheide and Hall, 1991; Hess et al., 1991; Johnson et al., 1991; Nakai et al., 1992; Richburg et al., 1994).

Microtubules are ubiquitous cytoskeletal elements, with numerous structural and functional roles, including nuclear division, movement, cell shape, and secretion (Dustin, 1984). They are polymers of the core protein tubulin, a 100 kDa α-β heterodimer, with attached microtubule associated proteins (Dustin, 1984). Sertoli cells produce a nurturing environment for germ cells within the seminiferous epithelium by forming inter Sertoli cell junctions, known as the blood-testis barrier. This special environment is maintained structurally by microtubules that are highly concentrated in radially oriented cytoplasmic trunks of the Sertoli cells. Exposure to microtubule disrupters which are known testicular toxicants consistently results in impaired Sertoli cell function and germ cell loss, indicating that Sertoli cell microtubules are critical to normal testicular homeostasis (Boekelheide et al., 2003).

2,5-HD is the toxic metabolite of the common industrial solvents n-hexane and methyl n-butyl ketone (Boekelheide et al., 2003). Chronic exposure to these solvents induces peripheral neuropathy (Spencer and Schaumburg, 1977) and testicular injury (Krasavage et al., 1980) after metabolism to the ultimate testicular toxicant, 2,5-HD. One unusual feature of 2,5-HD-induced testicular injury is the long latency between initiation of exposure and the appearance of histopathologic alterations. In fact, a two-week exposure to a 1% solution of 2,5-HD in the

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Toxicological Sciences vol. 80 no. 1 © Society of Toxicology 2004; all rights reserved.
drinking water of rats is sufficient to produce injury. However, morphological alterations in the seminiferous epithelium are not apparent until 1–2 weeks postexposure (Boekelheide, 1988a,b).

The delay in toxic effect is partially explained by the complex chemistry of the interaction of 2,5-HD with tissue nucleophiles that requires sequential steps of pyrrole formation, oxidation, and crosslinking (Boekelheide et al., 1988; DeCaprio et al., 1988; Genter et al., 1987, 1988; Graham et al., 1982; Rosenberg et al., 1987; Sayre et al., 1986). About 3–4 weeks after initiating exposure, large basal vacuoles appear in the Sertoli cell cytoplasm (Boekelheide, 1988a,b). This is soon followed by cessation of seminiferous tubule fluid secretion, a Sertoli cell function, and germ cell apoptosis (Blanchard et al., 1996). Progressive loss of spermatocytes and spermatagonia and sloughing of seminiferous epithelium continues for several weeks after the initial germ cell manifestations of injury (Boekelheide, 1988a,b).

At the molecular level, a dramatic alteration is observed in the assembly of testis tubulin purified from 2,5-HD exposed rats (Boekelheide, 1987a,b; Boekelheide, 1988a,b; Boekelheide and Eveleth, 1988) and tubulin treated with 2,5-HD in vitro displays the same altered assembly behavior (Boekelheide, 1987a,b). The alteration in microtubule assembly is taxol-like, characterized by more rapid assembly and enhanced microtubule stability (Boekelheide, 1987a,b). Given this microtubule effect, Sertoli cell microtubule-dependent functions—such as seminiferous tubule fluid secretion—have been proposed as a subcellular target for 2,5-HD (Boekelheide, 1988a,b; Chapin et al., 1983; Johnson et al., 1991).

CBZ is the active toxic metabolite of its less potent parent compound, benomyl, a benzimidazole fungicide (Lim and Miller, 1997). Subchronic or chronic exposure of animals to benomyl or CBZ produces selective alterations in the male reproductive tract, including decreased fertility, decreased epididymal sperm counts, decreased caudal epididymal weights, and histopathologic changes in the testis (Barnes et al., 1983; Carter et al., 1987; Carter and Laskey, 1982; Linder et al., 1988). Detailed histopathological analysis of the testicular effects of CBZ exposure has been facilitated by a dosing regimen of a single high dose exposure. Such exposures produce direct effects on dividing germ cells and on spermiogenesis, and significantly alter Sertoli cell structure and function. Sloughing of the apical seminiferous epithelium is observed due to cleavage of the apical Sertoli cell cytoplasm, resulting in a cluster of sloughed spermatids with an attached portion of Sertoli cell cytoplasm (Nakai and Hess, 1994). The sloughed fragments of seminiferous epithelium block the efferent ducts; acutely, this results in an increase in both testis weight and seminiferous tubule diameters with an eventual evolution to profound testicular atrophy.

At the molecular level, benzimidazoles bind to the β-tubulin subunit of the α-β-tubulin heterodimer, inhibiting microtubule polymerization (Howard and Aist, 1980; Quinlan et al., 1980). Studies of the effects of CBZ on microtubule assembly demonstrate a colchicine-like decrease in the rate of assembly and decreased microtubule stability (Correa and Miller, 2001; Winder et al., 2001).

Surgical efferent duct ligation (Setchell, 1970) is a commonly employed in vitro technique for the determination of seminiferous tubule fluid formation. The increased weight of the ligated testis compared to the unligated testis reflects the accumulation of seminiferous tubule fluid; alternatively, a decreased weight of the ligated testis following toxicant exposure would indicate inhibition of seminiferous tubule fluid formation. Toxicant induced sloughing of the seminiferous epithelium and subsequent occlusion of the efferent ducts partly mimics the changes observed in the surgical efferent duct ligation procedure.

Little is known at a mechanistic or practical level about the effects of coexposures on the testis. Given their opposing molecular mechanisms of action on microtubules in Sertoli cells, the purpose of these experiments was to determine if CBZ would antagonize or exacerbate the effects of an initial 2,5-HD exposure. Both in vitro (spectrophotometric microtubule assembly behavior of purified bovine brain tubulin) and in vivo (surgical ligation of the efferent ducts in rats; toxicokinetic profiling of testicular CBZ levels using HPLC; histopathology by light microscopy) techniques were used to analyze the testicular effects of individual compared to combined exposure to 2,5-HD and CBZ.

**MATERIALS AND METHODS**

**Chemicals.** All chemicals were obtained from the Sigma-Aldrich Chemical Company (St. Louis, MO) unless otherwise indicated and were of the highest purity available.

**Tubulin preparation.** Tubulin was purified according to Boekelheide (1987a,b) with some modifications. Bovine brains were homogenized for 1 min in a Waring blender, 100 g at a time, in 75 ml of 0.1 M 2-(N-morpholino)ethane sulfonic acid, 1 mM ethylene glycol bis(β-aminoethyl ether)N,N,N',N'-tetra-acetic acid, 0.5 mM MgCl2, 4 M glycerol, pH 6.75, at 4°C. The supernatant from a cold ultracentrifugation at 100,000 × g at 4°C was frozen in liquid nitrogen and stored at −80°C until needed. The buffer for the remaining purification steps was 1 M sodium glutamate, 0.1 M guanosine 5′-triphosphate (GTP), pH 6.6. DEAE-Sephadex (Pharmacia Inc., Piscataway, NJ) was repeatedly separated for washing and elution steps by gently sedimentation in a clinical centrifuge rather than by filtration. Tubulin, eluted with 1 M NaCl in glutamate buffer, was further purified by two cycles of temperature-dependent assembly and disassembly to give the starting material for incubation with 2,5-HD.

**2,5-HD incubation.** 2,5-HD was incubated with purified tubulin as described (Boekelheide, 1987a,b) with minor modifications. Twice-cycled bovine brain tubulin was adjusted to 1–2 mg/ml and incubated for 16 h at 37°C in 1 M sodium glutamate, 1 mM GTP, pH 6.6, and 100 mM 2,5-HD, yielding 2,5-HD-treated tubulin. Protein concentrations were determined by a dye binding assay using the BioRad DC Protein Assay Kit (BioRad, Rockville Centre, NY) with bovine serum albumin as the standard. Control tubulin was prepared in the same manner; however, control tubulin was incubated in parallel for the last 30 min without the addition of 2,5-HD. Following a 30 min warm ultracentrifugation at 100,000 × g at 29°C, pellets were resuspended in cold glutamate buffer at 0°C without GTP. The solution was then homogenized by a Teflon-glass homogenizer by hand three times and incubated at 0°C for 30 min to disassemble the microtubules. Cold ultracentrifugation at 100,000 × g at 4°C for 30 min gave yield to soluble, active tubulin in the supernatant.
Absorbance at 350 nm was monitored over a period of 25 min at 37°C with a photometer equipped with a thermally jacketed cuvette holder. The change in absorbance was measured spectrophotometrically using a Shimadzu UV-240 recording spectrophotometer equipped with a thermally jacketed cuvette holder. The change was monitored over a period of 25 min at 37°C. After pre-equilibration of semimicro quartz cuvettes, the standard assembly reaction was initiated by addition of ice-cold tubulin at a concentration of 0.4 mg/ml in glutamate buffer, GTP to a final concentration of 250 mM, and coreactants in a final volume of 1.5 ml. Zero time was taken as the time of initiation of recording and excludes a 5–15 s delay involved in the addition and mixing of the reagents. For inhibition studies (Lim and Miller, 1997), a stock solution of CBZ dissolved in dimethyl formamide (DMF; final concentration 1% [v/v]) was added to the final reaction volumes at 0, 4, 37, 8, 7, 15, 7, 35, and 70 μM for control tubulin and 0, 70, and 140 μM for 2,5-HD-treated tubulin.

Animals. Adult male Fischer rats (Charles River Laboratories, Wilmington, MA) weighing 150–175 g were housed on a 12-h alternating light-dark cycle and ad libitum drinking water and fed Purina Rodent Chow 5001 (Farmer’s Exchange, Framingham, MA) and water ad libitum. Animals were acclimated to housing conditions for at least one week prior to use. All procedures involving animals were performed in accordance with the guidelines of Brown University’s Institutional Animal Care and Use Committee in compliance with National Institutes of Health guidelines.

Toxicant exposure. 2,5-HD was administered as a 1% solution in the drinking water ad libitum for 2.5 weeks (Fig. 1). The control for 2,5-HD treatment was drinking water ad libitum for 2.5 weeks. CBZ was administered by gavage, at a dose of 200 mg/kg body weight, in a corn oil emulsion at a volume of 2 ml corn oil/kg body weight. The control for CBZ exposure was gavage of the corn oil vehicle at a volume of 2 ml corn oil/kg body weight.

Surgical ligation of the efferent ducts. Animals were anesthetized with Ketamine (Ketaset, Wyeth Inc., Fort Dodge, IA) and Xylazine (Anased, Lloyd Laboratories, Shenandoah, IA), ip, 100 mg/kg and 13 mg/kg, respectively. Unilateral surgical ligation of the efferent ducts of the right testis was then performed as described by Johnson et al. (1991). Briefly, the right testis was exposed through an abdominal incision, the efferent ducts of the testes were visualized, ligated, and the wound was sutured closed. After 24 h, the rats were killed by carbon dioxide inhalation, and both left and right testes (including ligated ducts) were immediately individually removed, weighed, and immersion fixed in 10% neutral-buffered formalin.

Histopathology and seminiferous tubule diameters. A testis cross section of the left (unligated) fixed testicle was embedded in glycol methacrylate (Technovit 7100, Heraeus Kulzer GmbH, Wehrheim, Germany) sectioned (3 μm), and stained with periodic acid-Schiff reagent followed by a hematoxylin counterstain (PASH). All slides were blinded and coded before evaluation of histopathology and seminiferous tubule diameters. Fifty seminiferous tubules from each unligated left testis of each animal were selected using randomly chosen coordinates from a stage engraved Vernier scale. To be acceptable for evaluation, seminiferous tubule cross sections were required to be nearly round (major:minor axis less than 1.5:1). Each seminiferous tubule was evaluated for its minor diameter and the presence or absence of vacuolization and sloughing. Vacuolization was defined as the presence of one or more basally located vacuoles >16 μm in greatest dimension within the seminiferous epithelium. Sloughing was defined as the presence of one or more groupings of luminal cellular material detached from the seminiferous epithelium of >24 μm in greatest dimension. The percentage of tubules demonstrating these described histopathological endpoints was assessed. Light microscopic images were obtained on a Zeiss Axiosvert 35 microscope (Carl Zeiss, New York, NY) and connected to a Spot RT camera (Diagnostic Instruments Inc., Sterling Heights, MI).

Testicular CBZ levels. CBZ levels in the tests were determined using HPLC preparation and analysis as previously described by Correa et al. (2002) with modifications. Testicular CBZ levels were determined in both control and 2,5-HD-treated rats (three rats per group per time point: 15, 30, 60, 90, and 150 min) after CBZ exposure by gavage (200 mg/kg). At the designated time points, rats were killed by carbon dioxide inhalation and their testes were removed, weighed, digested, and analyzed in liquid nitrogen and stored at −80°C for HPLC analysis. The body weights for control (n = 15) and 2,5-HD-treated (n = 15) rats were 255 ± 4.10 g and 184 ± 3.14 g, respectively. The mean testis weight per animal for control (n = 15) and 2,5-HD-treated (n = 15) rats were 1.32 ± 0.02 g and 1.29 ± 0.02 g, respectively. Testes were homogenized in two volumes of 50 mM potassium phosphate buffer, pH 7.4, using a glass homogenizer and a Teflon pestle. Homogenates were mixed with two volumes of methanol, vortexed for 1 min, and centrifuged at 2000 × g for 10 min at 4°C. The supernatants were removed and 100 μl aliquots were analyzed by HPLC. Carbendazim was separated by reverse-phase HPLC on a C18 column using a gradient solvent system. The flow rate was 1 ml/min, and detection was by UV absorption at 280 nm. Initial conditions in the gradient solvent system were 70% water:30% methanol, followed by a linear gradient to 30% water:70% methanol over 5 min. This ratio of solvents was maintained for 5 min, followed by a linear gradient back to 70%:30% over 5 min and subsequent equilibration for an additional 15 min. The retention time for CBZ was approximately 9.4 min. Standards ranging in concentration from 50 to 300 ppm CBZ were prepared in the same buffer: methanol ratio as testes samples, and used to construct standard curves of concentration versus area under the curve. The concentration of CBZ in testis samples was calculated from these standard curves.

The two CBZ levels from each test of each animal were averaged to provide a mean testis CBZ level per animal. The area under the curve (AUC) was calculated using the trapezoid rule of the observed area without extrapolation. Three curves for each group: mean testis CBZ level per animal. The AUC was calculated for control tubulin and 0, 70, and 140 μM for 2,5-HD treated tubulin.

Statistical analysis. Levels of statistical significance were analyzed by ANOVA, followed by post hoc Scheffe’s F test to compare the means between different treatment groups. All values are expressed as the mean ± standard error of the mean (SEM). Differences were considered significant for p < 0.05.

RESULTS

Microtubule Assembly

To assess tubulin assembly dynamics in the presence of CBZ with or without 2,5-HD coexposure, microtubule assembly curves were analyzed spectrophotometrically (Fig. 2). 2,5-HD treatment altered microtubule assembly by reducing the lag time (tVmax) and increasing the maximal velocity of assembly (Vmax) when compared to control tubulin (Table 1). With increasing
CBZ concentrations, the \( tV_{\text{max}} \) became more delayed and the \( V_{\text{max}} \) decreased in the 2,5-HD-treated microtubule assembly, mimicking control-like microtubule assembly behavior (Fig. 2; Table 1). The concentration of CBZ that inhibited microtubule assembly by 50% (IC\(_{50}\)) for CBZ-induced inhibition of control and 2,5-HD-treated tubulin differed by an order of magnitude (14.63 \( \mu \)M vs. >140 \( \mu \)M CBZ, respectively). Therefore, 2,5-HD-treated tubulin is markedly resistant to the microtubule assembly inhibiting effects of CBZ.

### In Vivo Exposure

An in vivo exposure paradigm was developed to test for interacting effects of combined exposure to 2,5-HD and CBZ (Fig. 1). Because of the slow onset of toxicity with 2,5-HD exposure, young adult rats were treated with 1% 2,5-HD in the drinking water for 2.5 weeks and then challenged with a single gavage dose of 200 mg/kg CBZ in corn oil. Including appropriate controls, this exposure paradigm resulted in four exposure groups: control, 2,5-HD alone (2,5-HD), CBZ alone (CBZ), and combined 2,5-HD and CBZ exposure (2,5-HD/CBZ). Immediately following CBZ (or corn oil control) exposure, the efferent ducts of the right testis were surgically ligated. The rats were killed 24 h after CBZ dosing.

In a preliminary experiment (Experiment 1, Table 2), significant differences were observed in the testis and body weights among the groups. The mean weight of the unligated testes from the combined 2,5-HD/CBZ-exposure group was significantly increased compared to all other groups. Contrastingly, the mean weight of the ligated testes, from the combined 2,5-HD/CBZ-exposure group, was significantly decreased compared to all other groups. In addition, the mean body weights of animals receiving 2,5-HD treatment were found to be significantly lower than those of control or CBZ-exposed animals.

The significant difference in body weight in the 2,5-HD-exposed animals raised the concern that CBZ toxicokinetic parameters might differ between the control and 2,5-HD-exposed groups. Testicular CBZ levels in control and 2,5-HD-exposed rats over an appropriate postexposure time course (Fig. 3) had similar maximal testicular CBZ concentrations at 30 min (242 ± 12.0 and 231 ± 6.74 nmol CBZ/g testis wet weight, respectively). The IC\(_{50}\) (the concentration of CBZ that inhibited microtubule assembly by 50%) for CBZ-induced inhibition of control and 2,5-HD-treated tubulin is 14.63 \( \mu \)M and >140 \( \mu \)M CBZ, respectively. Percent inhibition of assembly is based on final O.D.\(_{350}\).
respectively). No significant difference was observed between the areas under the curve (AUCs) for CBZ concentration in testes of control (14022.5 ± 6760.2 nmol ml⁻¹/C01 min/C01) and 2,5-HD-exposed (14285.0 ± 602.4 nmol ml⁻¹/C01 min/C01) rats over time. These results indicated that CBZ toxicokinetics were similar in control and 2,5-HD-exposed rats, and that the observed differences in testis effects among the groups were not caused by altered distribution of CBZ.

A second experiment using a larger number of animals was conducted with the same exposure paradigm (Experiment 2, Table 2). This experiment verified the significant increase in unligated testis weight with combined exposure (2,5-HD/CBZ) compared to control and single toxicant-exposed animals (Table 2). Further, the combined exposure (2,5-HD/CBZ) testis weight change (ligated minus unligated testis weight) was significantly decreased when compared to the other groups (Table 2).

**DISCUSSION**

Most often, people are exposed to complex hazardous chemical mixtures, rather than to single chemicals. Chemicals may interact in a number of ways, resulting in antagonistic, additive, or synergistic effects (Carpenter et al., 2002; Groten et al., 2001;
Teuschler et al., 2002). One well-described approach to exposure assessment of mixtures is the use of toxic equivalency factors for dioxins, furans, and polychlorinated biphenyls (Safe, 1990). Because these compounds interact with the aromatic hydrocarbon receptor (AhR), the degree of activation or inhibition of this receptor integrates the response across this whole class of compounds. However, for many compounds, particularly compounds with broad chemical reactivity such as 2,5-HD, multiple targets and mechanisms of action are likely and the contributions of these targets and mechanisms to toxicity are likely to vary with dose. Despite this complexity, understanding the toxicity of coexposures in relation to the toxicity of the constituent single compound exposures is an important step forward.

Testicular toxicity has been evaluated after exposure to mixtures of closely related chemicals, and after exposure to chemicals presumed to have a similar mechanism of action. The following examples illustrate these kinds of studies: (1) Aroclor 1248 is a complex commercial mixture of polychlorinated biphenyls that inhibits rat testicular steroidogenesis (Andric et al., 2000); (2) coadministered metals may interact by altering tissue uptake (Waalkes and Poirier, 1985) or detoxification pathways (Iscan et al., 2002); and (3) endocrine disrupting chemicals have been coadministered to evaluate potential interactions on a common pathway (Carpenter et al., 2002). Our study is unique in examining two very different compounds that have some molecular, cellular, and tissue targets in common.

At the molecular level, 2,5-HD and CBZ have opposite effects on microtubule assembly with 2,5-HD acting as a promoter and CBZ acting as an inhibitor (Boekelheide, 1987a,b; Lim and Miller, 1997). At the tissue level, both 2,5-HD and CBZ cause sloughing of the seminiferous epithelium due to a weakening of microtubule-dependent structural support within Sertoli cells (Boekelheide, 1988a,b; Nakai and Hess, 1994). It is important to note that the sloughing processes induced by 2,5-HD and CBZ exposure are quite different. CBZ produces a rapid

FIG. 4. Testis histopathology of rats treated with 2,5-HD alone (A) and combined 2,5-HD/CBZ (B). Sloughing (S) and vacuolization (V) are more prominent in the combined exposure than with 2,5-HD alone. Roman numerals, stage of the seminiferous tubule; bar, 50 μm.

FIG. 5. Histopathology of (A) seminiferous tubule terminal segments and rete, and (B) efferent ducts in combined 2,5-HD/CBZ exposed rats. Sloughed seminiferous epithelial debris fills these structures, causing an acute inflammatory response. Polymorphonuclear leukocytes (arrows, inset C), are noted along the basement membrane of the efferent ducts. Bar, 50 μm.
and dramatic fragmentation of the adluminal seminiferous epithelium with sloughing of portions of the Sertoli cell with adherent germ cells (Nakai and Hess, 1994). On the other hand, 2,5-HD-induced testicular injury results in a shedding of germ cells from the seminiferous epithelium that is slowly progressive, less dramatic, and does not occur until several weeks after beginning 2,5-HD treatment (Chapin et al., 1983). Assembly of 2,5-HD-treated tubulin occurs earlier and more rapidly than control tubulin, an effect ascribed to tubulin crosslinking by 2,5-HD (Sioussat and Boekelheide, 1989). As shown here, inclusion of CBZ in the reaction normalized 2,5-HD-treated microtubule assembly by increasing the lag time and slowing the assembly (Fig. 2), confirming the molecular antagonism of these two toxicants at their microtubule target.

In vivo, however, combined 2,5-HD and CBZ coexposure was clearly synergistic in its ability to produce testicular injury compared to single toxicant exposures. A careful evaluation of CBZ toxicokinetics confirmed that the distribution of CBZ to the testis was the same in control and 2,5-HD-treated rats; therefore, an altered distribution did not confound the results. Testis weight measurements showed that combined exposure to 2,5-HD and CBZ was significantly different from single toxicant exposures or control. The testis weight measurements in this experiment reflected complex physiologic changes, including alterations in seminiferous tubule fluid formation and occlusion of the efferent ducts by sloughing. The efferent ducts of the right testis of each rat were occluded by ligation at the time of CBZ exposure, 24 h before the end of the experiment. Therefore, a decreased weight of the ligated testis would indicate inhibition of seminiferous tubule fluid formation. Alternatively, sloughing of the seminiferous epithelium resulting in occlusion of the efferent ducts could result in an increased weight of the unligated testis due to fluid backup. Overall, the combined 2,5-HD and CBZ exposure group had an increased unligated testis weight compared to single toxicant exposures and control, indicating enhanced sloughing and occlusion of the efferent ducts.

Quantitative histopathological studies confirmed that the combined 2,5-HD and CBZ exposure produced significantly more testicular damage than either single toxicant exposure alone. The percentage of seminiferous tubules with sloughing and vacuolization was significantly increased with the combined exposure. In addition, seminiferous tubule diameters were markedly increased in the combined exposure group. Logically, the sloughing of the adluminal seminiferous epithelium resulted in occlusion of the efferent ducts, causing a backup and accumulation of seminiferous tubule fluid and an increased seminiferous tubule diameter. The observed accumulation of fragments of seminiferous epithelial debris within the terminal segments of the seminiferous tubules, the rete, and the efferent ducts (Fig. 5) confirmed the etiology of obstruction in the combined exposure group.

A major role for microtubule integrity in maintaining homeostasis in the seminiferous epithelium is supported by recent experiments that used a molecular approach to microtubule disruption (Fleming et al., 2003a,b). In this approach, γ-tubulin was overexpressed in Sertoli cells using an adenoviral vector to selectively disrupt microtubule networks in vivo (Fleming et al., 2003a). γ-Tubulin over-expression resulted in a redistribution of microtubules within the Sertoli cells, disorganization of the seminiferous epithelium, retention of elongate spermatids and residual bodies, and increased germ cell apoptosis (Fleming et al., 2003b). Therefore, both toxicant-induced and molecularly induced Sertoli cell microtubule disruptions alter the structure and function of the seminiferous epithelium.

In conclusion, based on the results herein, CBZ coexposure markedly exacerbates the toxic effects of an initial 2,5-HD exposure in vivo, even though the effects of either 2,5-HD or CBZ exposure alone are modest. This suggests that the initial 2,5-HD
exposure renders the seminiferous epithelium vulnerable to injury, without producing marked alterations by itself. In these experiments, this 2,5-HD-induced susceptibility to injury was revealed by exposure to a second toxicant (CBZ) that targets the same testicular cell type (Sertoli cells) but has opposing molecular effects on microtubules. Further investigation is needed to determine whether an initial 2,5-HD exposure also sensitizes the seminiferous epithelium to coexposure toxicants with different testicular cellular targets.

ACKNOWLEDGMENTS

This work was supported in part by the Public Health Service NIEHS grant RO1 ES05033.

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