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Liver Histopathology of the Southern Watersnake, *Nerodia fasciata fasciata*, Following Chronic Exposure to Trace Element-Contaminated Prey from a Coal Ash Disposal Site

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ABSTRACT.—Previous studies have demonstrated the accumulation of arsenic, cadmium, selenium, strontium, and vanadium in livers of Southern Watersnakes fed fish from a coal-ash contaminated site. Our study is the first to investigate effects of trace element accumulation on cytology of snake liver. Snakes were born in the laboratory and raised for one or two years on diets consisting of varying proportions of contaminated fish. The majority (71%) of snakes fed contaminated prey did not exhibit any differences in liver histology when compared to control snakes fed an uncontaminated diet. In the remaining contaminant-exposed snakes, some aberrations were noted. The most prevalent pathology involved the proliferation of collagen fibers that resulted in narrowing or occlusion of sinusoids and increasing the mass of the intersinsuoidal parenychma. Fibrosis of the liver as a result of chronic injury has been reported previously in reptiles, but this is the first report that links such tissue damage to dietary contamination.

As in other vertebrates, the liver of snakes is the largest extrinsic digestive gland and is the site of initial processing of materials absorbed by intestinal capillaries and transported via tributaries of the hepatic portal vein (Telford and Bridgman, 1995; Schaffner, 1998). Thus, toxic materials in absorbed foodstuffs can enter the liver and affect its structure and functions (Schaffner, 1998). Few studies have examined the effects of ingested contaminants on liver histopathology in reptiles, despite the fact that the primary route of pollutant exposure in many carnivorous reptiles is ingestion of contaminated prey (Hopkins, 2000).

In this study, we examine the livers of Southern Watersnakes, *Nerodia fasciata fasciata*, raised for one or two years on a diet of fish from a coal-ash contaminated site. A previous study (Hopkins et al., 2002a) demonstrated that a subset of these snakes had significantly higher levels of several potentially toxic trace elements, most notably selenium, arsenic, and cadmium, in their livers and other target organs (kidneys, gonads) compared to snakes raised on fish from an uncontaminated site. Using light and transmission electron microscopy we determined whether chronic exposure to the contaminated diet alters the normal liver structure of snakes.

MATERIALS AND METHODS

Snake Maintenance.—Gravid watersnakes ($N = 3$) were collected from an uncontaminated area on the Department of Energy's Savannah River Site (SRS), Aiken County, South Carolina, and kept in captivity until giving birth. Offspring were randomly divided into three groups. One group (the control) was fed prey items collected from a reference site (Fire Pond, on the SRS) that has low sediment concentrations of trace elements (Hopkins et al., 1999). The other two groups were fed contaminated fish from a drainage swamp downstream from two coal ash settling basins also located on the SRS. One of the treatment groups served as an intermediate exposure treatment (Treatment 1) and was fed fish from the reference site and the coal-ash contaminated site on alternating weeks. The other group of snakes served as a high exposure treatment (Treatment 2) and was fed only fish from the contaminated site. Trace elements elevated in prey items included: As, Cd, Cu, Se, Sr, and V. In particular, As and Se levels in the contaminated prey were 8.3 and 22.8 times greater (respectively) than in control prey. In addition to examining varying proportions of contaminated prey in the diet, the current histopathological investigation also considered the duration of contaminant exposure. In each of the three treatment groups, a subset of snakes was maintained on their respective diet for one year and the remaining subset for two years. Sample sizes of snakes used in each group are reported in Table 1. The original trace element concentrations in the experimental diet (Hopkins et al., 2001) as well as a detailed discussion of snake husbandry and experimental protocols (Hopkins et al., 2002a) were previously published. The protocol for the maintenance and sacrifice of snakes was approved by the Institutional Animal Care and Use Committee of the Savannah River Ecology Laboratory.

Tissue Preparation.—After snakes were sacrificed with an overdose of ether, livers were removed and portions were cut into small blocks (5 mm^2) for preparation for light (LM) and transmission electron (TEM) microscopy. For LM, liver blocks were fixed in 10% neutral buffered formalin. Subsequently, tissues were rinsed in water, dehydrated in ethanol, cleared in Histosol (National Diagnostics, Inc., Atlanta, GA), and embedded in paraffin. Paraffin sections (10 μm)

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TABLE 1. Control and experimental groups, and the numbers of these snakes with hepatic fibrosis.

Group	Age	Sex	Total	Normal	Fibrosis
Control	1 yr	M	3	3	0
		F	5	5	0
	2 yr	M	4	4	0
		F	6	6	0
	TOTAL		18	18	0
Treatment 1	1 yr	M	3	2	1
		F	4	3	1
	2 yr	M	4	3	1
		F	6	4	2
	TOTAL		17	12	5
Treatment 2	1 yr	M	2	2	0
		F	3	2	1
	2 yr	M	4	2	2
		F	5	4	1
	TOTAL		14	10	4

were affixed to prealbuminized slides, and alternate slides were stained or treated with the following procedures: hematoxylin-eosin (general histology), periodic acid-Schiff's procedure with amylase (removal of glycogen) and without amylase treatment (neutral carbohydrates including glycogen), Gomori's reticulum stain, and the Prussian Blue stain for iron. Procedures followed Humason (1979) and Kiernan (1990).

Tissue for TEM was fixed in a 1:1 mixture of 2.5% formaldehyde and 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer at pH 7.4. After initial fixation, tissues were rinsed in distilled-deionized water, postfixed in 2% osmium tetroxide, dehydrated through a graded series of ethanol, cleared in propylene oxide, and polymerized in an epoxy resin (Embed 812, Electron Microscopy Sciences, Port Washington, PA). Plastic sections were cut with an RMC MT7 ultramicrotome (Research and Manufacturing Co., Tucson, AZ) and DiATOME (Biel, Switzerland) diamond knives. Semithin sections (0.5–1 μm) were placed on microscope slides and stained with toluidine blue. Ultrathin sections (70 nm) for TEM were collected on uncoated copper grids and stained with solutions of uranyl acetate and lead citrate. Ultrathin sections were viewed with a Hitachi H-300 transmission electron microscope (Nissei Sangyo America, Mountain View, CA).

Small sample sizes (only three sibships were available) precluded statistical comparisons of age classes and sexes, but we compared the proportion of individuals exhibiting fibrosis among control, Treatment 1, and Treatment 2 using a series of Fisher Exact Tests (Zar, 1996).

RESULTS

Liver histology and cytology were normal in all 18 of the control snakes, in 12 of the 17 snakes from Treatment 1, and in 10 of the 14 snakes from Treatment 2 (Table 1). No statistical difference was found in the proportion of individuals exhibiting liver fibrosis in Treatment 1 ($N = 17$) and Treatment 2 ($N = 14$; $P = 1.000$; $\alpha = 0.05$), but the occurrence of fibrosis was elevated above controls ($N = 18$) in both of the treatments (Treatment 1: $P = 0.0196$, $\alpha = 0.05$; Treatment 2: $P = 0.0278$, $\alpha = 0.05$).

In the normal (control) condition, the parenchyma of the liver consisted of intersinusoidal cords of hepatocytes two to three layers thick (Fig. 1A–B). Organization of the hepatic triad (hepatic portal vein, hepatic artery, and bile duct) and cytology of hepatocytes were similar to that described in other reptiles (Schaffner, 1998) and other vertebrates (Telford and Bridgman, 1995). Occasional ''light cells'' were found, and these appear to be degenerating cells that were part of a normal cycle of apoptosis of hepatocytes (Fig. 1B). The space of Disse separating the squamous endothelial cells that line the sinusoids contained some collagen fibers, but the fibers were not dense in the controls (Fig. 1C).

Several aberrations from what is generally considered healthy vertebrate liver tissue occurred in some individuals in the control treatment. In many individuals, large clusters of vacuoles were abundant (Fig. 1B), especially along the sinusoidal borders (Fig. 1D). These vacuoles may be indicative of extensive cytotic activity. Also, small clusters of lipoid material (Fig. 1D) as well as larger lipid droplets were numerous in the livers of many controls. Steatosis, or fatty infiltration, is often associated with reptilian captivity when feeding is frequent and exercise is lacking (Schaffner, 1998).

Among snakes in the two treatment groups, the livers that we considered aberrant exhibited an increase in the extent of the parencyhmal, intersinusoidal mass that resulted in narrowing of the sinusoids (Figs. 2A–B, 3A–B). This condition results from increased fibrosis, not from a proliferation of hepatocytes. Dense mats of collagen fibers occurred in the space of Disse between the endothelial lining of the sinusoids and the bordering hepatocytes (Figs. 2B, 3C), and in severe instances, fibers invaded the sinusoids and obliterated the lumen (Fig. 3D).

Other noteworthy histopathologies occurred in contaminant-fed snakes, but were not as consistently pronounced among snakes as was fibrosis. Similar to controls, steatosis was common in the two contaminant treatment groups, independent of fibrosis. One specimen from Treatment 1, however, displayed a decrease in the amount of lipid material (compare Figs. 1B–C with 2B–C). In this specimen, the hepatocytes contained much glycogen (Fig. 2D), whereas in all snakes with fatty livers (regardless of experimental group) glycogen was not abundant. Most treated snakes with fibrous livers had larger and more intensely electron-dense lipid droplets when compared to controls (compare Figs. 1B–C with Figs. 3C, 4). In addition, fibrous livers often exhibited extensive lipofuscin deposits (Figs. 3D, 4A) and large clusters of mylenic figures within lipid droplets (Fig. 4B). Whether the liver contained abundant lipid material, however, the endoplasmic reticulum of hepatocytes appeared normal in snakes with fibrosis of the liver (Figs. 2D, 4D). Mitochondria within hepatocytes were numerous in livers of both control and treated snakes, although they appeared more dense in the livers of treated snakes (compare Figs. 1C, 2D, 4D).

DISCUSSION

The most remarkable pathology present in snakes that ingested contaminated prey was liver fibrosis,

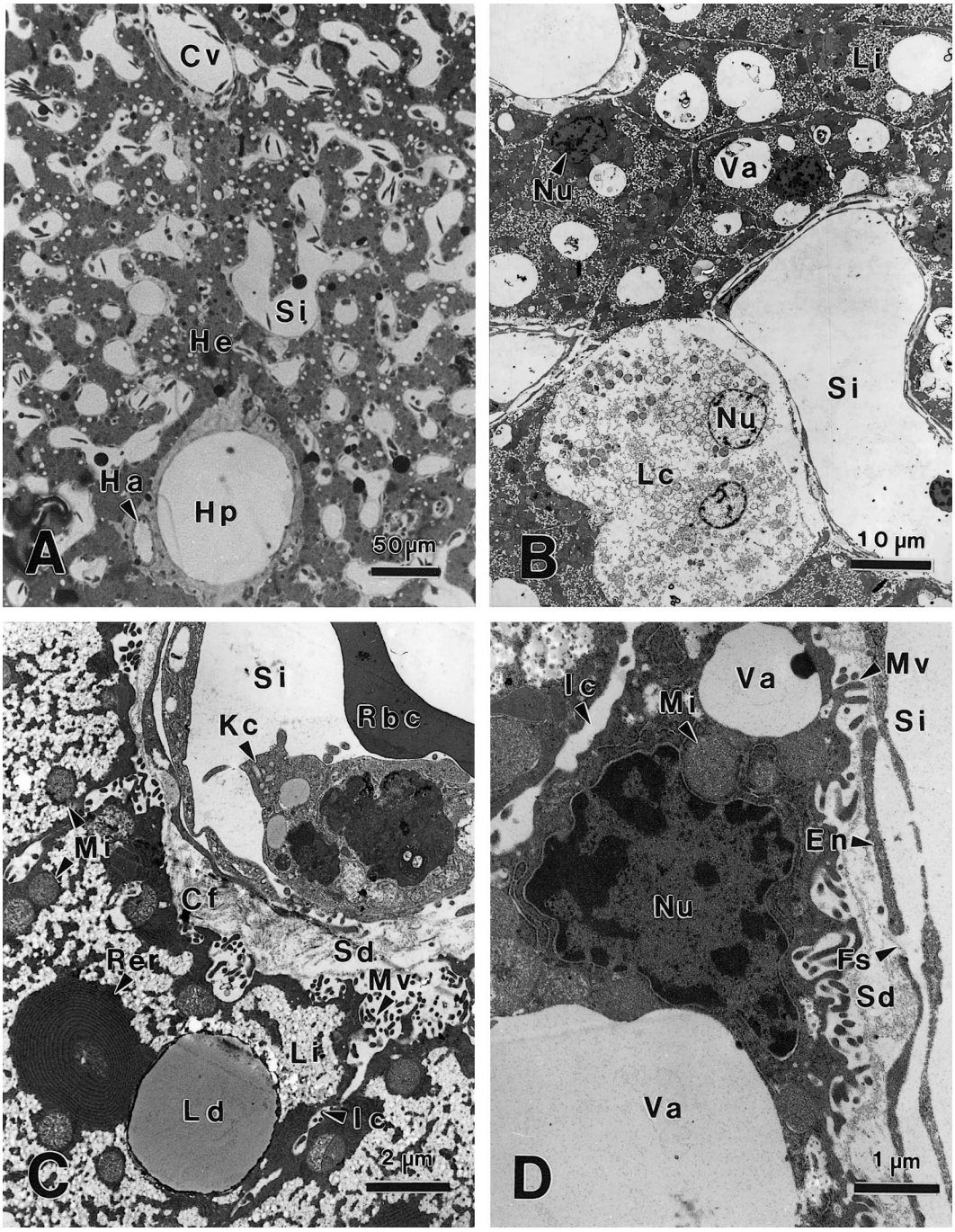

FIG. 1. Light (A) and electron (B–D) micrographs of liver tissue from control snakes. (A) Semithin section stained with toluidine blue showing the hepatic triad with its three tightly associated components; hepatic portal vein, hepatic artery, bile duct. (B) Cord of hepatocytes and associated sinuoids. (C) Sinusoidal border. (D) Vacuolated hepatocyte along sinusoidal border. Cf, collagen fibers; Cv, central vein; En, endothelial cell; Fs, filtration slit; Ha, hepatic artery branch; He, hepatocyte; Hp, hepatic portal vein branch; Ic, intercellular canaliculus; Kc, Kupffer cell; Ld, lipid droplet; Li, lipid material; Mi, mitochondria; Mv, microvilli; Nu, hepatocyte nucleus; Rbc, red blood cell; Rer, rough endoplasmic reticulum; Sd, space of Disse; Si, sinusoid; Va, vacuoles.

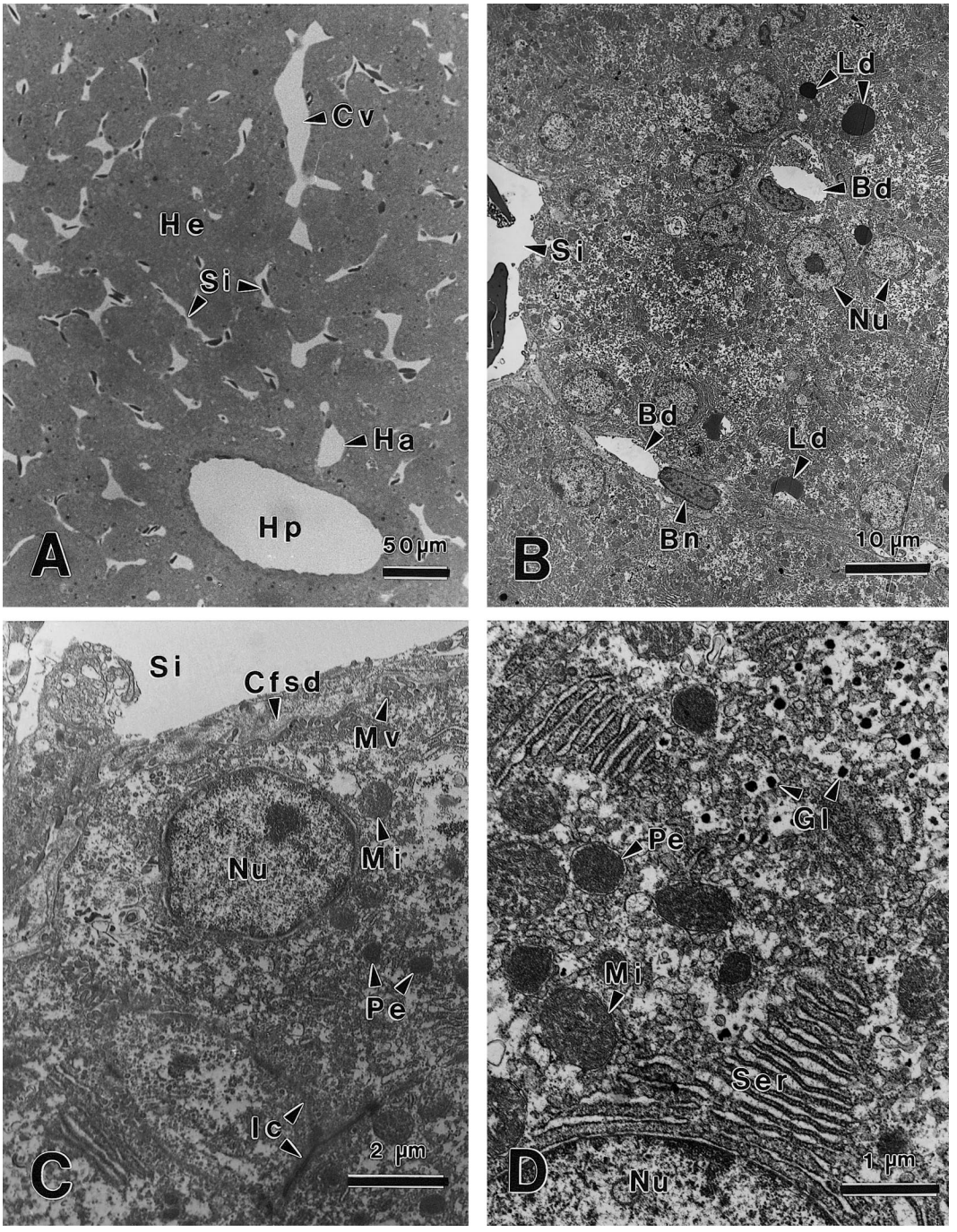

FIG. 2. Light (A) and electron (B–D) micrographs of the liver from a male snake fed a 50:50 normal:contaminated diet (Treatment 1) for one year. (A) Semithin section stained with toluidine blue showing the widened intersinusoidal parenchyma and associated loss of integrity of the hepatic triad. (B) Liver parenchyma adjacent to sinusoid. (C) Sinusoidal border. (D) Perinuclear hepatocyte cytoplasm. Abbreviations same as for Figure 1 plus: Bd, bile duct; Bn, nucleus of bile duct epithelial cell; Cfsd, collagen fibers in the space of Disse; Gl, glycogen; Pe, peroxisomes; Ser, smooth endoplasmic reticulum.

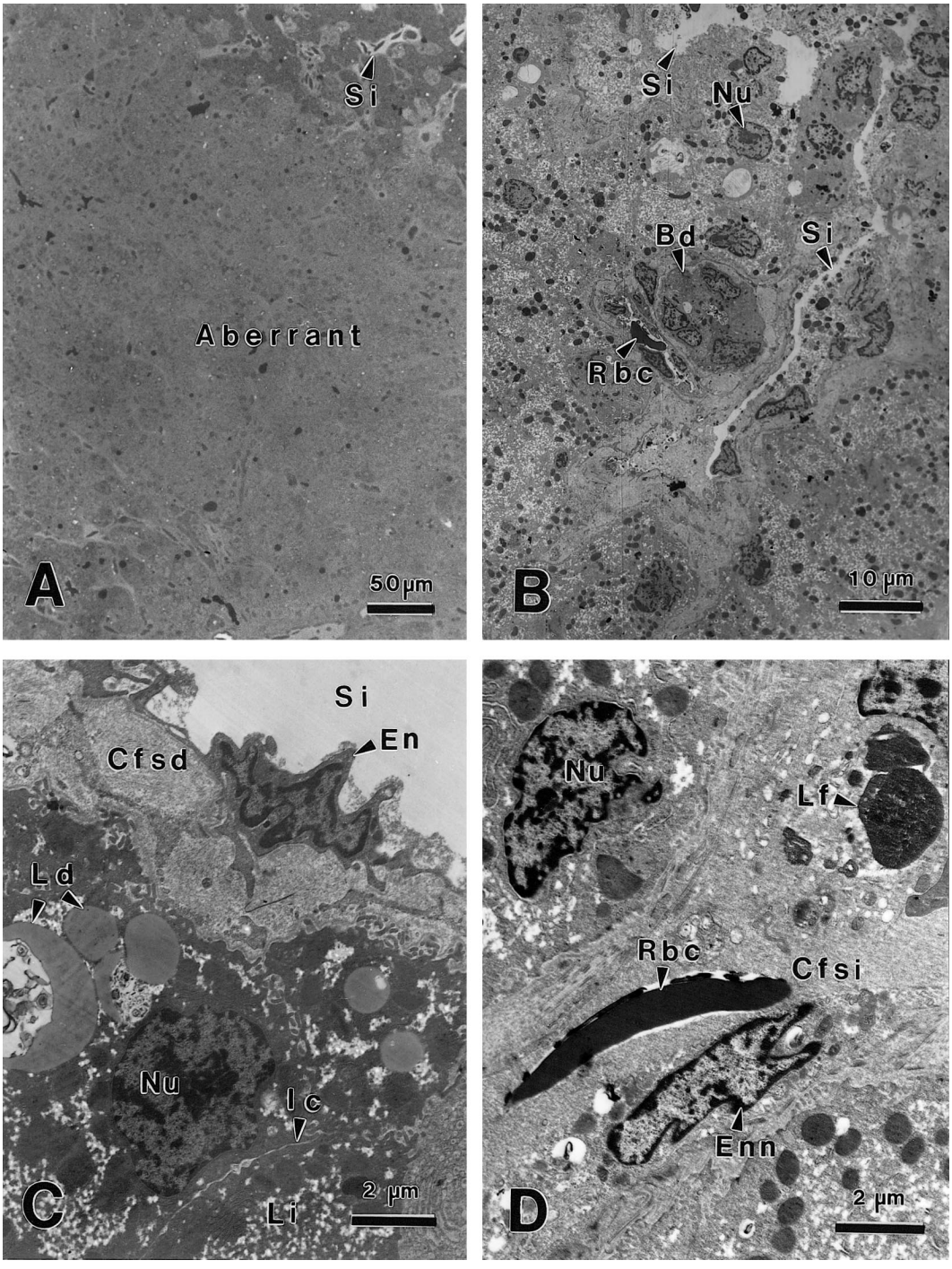

FIG. 3. Light (A) and electron (B–D) micrographs of the liver from a female snake fed a completely contaminated diet (Treatment 2) for two years. (A) Semithin section stained with toluidine blue showing liver parenchyma. (B) Hepatocytes associated with nearly obliterated sinusoids. (C) Sinusoidal border. (D) Sinusoid invaded with collagen fibers. Abbreviations the same as for Figures 1 and 2 plus: Aberrant, area of extensive fibrosis; Cfsi, collagen fibers in a sinusoid; Enn, endothelial cell nucleus; Lf, lipofuscin particles.

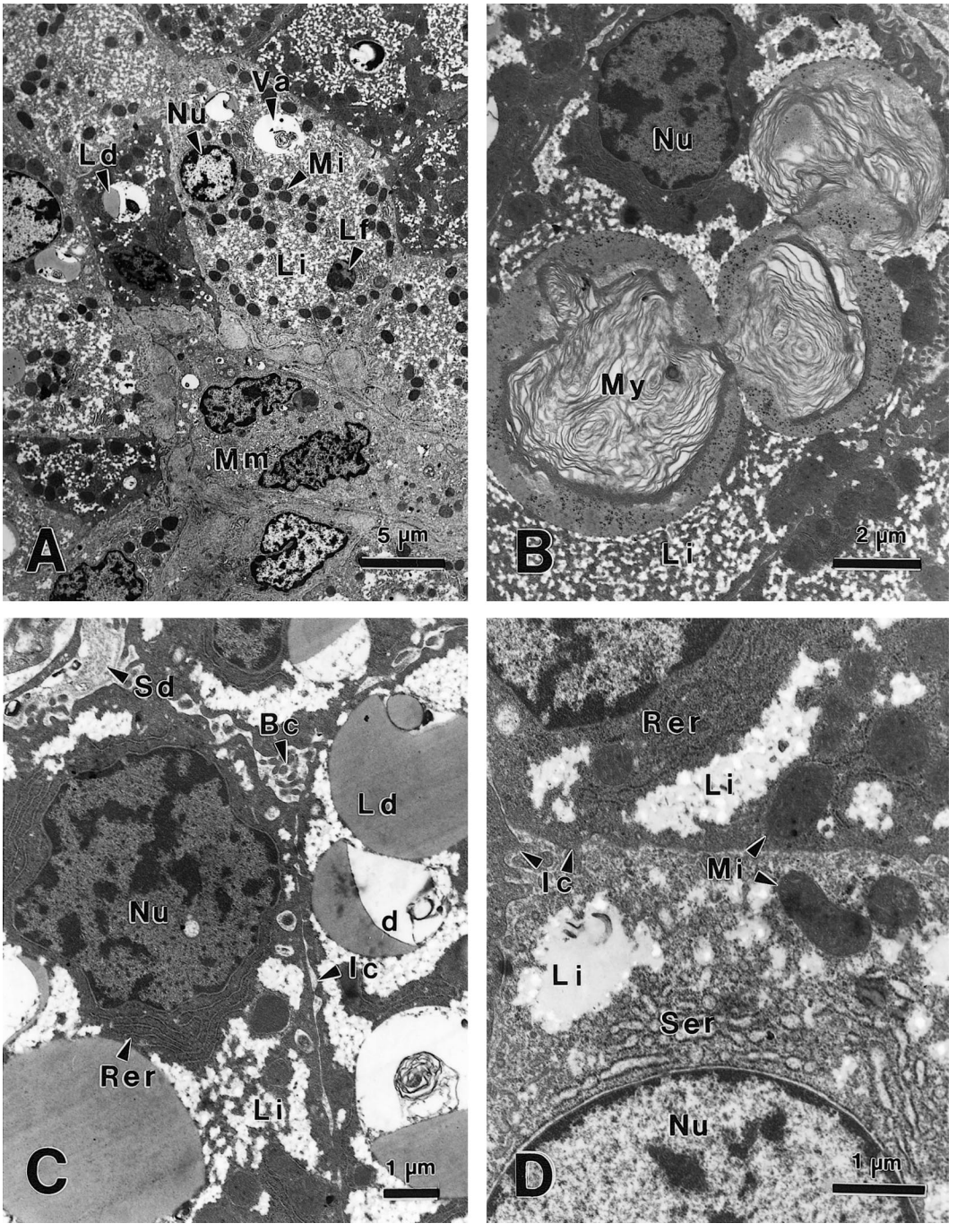

FIG. 4. Electron-micrographs of the liver of the same specimen used for Figure 3. (A) Overview of liver parenchyma. (B) Mylenic structures and associated hepatocyte. (C) Numerous large lipid droplets in hepatocytes. (D) Perinuclear hepatocyte cytoplasm. Labels same as for Figures 1–3, plus: Bc, bile canaliculus; Mm, melanomacrophages.

caused by proliferation and infiltration of collagen fibers into vascular and other interhepatocyte areas. Schaffner (1998) stated that chronic toxic injury resulting from environmental pollutants usually leads to fibrosis of the liver in reptiles. However, we are not aware of any previous report that links fibrosis of the liver in reptiles to a diet of trace element-contaminated prey.

Liver fibrosis similar to that observed in snakes that ingested contaminated prey also was reported in sunfish (*Lepomis* sp.) inhabiting a Texas reservoir contaminated with selenium-enriched power plant effluents (Sorenson, 1988; Sorenson et al., 1982; Sorenson et al., 1983a,b). However, fish exposed to effluents exhibited other cellular abnormalities not present in snakes, including reduced quantities of rough endoplasmic reticulum and glycogen particles as well as increased numbers of Kuppfer cells and lysosomes. Such additional histopathologies present in fish may result from species specific differences in susceptibility to trace elements but could also result from differences in quantity, route, and duration of trace element exposure.

Steatosis was common among snakes in all treatment groups. Fatty necrosis was also a common pathology in fish exposed to power plant effluents and is believed to be associated with exposure to Se (Sorenson et al., 1983a,b). Because steatosis is also frequently associated with captivity in reptiles when feeding is frequent and exercise is lacking (Schaffner, 1998), the connection between contaminants and steatosis in experimental snakes cannot be ascertained. However, lipid droplets in fibrous snake livers differed from droplets in normal snake livers; abnormal livers tended to have larger, more densely staining lipid droplets, many of which contained membranous structures.

The frequency of liver necrosis among snakes exposed to contaminated prey was similar among males and females, the intermediate- and high-dietary exposure groups, and the one- and two-year exposure groups. Such a result suggests that approximately one-third of contaminant-exposed individuals are more sensitive than others and respond adversely to the pollutants regardless of the duration of exposure (i.e., at least within the two-year span of the study). The observation that more than two-thirds of snakes exposed to the contaminated diet show no liver damage further suggests substantial individual variability in response to trace element exposure. Similar interindividual variability in physiological responses has also been documented in fish and field-captured snakes exposed to coal ash (Hopkins et al., 1999; Hopkins et al., 2002b), suggesting that such variation may be a common phenomenon among ectotherms exposed to this complex contaminant mixture.

In conclusion, snakes exposed to inorganic contaminants associated with power plant effluents exhibited liver pathology consistent with abnormalities noted in other vertebrates exposed to Se from similar effluents. Most notably, liver necrosis caused by aberrant infiltration of collagen fibers resulted in altered hepatic architecture. Such abnormalities were conspicuous in nearly one-third of snakes exposed to the contaminated diet, yet exposed snakes otherwise appeared healthy (Hopkins et al., 2002a). More prolonged exposure could possibly result in increased accumulation of contaminants, more severe liver necrosis, and eventual alterations in organism-level responses (e.g., growth, reproduction, or survival). Indeed, previous work indicates that snakes collected from the contaminated field site have much higher tissue burdens than what was found in the laboratory, and such burdens are associated with abnormally high metabolic rates (Hopkins et al., 1999). Future investigations are needed to ascertain whether histopathological abnormalities are precursors of detrimental effects with clear fitness consequences for affected individuals.

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