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Abstract. Livers from 54 snow leopards, 4 days to 23 years old, that had died in 23 US zoos, were evaluated histopathologically to determine if the hepatic fibrosis, which has been noted to be prevalent in this species, was due to chronic active hepatitis from hepadnaviral infection, Ito cell proliferation, or hemosiderosis. Forty-two of 54 snow leopards had subintimal vascular fibrosis with partial or total occlusion of central and sublobular veins (veno-occlusive disease) of unknown origin. All 21 leopards older than 5 years were affected. Four leopards had chronic active hepatitis, and 12 leopards had cholangiohepatitis; but these lesions were not connected anatomically to central and sublobular venous fibrosis. Hepatocellular and Kupffer cell siderosis and Ito cell proliferation were prevalent and often coexisted with perisinusoidal, central, and sublobular venous fibrosis; but fibrosis was present in leopards without siderosis or Ito cell proliferation. The pattern and prevalence of veno-occlusive disease in these leopards was similar to that reported in captive cheetah *(Acinonyx jubatus),* suggesting that a common extrinsic factor may cause the majority of hepatic disease in these large felid animals in captivity.

Key words: Chronic active hepatitis; fibrosis; liver; snow leopard; veno-occlusive disease.

Global populations of snow leopards *(Panthera uncia)* are endangered, and extinction is possible due to widespread poaching. This crisis has increased the value of snow leopard populations in zoological parks and intensified interest in their diseases. Liver fibrosis and cirrhosis have been identified at necropsy in several snow leopards in zoological parks.[24,25,32] Hepadnavirus infection has been suggested as a cause of hepatic fibrosis, because of a high prevalence in the snow leopard population (71% of 69) of low-titered serum antibodies that bind to human hepatitis B viral surface antigen (HBsAg) in solid-phase radioimmunoassaysv-" (M. Worley, personal observation). Also, 22-nm diameter spherical particles similar in structure to HBsAg have been identified in the serum of two snow leopards with clinical hepatitis." Hemosiderosis and Ito cell proliferation also have been noted in snow leopards (L. Munson, personal observation). The purposes of this study were to determine the histomorphologic pattern of hepatic fibrosis in snow leopards from zoological parks in the United States, to assess if snow leopards had histologic evidence of hepadnaviral infection, and to evaluate the relationship of hepatic fibrosis to hepadnaviral lesions, Ito cell proliferation, or hemosiderosis.

Materials and Methods

Survey population

Liver samples from 54 snow leopards that had died before 1988 were provided by 23 cooperating zoos throughout the United States. These represent the majority of snow leopards that had died in the United States before 1988. Samples were obtained at necropsy and provided regardless of clinical signs or cause of death. Liver samples were submitted as formalin-fixed tissue, as paraffin blocks, or as unstained histologic sections. Age and sex were known for 47 leopards. The resulting population had the following distribution: < 1 year, four males, six females; I to 5 years, eight males, eight females; 6 to 10 years, zero males, five females; and > 10 years, eight males, eight females. Because data was not available for most cases, information on diets and medical histories was excluded from the study.

Tissue processing

Fifty-four samples were stained with hematoxylin and eosin. Enough tissue samples were available to stain 52 cases with Masson's trichrome, 46 cases with Victoria blue (for detection of hepatitis B virus surface antigen)," 44 with periodic-acid Schiff(PAS) after diastase pre-digestion, or Gridley's modified silver stain for reticulum, and 46 cases with Perl's Prussian blue. Twenty-six samples that had been par-

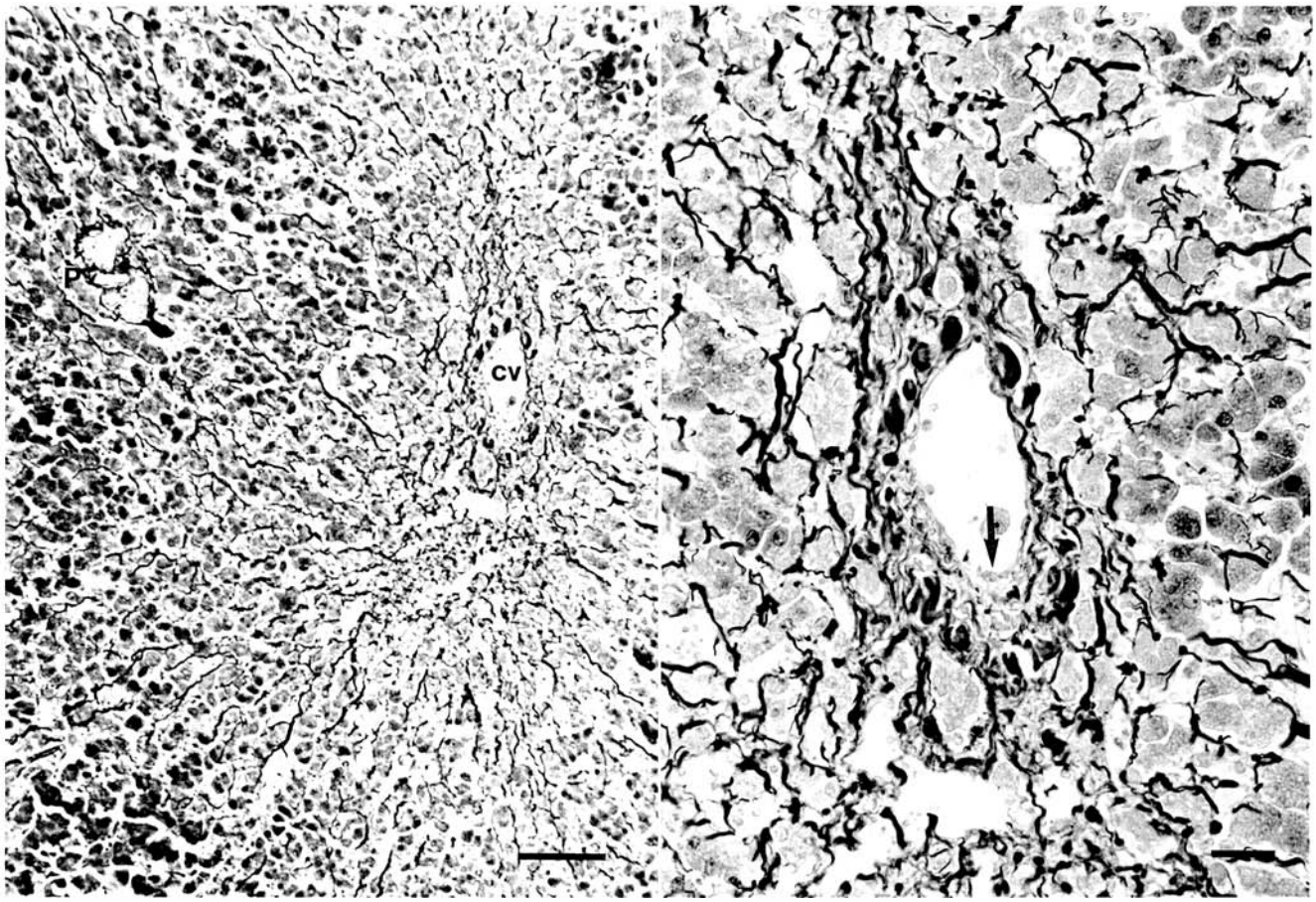

Fig.I. Fig. lao Liver, centrilobular fibrosis; snow leopard. Subendothelial collagen deposition surrounds the central vein (CV) and extends along sinusoids. Note that perisinusoidal fibrosis increases from portal areas (P) to central veins, and portal areas do not have increased fibrosis. Bar = 100 μm. Fig. 1b. Higher magnification of subendothelial fibrosis (arrow) that surrounds the central vein and thick collagenous bands along sinusoids. Gordon and Sweet's reticulum. Bar = 25 μm.

affin-embedded within 1 month of fixation were stained for copper with rubeanic acid."

Liver from one leopard was fixed in glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3), post-fixed in 2% osmium tetroxide in distilled water, and embedded with Epon. Thin sections were cut at approximately 50 nm and stained with lead citrate and uranyl acetate.

Definition of terminology

Fibrosis. Patterns of fibrosis were assessed in Masson's trichrome-stained sections and were defined and graded by the following criteria. The patterns of hepatic fibrosis were defined as follows:

1) Perisinusoidal fibrosis: deposition of collagen in the space of Disse. Mild = thin fibril deposition primarily centrilobular; Moderate = fibril deposition along sinusoids throughout lobules; Severe = thick fibril deposition throughout lobules.
2) Veno-occlusive disease: Subtotal or total obliteration of central or sublobular veins (sublobular veins had larger diameters than central veins and were not accompanied by other vessels or bile ducts") by subintimal accumulation of collagen and fibrous tissue. This category included two subsets: a) Subtotal occlusion: Subendothelial fibrosis around central and sublobular veins, resulting in partial luminal occlusion. Mild = thin band of fibrosis surrounding veins without impinging on the lumen. This group included those cases with accumulation of fibrin and edema in the subendothelial space of central veins; Moderate = Fibrosis with slight narrowing of vein lumens; and Severe = Subintimal fibrosis of hepatic veins with severe luminal narrowing, but lumen still patent. b) Total occlusion: Total obliteration of central or sublobular vein lumens by subendothelial fibrosis. Graded by proportion of vessels affected within a tissue section.
3) Bridging fibrosis: Connecting fibrous septa between adjacent central veins or central veins and portal areas (periacinar fibrosis). Lesions were graded by amount of liver affected within a sample.
4) Cirrhosis: Complete disruption of normal lobular

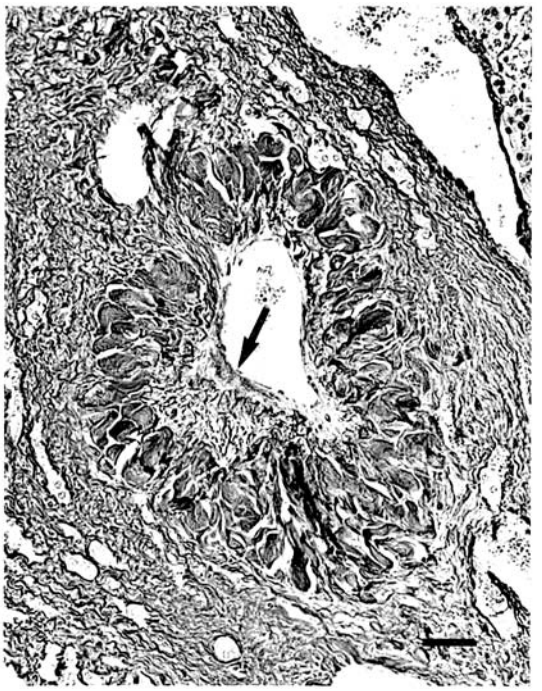

Fig. 2. Liver, veno-occlusive disease; snow leopard. Partial occlusion of a sublobular vein by an accumulation of loose fibrocollagenous tissue in the subintimal space (arrow). Gordon and Sweet's reticulum. Bar = 50 μm.

architecture, presence of regenerative nodules and extensive fibrosis involving all parts of the liver.t-!'

Inflammation. Inflammatory lesions were defined and graded by the following criteria: Chronic active hepatitis: Infiltration of most or all portal areas by a lymphoplasmacytic population that disrupted the terminal plate, and piecemeal necrosis of hepatocytes around portal areas. Cholangitis and cholangiohepatitis: Damage to bile duct epithelium, and inflammatory infiltrates around bile ducts and extending into periportal parenchyma in some areas. This category was distinguished from chronic active hepatitis by the absence of piecemeal necrosis and by damage to bile ducts from the inflammatory process.

Fig. 3. Liver, veno-occlusive disease; snow leopard. Total occlusion of a sublobular vein (SL) by fibrosis. Gordon and Sweet's reticulum. Bar = 31 μm.

Table 1. Prevalence and grade of hepatic lesions in 54 snow leopards from U.S. zoological parks.

	Number of Animals					
	Grade*				Total Affected	Percentage
	None	Mild	Moderate	Severe		
Fibrotic lesions						
Perisinusoidal fibrosis	15	20	17	2	39	72
Subtotal occlusiorr]	13	15	21	5	41	76
Total occlusiorr]	33	13	5	3	21	39
Bridging fibrosis	42	6	3	3	12	22
Cirrhosis	48	3	2	I	6	I I
Inflammatory lesions						
Chronic active hepatitis	50	4	0	0	4	7
Cholangitis/hepatitis	42	9	2	1	12	22

* Graded by most severe lesion in an individual.
t Of central or sublobular veins.

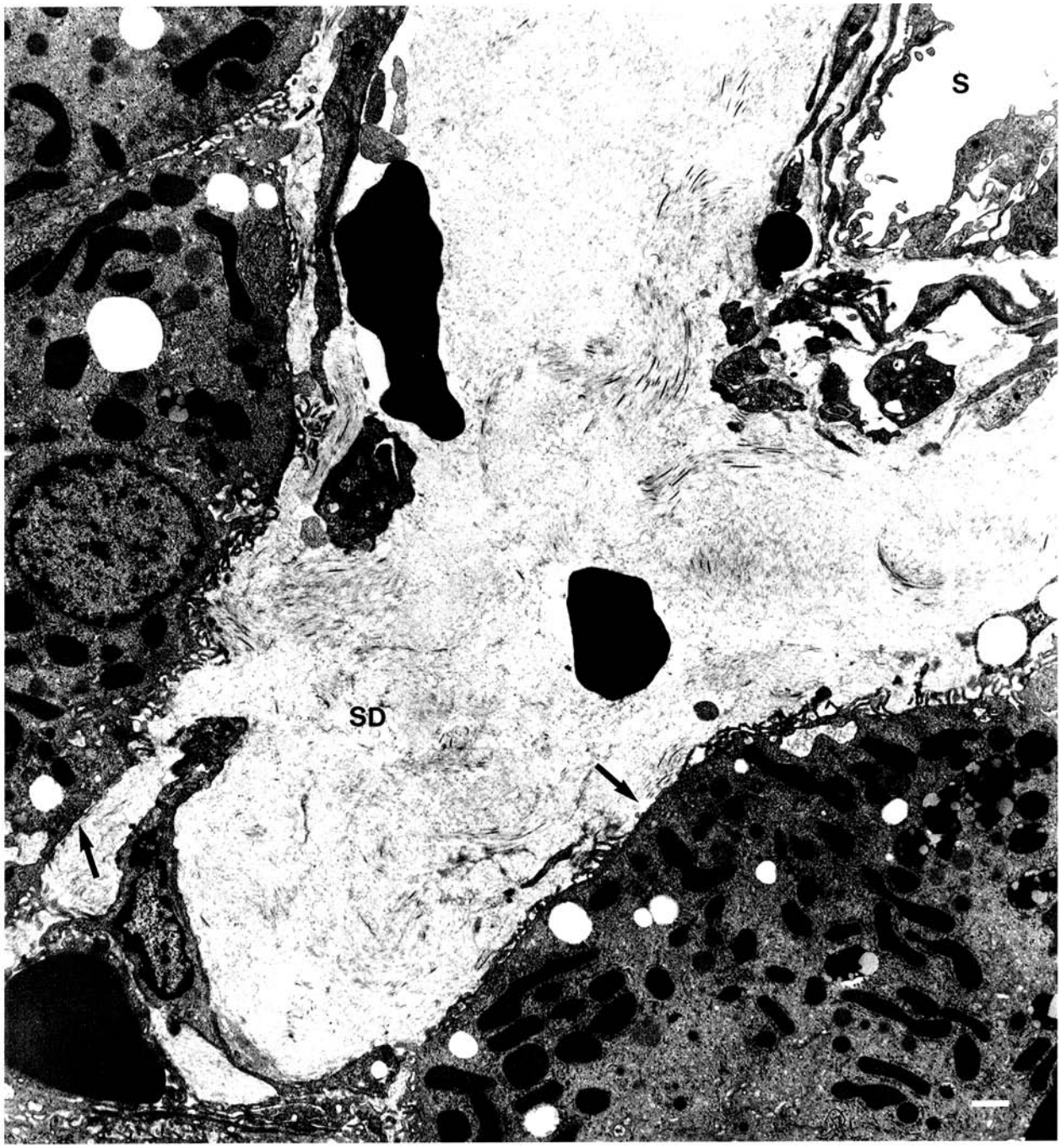

Fig. 4. Electron micrograph. Liver; snow leopard. Ultrastructural changes along a sinusoid (S) include mild sinusoidal endothelial swelling, accumulation of fibrin, collagen, and red blood cells in the space of Disse (SD), and focal loss of hepatocytic microvilli (arrows). Note Ito cell (I). Bar = I μm.

Results

Veno-occlusive disease was the predominant lesion in snow leopard livers (Table 1). The degree of fibrosis varied between and within individual leopards and ranged from mild perisinusoidal fibrosis with subtotal occlusion of central veins (Fig. 1) or sublobular veins (Fig. 2) to total occlusion of central or sublobular veins (Fig. 3). **In** most cases, the severity of perisinusoidal fibrosis increased from portal areas to central veins (Fig. 1).

Ultrastructural changes in the liver of one case with veno-occlusive disease included endothelial swelling, red blood cells in the space of Disse, deposition of collagen and fibrin in the space of Disse, and foci of hepatocyte microvillous loss (Fig. 4).

Either subtotal or total occlusion of central or sublobular veins (veno-occlusive disease) affected 42/54

Table 2. The effect of increasing age on the grade of severity of veno-occlusive disease in 47 snow leopards divided into four age groups.

Age (Years)	Total Number	Grade: None Number	None Per-centage	Mild Number	Mild Per-centage	Moderate Number	Moderate Per-centage	Severe Number	Severe Per-centage	Total Affected	Per-centage
<I	10	8	80	I	10	I	10	0	0	2	20
I to 5	16	2	13	5	31	6	37	3	19	14	88
6 to 10	5	0	0	2	40	3	60	0	0	5	100
> 10	16	0	0	4	25	9	56	3	19	16	100

snow leopards (78%), and the fibrosis was moderate to severe in 28 leopards (52% of total population). The trend of increasing fibrosis with age is presented in Table 2. Some form of fibrosis (perisinusoidal fibrosis or veno-occlusive disease) was present in 43 leopards (80%).

Four leopards had mild lesions of chronic active hepatitis (Fig. 5). Three of four leopards with chronic active hepatitis had coexisting veno-occlusive disease/ centrilobular fibrosis, and two of these three had a mild diffuse lymphocytic infiltrate associated with centrilobular fibrosis. Leopards with chronic active hepatitis were from 2.5 to 13.5 years old, and none had contact (past or present) with the other leopards with these lesions.

Thirty-eight of 46 leopards had granular or focal Victoria blue positive material in Kupffer cells and in hepatocytes. In all but three leopards, this material also was periodic acid-Schiff (PAS) positive after diastase pre-digestion, consistent with the appearance of lipofuscin. In no case of chronic active hepatitis was material in Kupffer cells or hepatocytes Victoria blue pos-

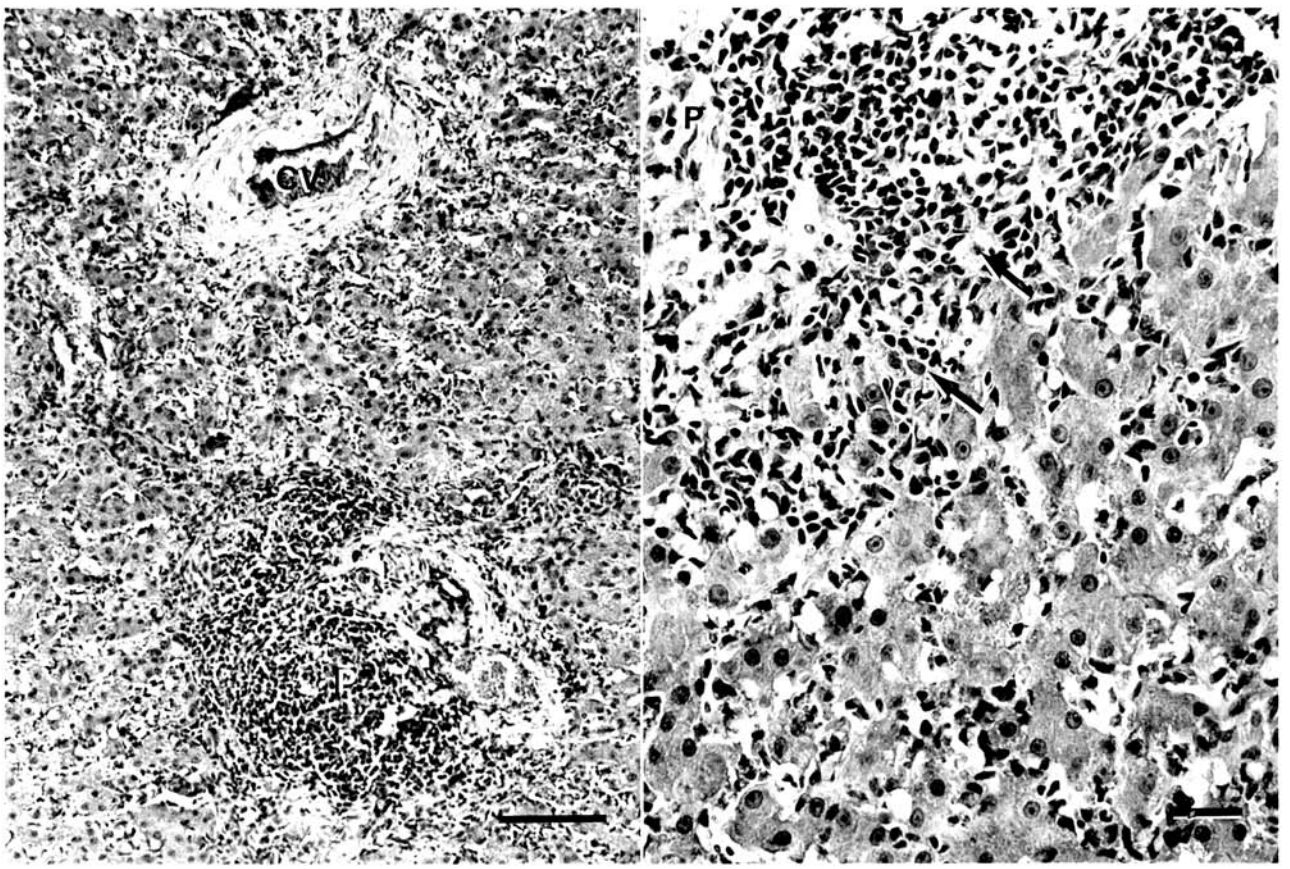

Fig. 5. Fig. Sa. Liver, chronic active hepatitis; snow leopard. Lymphocytic accumulations in a portal area (P) extend through the limiting plate into the parenchyma. Note fibrosis around adjacent central vein (CV). Bar = 110 μm. Fig. 5b. Higher magnification of lymphocytic infiltrate extending from portal area (P). Note piecemeal necrosis of hepatocytes (arrows). HE. Bar = 30 μm.

itive and PAS negative after diastase predigestion, as is characteristic of hepadnaviral surface antigen.'?

A diffuse lymphocytic infiltrate was present in areas of fibrosis in only 16/43 leopards (37%). Hepatic necrosis was associated with fibrosis in only 5/43 leopards (12%).

Thirty of 46 Prussian blue-stained livers (65%) had iron accumulations (siderosis) in Kupffer cells or hepatocytes. Siderosis increased from portal to centrilobular areas. Twenty-four of these 30 leopards had moderate to severe siderosis. Hepatic siderosis was less prevalent in leopards less than 1 year old (3/10 leopards) than in leopards over 1 year old, although there was not an increased prevalence with age in leopards greater than 1 year (11/16 leopards 1 to 5 years old, 3/5 leopards 6 to 10 years old, and 10116 leopards older than 10 years). Twenty-eight of 30 (93%) snow leopards with siderosis had concurrent subintimal fibrosis of sinusoids, central veins, and sublobular veins; however, 23% of 43 cases with subintimal fibrosis showed no evidence of siderosis. Also, the degree of siderosis was not associated necessarily with the severity of vascular fibrosis. Four of 26 cases stained for copper had mild rubeanic acid-positive material in Kupffer cells, and 3/4 also had severe Kupffer cell siderosis.

Twenty of 54 cases (37%) had Ito cell (also called lipocyte, stellate cell) proliferation. In most cases, Ito cell proliferation was mild, and a trend toward an increased prevalence with age was noted (10% of leopards less than 1 year, 31% of leopards 1 to 5 years, and 62% of leopards greater than 6 years). Nineteen of 20 (95%) snow leopards with Ito cell proliferation had concurrent subintimal fibrosis of sinusoids, central veins, and sublobular veins; however, 56% of 43 cases with subintimal fibrosis did not have Ito cell proliferation.

Discussion

This study was conducted to determine the histomorphologic pattern of hepatic fibrosis in captive snow leopards, to assess if these snow leopards had histologic evidence of hepadnaviral infection, and to evaluate the relationship of hepatic fibrosis to hepadnaviral lesions, Ito cell proliferation, or hemosiderosis. Veno-occlusive disease from partial or total occlusion of central or sublobular veins was the cause of hepatic fibrosis and was very prevalent in this captive population (78% of 54 leopards). Although the severity of veno-occlusive lesions varied within and between individuals, the subintimal fibrosis had a similar histopathologic pattern, suggesting a common pathogenesis. In all cases, collagen accumulated in the spaces of Disse and in subendothelial spaces around central and sublobular veins, resulting in attenuation or obliteration of venular lumens. Collagen accumulations increased from portal areas to central veins, suggesting that the primary site of injury was centrilobular. Bridging fibrosis from central veins to portal areas likely resulted from extension of centrilobular fibrosis in adjacent lobules. This pattern of bridging fibrosis followed the periphery of the simple liver acinus.[12]

In human beings, veno-occlusive disease is thought to develop from damage to the sinusoidal endothelium, resulting in accumulations of red blood cells and fibrin in spaces of Disse. These materials drain towards central veins, but because these veins lack fenestrations, red blood cells and fibrin--" progressively accumulate in the subintimal space. This induces subsequent subintimal fibroplasia and collagen deposition and results in progressive obliteration of central and sublobular veins. The cell of origin of the collagen is not known. Similar stages of progressive veno-occlusive lesions were noted in individual snow leopards, suggesting a similar pathogenesis.

Ultrastructural changes of veno-occlusive disease in human beings include sinusoidal endothelial damage, loss of hepatocyte microvilli, and accumulation of collagen and red blood cells in the space of Disse." These ultrastructural changes also were noted in the snow leopard, supporting the hypothesis that sinusoidal endothelial damage is the primary lesion.

Veno-occlusive disease was prevalent also in captive cheetahs.[10,19] This suggests that veno-occlusive disease in snow leopards and cheetahs may be caused by an extrinsic toxin or dietary deficiency and is not a species-specific disease. Because veno-occlusive disease is more prevalent in snow leopards and cheetahs than in other large, exotic-feline animals, it may be that the snow leopards and cheetahs are more susceptible (L. Munson, personal observation). Variation in lesion severity within livers of individual snow leopards and cheetahs may indicate that exposure to the cause is not constant. Diets, which usually are similar for both large cats, would be likely sources of causative factors (toxin or deficiency).

In human beings, several toxins have been associated with veno-occlusive disease, including pyrrolizodine alkaloids, dimethylnitrosamines, and aflatoxins.[4,5,6] Domestic cats are highly susceptible to the toxic effects of many drugs and exogenous compounds, because of a deficient level of hepatic glucuronide transferase that functions in the biotransformation of drugs and endogenous steroids for excretion.v" It is not known if snow leopards and cheetahs also have low levels of glucuronide transferase. Domestic cats have been shown to be particularly sensitive to the hepatotoxic effects of dimethylnitrosamines." Nitrosamines were determined to be low, however, in commercial exotic feline diets.[11] In human beings, exogenous estrogens in contraceptives have been associated with Budd-Chiari syndrome, peliosis hepatis,

and rarely veno-occlusive disease,' and in cheetahs, phytoestrogens have been proposed as the cause of the disease.v-' A direct association between phytoestrogen levels and veno-occlusive disease in cheetahs has not been reported, and the more common estrogen-associated hepatic lesion of hepatic vein thrombosis (Budd-Chiari syndrome) has not been identified in cheetahs or snow leopards.

No leopards had prominent hepatocellular degeneration or necrosis in association with perisinusoidal, central vein, or sublobular vein fibrosis, suggesting that this fibrosis was not secondary to recent hepatocellular injury; furthermore, cardiac failure is an unlikely cause of hepatic fibrosis, because there was no generalized dilation of central veins or fatty change in centrilobular hepatocytes.

Stimulation onto cell collagen synthesis is a possible mechanism leading to hepatic fibrosis, because Ito cells are considered fibroblast precursors" and are a major source of collagen in the liver.[8,13,18] Ito cells proliferate under some toxic conditions, particularly hypervitaminosis A,26 and hypervitaminosis A can result in Ito cell proliferation and perisinusoidallcentrilobular fibrosis in human beings." The presence of abundant Ito cells in the snow leopard population and concentration of these cells in areas of fibrosis suggest that they may contribute to vascular fibrosis; yet, more than half of the leopards with peri sinusoidal fibrosis and veno-occlusive disease did not have associated Ito cell proliferation. Ito cell proliferation without hepatic fibrosis is a common finding in many species of Felidae (L. Munson, personal observation). Vitamin A excess was associated with only 26% of veno-occlusive disease cases in cheetahs,'? although levels of vitamin A, that would be considered toxic, were found in 3/8 diets."

Because collagen can accumulate around macrophages with hemosiderin." the association of Kupffer cell siderosis and subintimal perisinusoidal fibrosis was evaluated. Siderosis was prevalent in this snow leopard population and often, but not always, was associated with hepatic venular fibrosis. Siderosis in hepatocytes always was accompanied by siderosis in Kupffer cells and was more dense in centrilobular areas, indicating that snow leopards did not have primary hemochromatosis in which hepatocellular iron accumulations exceed Kupffer cell iron levels and iron accumulations are most severe around portal areas. It should be noted that hepatocellular and Kupffer cell siderosis of unknown cause is common in many zoo species and is not associated with fibroplasia in most cases (L. Munson, personal observation). Also, siderosis may have been secondary to the effects of fibrosis (hemostasis or hepatocellular damage from entrapment in areas of fibrosis), resulting in this close correlation between lesions. The high prevalence of lipofuscin in Kupffer cells and concurrent presence of copper in some cases support the latter conclusion.

In most cases, fibrosis of the central and sublobular veins did not coexist with active inflammatory lesions, and those cases with inflammation had a mild response associated with damaged hepatocytes entrapped in areas of fibrosis. Lesions of chronic active hepatitis in four snow leopards were not associated with portal fibrosis and were not connected to areas of centrilobular and sublobular venous fibrosis. This suggests that hepadnaviral infection was not the cause of hepatic fibrosis in snow leopards.

Lesions of chronic active hepatitis do support, however, serologic evidence of hepadnavirus infection in the snow leopard population, which is based on a high prevalence of serum antibodies that bind to human hepatitis B viral surface antigen (HBsAg) in a solid-phase radioimmunoassay." Specificity of these antibodies for HBsAg has been demonstrated by blocking the binding of these antibodies to HBsAg by preincubation with a panel of infected human plasma representing nine subtypes of HBsAg (M. Worley, unpublished data). Further evidence that hepadnavirus is present in snow leopards is the presence of particles similar in size and structure to HBsAg in liver homogenates of two snow leopards after ultracentrifugation on cesium chloride gradients (M. Worley, personal observation). One of these two leopards had chronic active hepatitis, and one had veno-occlusive disease and HBsAG-like particles in its serum. All four snow leopards with chronic active hepatitis had Victoria blue-positive material in hepatocytes. This material also was PAS positive after diastase pre-digestion (compatible with lipofuscin). This suggests that viral antigens were not identified in snow leopard livers by Victoria blue staining, contrary to what has been demonstrated in human livers.'?

Low lesion (chronic active hepatitis) prevalence in a population with a high hepadnaviral seroprevalence, as was noted in snow leopards, also is characteristic of hepadnaviral infections in human beings." In human beings, chronic active hepatitis is an immune-mediated disease, associated with hepadnaviral replication in hepatocytes and cell-mediated immune responses." Many patients with high levels of HBsAg and anti-HBsAg have mild or no hepatic lesions, suggesting that a humoral response is not associated with the development of hepatitis.

No lesions of acute hepadnaviral hepatitis that have been noted in human beings (such as acidophilic degeneration of hepatocytes, ground glass hepatocellular cytoplasm, and acute lobular disarray) were noted in snow leopards in this study. A bias in a necropsy-based study toward cases in which lesions were resolving could account for this lack of acute lesions. Lesions of chronic active hepatitis in the snow leopards also were

not as severe or diffuse as hepadnaviral-induced chronic active hepatitis in human beings." or woodchucks.[22,28,29] Hepadnaviral-associated lesions in species other than woodchucks or human beings have been mild;? however, they were found in snow leopards in this study. In individual ducks *(Anus domesticus)* with duck hepatitis B virus (DHBV), lesions of chronic active hepatitis, chronic persistent hepatitis, and cirrhosis were not associated necessarily with the presence of DHBV in serum." Beechly ground squirrels *(Spermaphilus beecheyi)* infected with ground squirrel hepatitis virus either had mild hepatitis or were without evidence of hepatitis." It also is possible that the lesions of chronic active hepatitis in snow leopards were not related to a viral infection. Studies confirming the presence of hepadnavirus in snow leopards using serologic and molecular virologic methods are in progress.

Veno-occlusive disease was the major cause of hepatic fibrosis in snow leopards in this study. The cause of this disease could not be determined from the material available, as also was true for studies on veno-occlusive disease in captive cheetahs.[10,19] Fibrosis due to chronic hepadnavirus infection, Ito cell proliferation, or siderosis is not likely, because the relationship between these lesions and fibrosis was not conserved in cases of veno-occlusive disease in this study. Some toxin or deficiency in diets, common to many species of large exotic cats, is likely the cause of this lesion. Feline diets in zoological parks should be analyzed for toxic levels of vitamin A, iron, copper, nitrosamines, or other hepatotoxins or endothelial toxins and for deficiencies of essential nutrients, such as taurine.

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