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Cellular and matrix changes in drug abuser liver sinusoids: a semiquantitative and morphometric ultrastructural study

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Abstract. The aim of the present work was to analyse, at the ultrastructural level, the action of heroin of 150 centrilobular sinusoids from liver biopsies of five intravenous drug abusers, who presented clinical and biological manifestations of hepatic impairment. A comparative study of 90 sinusoids from liver biopsies of three control patients was performed. Electron microscopic observations showed a thickening of the sinusoidal wall related to endothelial cell hypertrophy and to fibrosis of the space of Disse. This was generally associated with basement-membrane-like material and hepatocyte microvilli flattening. In addicts, hepatic vascular pole changes were a constant finding, accompanied by interhepatocyte space disjunction and perisinusoidal collagenization. Morphometric assessment confirmed a significant increase of sinusoidal wall surface, endothelial cell body and processes and Ito cell process surface was significantly different between the patient groups. This cellular hypertrophy may represent hyperactivation of the sinusoidal cell functional capacity, triggering the fibrogenesis in the space of Disse. While this mechanical barrier might hinder the free exchange through the space of Disse, it may equally well protect the liver against heroin toxicity.

Key words: Heroin – Sinusoids – Fibrosis – Ultrastructure – Morphometry

Introduction

In recent years, drug consumption has become a major social and public health problem. Most organic dysfunctions accompanying drug taking are related to vascular disturbances. Anoxia and increased vagal tone have been shown to be responsible for myocardial arrhythmia, ischaemia and infarction (Coleman et al. 1982; Tazelaar et al. 1987; Tella et al. 1990), renal (Sharff 1984) and colonic (Nalbandian et al. 1985) infarction. Hypoperfusion has also produced congenital limb reduction defects and/or intestinal atresia or infarction (Hoyme et al. 1990). The pathophysiological mechanism for neurological non-infectious diseases is uncertain (Rodriguez et al. 1971), although it has been most often related to sympathomimetic effects (Rowbotham and Lowenstein 1990). Drug adulterants such as quinine, lactose, talc and mannitol have been implicated in the genesis of embolic disease, including foreign body reactions (Rosenow 1972) and septic embolism has been associated with intravenous drug administration (Bisbe et al. 1989; Endress et al. 1990; Geelhoed and Joseph 1974; Holzman and Bishko 1971). Liver dysfunction in addicts has been frequently related to viral hepatitis (Alter and Michael 1968; Gorodetzky et al. 1968; Levine and Payne 1960; Weller et al. 1984), human immunodeficiency virus (HIV) infection (Scoazec et al. 1988), malnutrition, alcoholism (Leevy et al. 1970; Stimmel et al. 1972) and iatrogenic factors (Bagheri and Boyer 1974; Zafrani and Feldmann 1988; Zafrani et al. 1983).

In previous work, we attempted to establish the direct hepatotoxicity of heroin by a semiquantitative and morphometric study on liver biopsies from intravenous addicts who presented biological liver abnormalities. To eliminate the possible role of infectious hepatitis in the pathogenesis of hepatic lesions in abusers, three groups of patients were examined: drug addicts considered as putative virus of C hepatitis (VHC)-infected patients, non-drug-addict chronic hepatitis patients and a control group who presented surgical biopsies without liver disease. We demonstrated that sinusoidal alterations (inflammation and increase in the volume sinusoidal density) and Disse's network and terminal hepatic vein fibrosis were well developed, mainly in zone III of liver acini. The disappearance of liver vascular lesions after drug withdrawal and its substitution by a fibrotic thickening of the terminal hepatic vein wall and perisinusoidal

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space constituted another argument for direct heroin-induced vascular hepatotoxicity (Trigueiro de Araujo et al. 1990).

The aim of the present work is to analyse, at a semiquantitative and morphometric ultrastructural level, these sinusoidal wall alterations, to check the involvement of sinusoidal cells in the process of vascular dysfunction and to determine how early the fibrotic apposition occurs in the perisinusoidal space.

Materials and methods

Percutaneous needle biopsies of liver were obtained from five male intravenous heroin abusers (age range 19–28 years). These were designated drug abusers (DA). Surgical biopsies performed in three other patients during extrahepatic abdominal surgery constituted the control goup (age range 43–71 years). The DA had been consuming heroin for at least 5 years. Their biopsies were performed for diagnostic purposes, since all of them had clinical elevation of transaminase levels, without hepatitis B surface antigen (HBsAg) and HIV antibodies. Clinical and biological liver function data and virus status of the drug addicts are presented in Tables 1 and 2. In the control biopsies, liver function tests and histology were normal.

A part of the liver biopsy was cut into 1 mm^3 blocks and fixed immediately in 1% osmium tetroxide in 0.15 M sodium cacodylate buffer, pH 7.4, at 4° C, for 1 h. Dehydratation through graded alcohols and propylene oxide was followed by embedding in epoxy resin. Sections 1 μm thick were stained in toluidine blue to select areas for ultramicrotomy. Ultra-thin sections (60–80 nm) were double-stained with methanolic uranyl acetate and lead citrate.

For the purposes of ultrastructural analysis the centrilobular zone of hepatic acini was recognized by the presence of terminal hepatic veins in semi-thin sections. In the absence of this feature, zone III was estimated by relevant sinusoidal dilatation remote from portal tracts (Motta et al. 1978). Grids were observed with an electron microscope (Philips EM 300). Two hundred and forty sinusoids (30 for each case) from three to four blocks per patient were studied. Three to five electron micrographs of sinusoids were necessary to make the reconstruction of the hepatic vessels, by using ×4500 initial and ×9000 final magnification.

A description of the sinusoidal inflammatory reaction and of the endothelial, perisinusoidal (fat-storing cells, Ito cells) and Kupffer cells was established. A semiquantitative score graded the degree of ultrastructural changes observed in the hepatic pole (Table 3) and the space of Disse. The evaluation of the fibrotic deposit in the space of Disse, in control and DA liver biopsies, was assessed according to Orrego et al. (1979), modified (scale of 0+ to 3+) as follows:

0+ : non-existent of minimal collagenization (rare fibrils) in a space of Disse of normal width
1+ : few collagen fibril bundles in a space of Disse of normal width
2+ : many collagen fibril bundles in a widened space of Disse, associated with a frequent discontinuous electron-dense material and with a multifocal flattening of hepatocyte microvilli
3+ : large collagen bundles in a widened space of Disse, associated with a discontinuous or continuous electron dense material, separating the sinusoidal barrier from the hepatocytes; hepatocyte microvilli almost completely absent

The quantitative data were assessed using a semiautomatic analysis system (Leits-Dialux microscope, Varioscan camera, Leitz ASM 68K computer with black and white monitor, digitizing table and Hewlett-Packard Thinkjet printer). The sinusoidal wall was assessed quantitatively on electron micrographs. The sinusoidal external and lumen outline, and the outlines of endothelial and Ito cells (cell body and processes) were traced with an electron pen and the following variables of measurements were automatically determined by the computer: lumen perimeter, lumen surface (LS), external perimeter and total surface (TS) of the sinusoids, perimeter and surface of endothelial and Ito cells (cell body and processes). The sinusoidal wall surface (SWS) was calculated by the difference between TS and LS. Stereological principles of Weibel et al. (1966) were used to determine the contribution to the sinusoidal wall of endothelial and perisinusoidal cells. Thus, the cellular surface density (CSD) represented the percentage area of sinusoidal wall occupied by the cell body and process surface of each of the mentioned cells. It was calculated by the equation: CSD (%) = 100 × cellular (cell body or processes) surface/SWS. In all cases, the section thickness was constant.

Computer software (SPSS, Chicago) was used for the statistical analysis of control and DA results. Relationships between semiquantitative variables studied (microvilli flattening, interhepatocyte space disjunction and fibrosis of the space of Disse) were evaluated by rank correlation of Kendal's tau C (τ). Morphometric variables (SWS, endothelial and Ito cell surface and perimeter, and CSD) were compared in the two groups of patients by Anova univariate analysis and an F-test was performed. The results were expressed as mean ± standard error of mean (SEM). A significance level of 0.05 was used in all tests.

Table 1. Clinical and biological data, at the time of biopsy, in heroin addicts

Patients	1	2	3	4	5
Age (years)	19	22	27	28	28
T/addict (months)	36	48	34	60	48
Bilir (N = 0–20 μmol)	48	5	9	11	6
SGOT (N = 5–30 units/l)	89	43	40	53	51
SGPT (N = 5–50 units/l)	768	117	110	129	111
AP (N = 0–90 units/l)	155	72	103	77	64
PT (N = 100%)	100	100	92	100	100

T/addict, Duration of addiction; Bilir, total bilirubin; SGOT, serum glutamic-oxalacetic transaminase; SGPT, serum glutamic-pyruvic transaminase; AP, alkaline phosphatase; PT, prothrombin time; N, normal levels

Table 2. Viral hepatitis – status in the heroin addicts

Patients		1	2	3	4	5
Serum	HBsAg	–	–	–	–	–
	Anti-HBs	+	+	–	–	–
	Anti-HBc	+	+	–	–	+
	Anti-HBe	–	+	–	–	+
HBV replication in serum	HBV-DNA	–	–	–	–	–
	HBV-DNA polymerase	–	–	–	–	–
HIV antigen (ELISA)		–	–	–	–	–

Table 3. Semiquantitative grading of the ultrastructural alterations on the vascular pole of the hepatocytes and interhepatocyte space, in biopsies from controls and drug abusers (scale 0+ to 3+)

Grade	Microvilli	Interhepatocyte space
0+	Normal[a]	Normal
1+	Focal flattening	Oedema
2+	Multifocal flattening	Fibrosis
3+	Diffuse flattening	Oedema and fibrosis

[a] According to Motta et al. (1978)

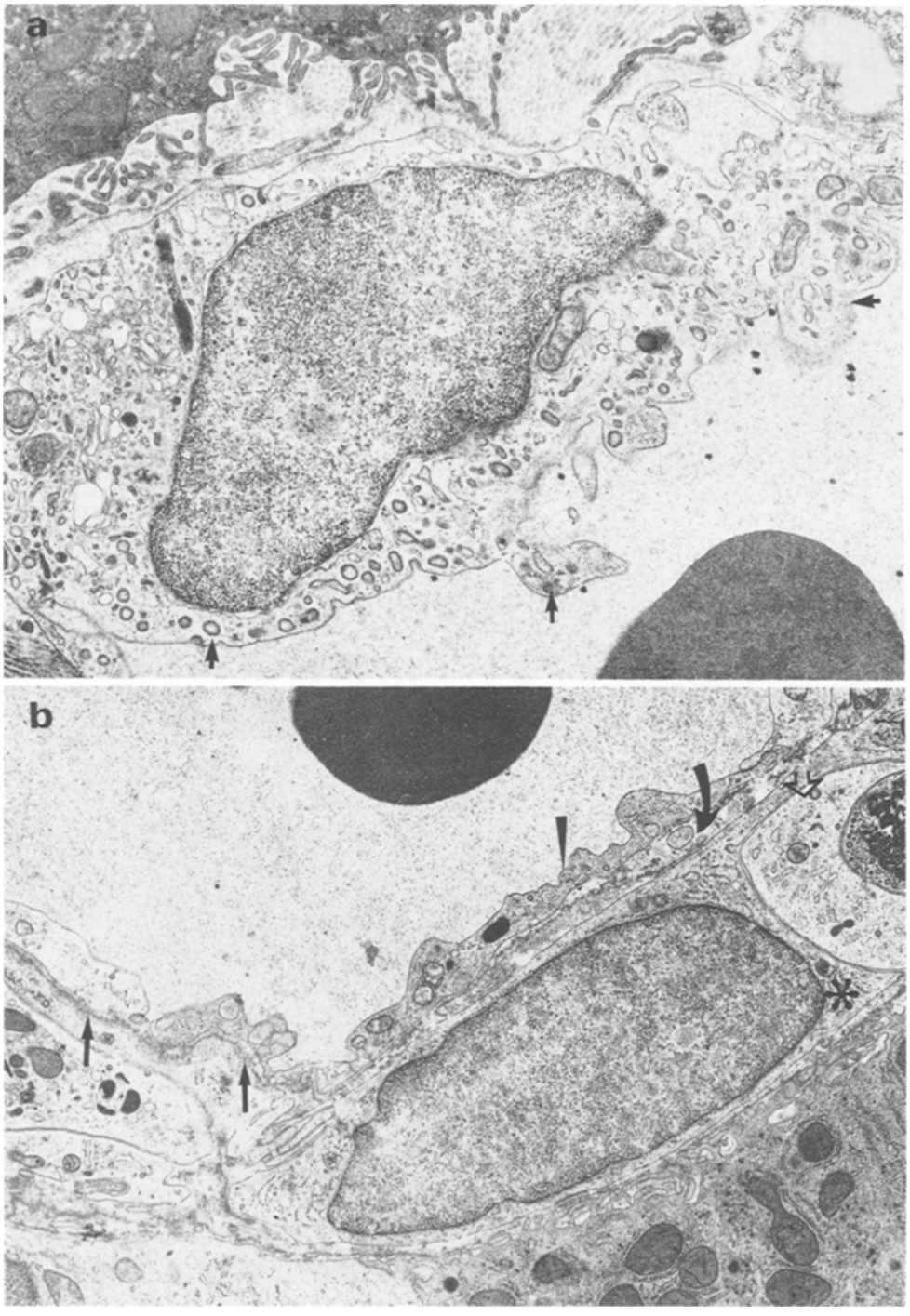

Fig. 1 a, b. Sinusoidal barrier changes. **a** Endothelial cell, with an increased number of pinocytic vesicles and dense bodies in the cytoplasm, bulging into the sinusoidal lumen (*arrows*). **b** Pluri-layered sinusoidal lining composed of thickened endothelial cell processes (*arrowhead*), basement-membrane-like material (*arrow*) and perisinusoidal cell process (*curved arrow*) and body (*asterisk*). Presence of Kupffer cell in the space of Disse (*open arrow*). **a** ×14600, **b** ×11000

Results

Electron microscopic observations from the liver biopsies of DA showed a thickening of the sinusoidal barrier mainly due to hypertrophic endothelial, Kupffer and perisinusoidal cells, and to a collagenous matrix deposit in the space of Disse. The poorly fenestrated endothelial cells bulged into the sinusoidal lumen. In the cytoplasm, numerous granules and dense bodies were disposed near the nucleus and micropinocytic vesicles were increased (Fig. 1a). Moreover, thickening of endothelial cytoplasmic processes was one of the most remarkable features. The pseudoduplication of sinusoidal lining cells was composed of two or three layers of Ito cell processes showing a great electron density and numerous lipid droplets, placed in the depth of the space of Disse in frequent relationship to basement-membrane-like material. The afore-mentioned cell expansions were close to endothelial cells (Fig. 1b). Two types of Ito cells were found: hyperactive cells with thick processes, a well-developed rough endoplasmic reticulum and lipid drops, closely related to bundles of collagen fibers and transi-

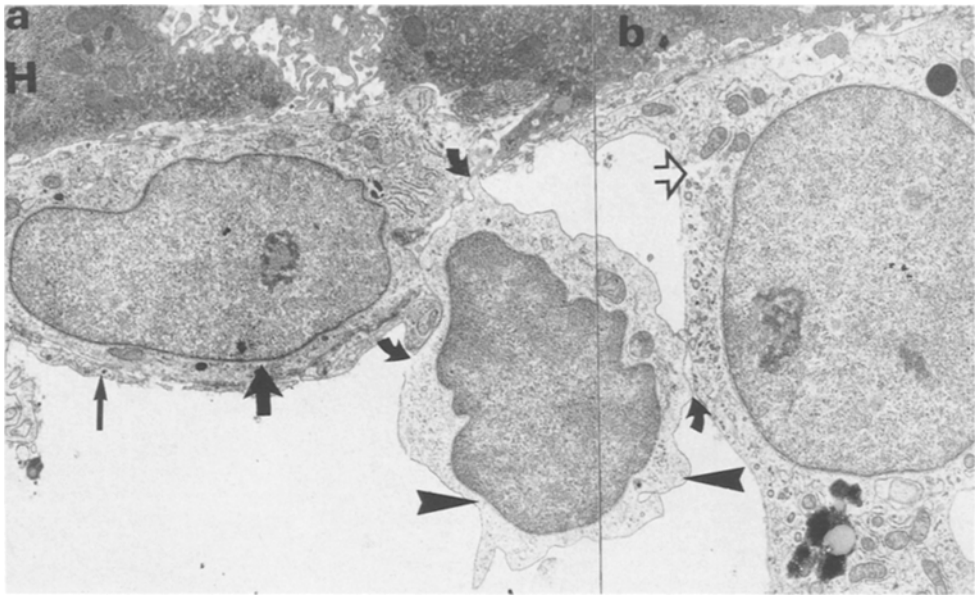

Fig. 2a, b. Inflammatory cell in sinusoidal lumen: intracellular contact (*curved arrows*) between **a** lymphocyte (*arrowhead*), endothelial (*thin arrow*) and Ito cell (*large arrow*); **b** lymphocyte (*arrowhead*) and Kupffer cell (*open arrow*); Hepatocyte (*H*). ×7600

tional cells devoid of lipid droplets, with an irregular nucleus and membrane densifications. The Kupffer cells were numerous and were largely responsible for the filling up of the sinusoidal lumen; their cytoplasmic expansions were sometimes found between endothelial cells obliterating the fenestration, or in the space of Disse. The phagocytic activity of Kupffer cells was expressed by the development of abundant and irregular cytoplasmic processes as microvilli, microplicae and invaginations; an increased number of lysosomes; and the presence of well-developed rough endoplasmic reticulum (RER) and Golgi apparatus. In DA, the sinusoidal lumen was partially obstructed by red blood cells, immunocompetent cells (granulocytes and lymphocytes) and Kupffer cells. Frequent cellular contacts between lymphocytes of granulocytes and sinusoidal cells (endothelial, Kupffer and Ito cells) were observed (Fig. 2a, b). Moreover, alterations of cellular wall components – principally the elongation of endothelial cytoplasmic processes and cell bodies bulging into the lumen – contributed to the obstruction of liver sinusoids.

The ultrastructural semiquantitative analysis of the hepatocyte vascular pole and sinusoidal collagenization showed significant differences between DA and control specimens. Hepatocyte microvilli flattening was an almost constant feature in DA (90.7% of the analysed sinusoids), in contrast with 30.9% of the control material ($P<0.0001$) (Fig. 3a). Variable amounts of collagen

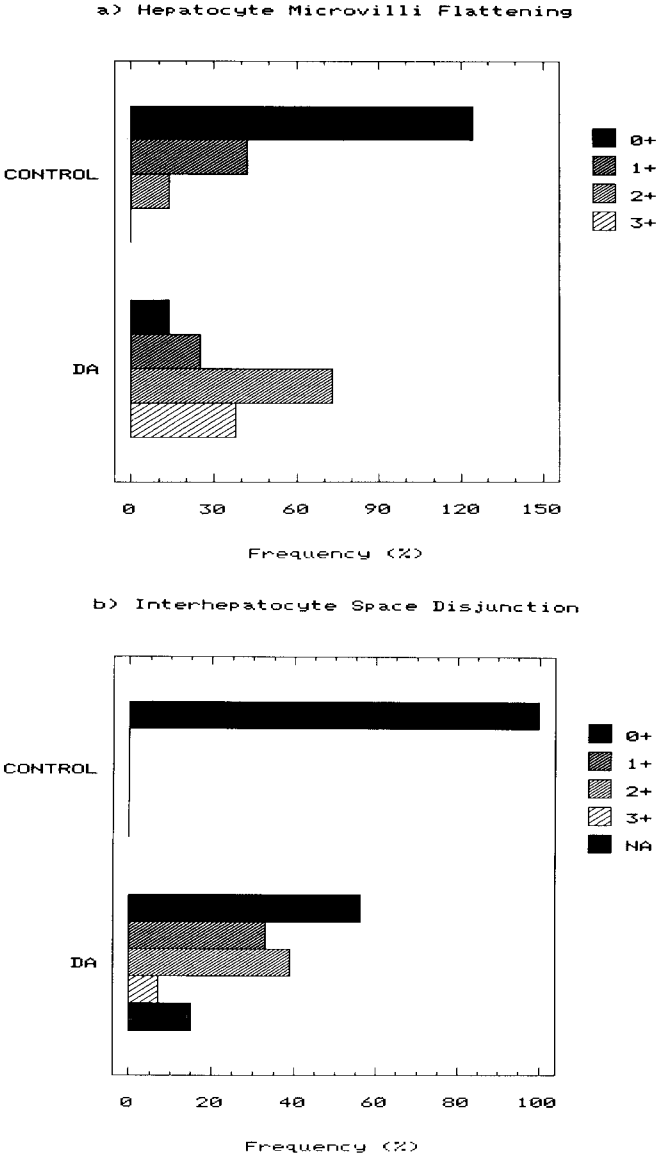

Fig. 3a, b. Frequency distribution of ultrastructural vascular pole alterations assessed by a semiquantitative analysis of liver sinusoids from controls and drug abusers (*DA*) respectively. Tested by rank tabulation of Kendal's tau C (τ). **a** Hepatocyte microvilli flattening graded on scale of 0 to 3+: 0 = normal, 1+ = focal flattening, 2+ = multifocal flattening, 3+ = diffuse flattening. $\tau=0.72$, $P<0.0001$. **b** Hepatocyte disjunction graded on scale of 0 to 3+: 0 = normal, 1+ = oedema, 2+ = fibrosis, 3+ = oedema and fibrosis; $\tau=0.60$, $P<0.0001$

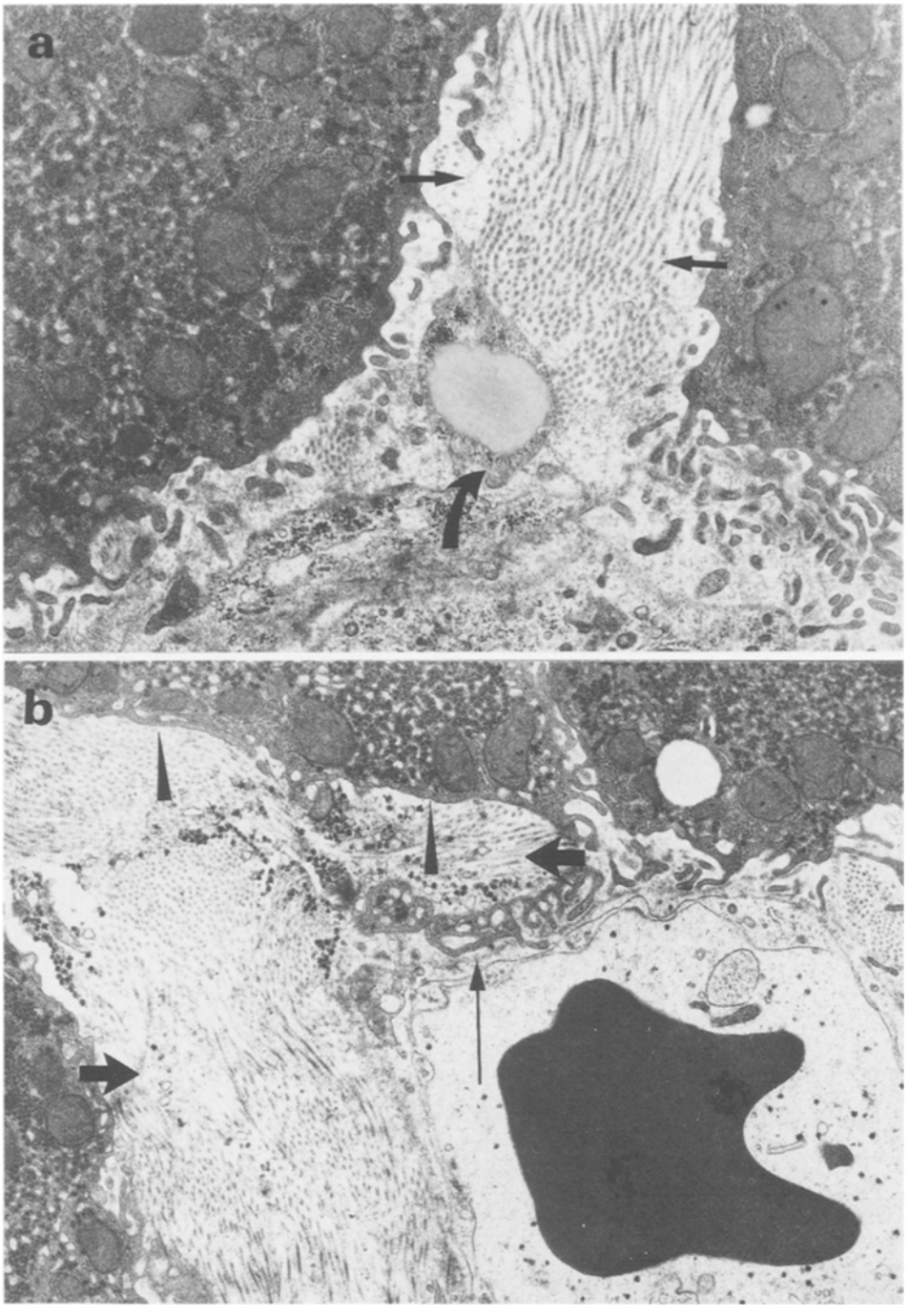

Fig. 4a, b. Interhepatocyte and space of Disse fibrotic reaction. **a** Hepatocyte disjunction due to collagen fibril deposit (*arrows*) close to Ito cell process (*curved arrow*). **b** Collagenization grade 3+ in a widened space of Disse: large collagen fibril bundles (*large arrows*), basement-membrane-like material (*thin arrow*), diffuse flattening of hepatocyte microvilli (*arrowhead*) and thinning of Ito cells (*open arrow*). **a** ×12100, **b** ×9000

fibrils, generally associated in bundles in the space of Disse, were related to microvillus flattening. Extension of fibrosis into the interhepatocyte space caused hepatocyte separation. Oedema (22.0%) or fibrosis (26.0%) causing this hepatocyte disjunction were rarely seen together (4.7%) and never found in control material ($P<0.0001$) (Figs. 3b, 4a). Sinusoidal collagenization (Orrego et al. 1979) was principally graded as 2+ in DA materials. Thus, more than half of the analysed sinusoids (56.7%) presented many collagen bundles in a widened space of Disse, associated with frequent basement-membrane-like material and an irregular flattening of the hepatocyte microvilli. Seventeen percent (17.3%) of the addict sinusoids showed severe collagenization (Figs. 4b, 5), results significantly different from control sinusoids which were abnormal in only 8.9% ($P<0.0001$). These generally exhibited no or minimal amounts of collagen fibrils, either isolated or in few bundles, randomly arranged around sinusoids.

Comparative analysis of sinusoidal wall surface (Table 4) showed a significant difference between DA and control material ($P<0.0001$). In DA, this result ($137.44\pm5.92\ \mu m^2$) was 1.7 times greater than that observed in the control ($82.50\pm4.17\ \mu m^2$). Table 5 presents the results of measured variables for sinusoidal wall cells. The area and perimeter of endothelial cells (body, pro-

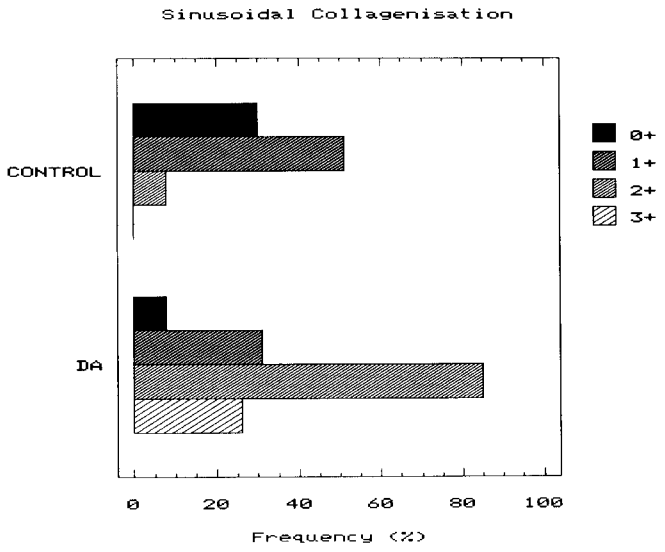

Fig. 5. Frequency of sinusoidal collagenization assessed by semi-quantitative ultrastructural analysis of 90 and 150 sinusoids from control and DA patients. Graded on scale of 0 to 3+ as: 0 = non-existent or minimal collagenization in a space of Disse of normal width: 1+ = few collagen fibrils in a space of Disse of normal width; 2+ = many collagen bundles and frequent basement membrane-like material in a widened space of Disse associated with a multifocal flattening of hepatocyte microvilli; 3+ = large collagen bundles and constant basement-membrane-like material in a widened space of Disse associated with diffuse flattening of hepatocyte microvilli. Tested by rank tabulation of Kendall's tau C. $\tau = 0.66$, $P < 0.0001$

Table 4. Sinusoidal wall surface assessed by morphometric data in controls and drug abusers (DA)

Patients	CONTROL ($n=90$)	DA ($n=150$)	F test: P values
Mean ± SEM (μm^2)	82.50 ± 4.17	137.44 ± 5.92	< 0.001 *
Range	27.72–223.51	26.69–454.72	

Tested by univariate analysis: * significantly different; n = number of sinusoids analysed

Table 5. Morphometric results of endothelial and Ito cells in control and DA patients: analysis of measured variables

		CONTROL ($n=90$)	DA ($n=150$)	F test: P values
Endothelial cell				
Cell body	Surface	5.60 ± 1.73	20.14 ± 2.40	< 0.0001 *
	Perimeter	3.82 ± 1.06	22.80 ± 2.64	< 0.0001 *
Processes	Surface	6.09 ± 0.41	12.58 ± 0.87	< 0.0001 *
	Perimeter	48.63 ± 2.17	44.67 ± 2.76	> 0.10
Ito cell				
Cell body	Surface	7.17 ± 1.65	11.22 ± 2.19	> 0.10
	Perimeter	5.36 ± 1.22	8.43 ± 1.72	> 0.10
Processes	Surface	8.69 ± 0.59	13.97 ± 1.00	< 0.001 *
	Perimeter	23.64 ± 1.26	35.62 ± 1.69	< 0.001 *

Values are mean ± SEM expressed in μm^2 for surface and μm for perimeter. Tested by univariate analysis; * significantly different; n = number of sinusoids analysed

Table 6. Surface density of endothelial and Ito cells in control and DA patients

	Control $n=90$	DA $n=150$	F test: P values
Endothelial cell			
Cell body	6.11 ± 1.85	14.41 ± 1.70	< 0.01 *
Processes	9.18 ± 0.75	11.34 ± 0.86	> 0.10
Ito cell			
Cell body	8.18 ± 1.92	9.48 ± 2.06	> 0.10
Processes	12.99 ± 1.18	12.38 ± 1.14	> 0.10

Values are mean ± SEM expressed in %. Tested by univariate analysis: * significantly different; n = number of sinusoids analysed

cesses) were significantly different when the two series of patients were compared ($P < 0.0001$). In DA, endothelial cell process and body surface was 2–3.6 times greater than that of controls. The endothelial cell body perimeter was 5.97 times greater in DA. In contrast, cytoplasmic process perimeter was non-significantly different in the two patient groups. Moreover, Ito cell process surface and perimeter were also significantly modified in DA (13.97 ± 1.00 μm^2 and 35.62 ± 1.69 μm, respectively; $P < 0.001$). However, the comparison between Ito cell body morphometric measurements showed slightly increased results in DA.

The study of CSD (Weibel et al. 1966) is presented in Table 6. Endothelial cell body occupied 14.41 ± 1.70% of sinusoidal wall, showing a significant difference from controls ($P < 0.01$). The other CSD values indicated no significant difference between endothelial cell processes and Ito cell body and processes when DA and controls were compared.

Discussion

The high incidence of liver dysfunction among heroin addicts has been attributed to a multifactorial aetiology (Alter and Michael 1968; Leevy et al. 1970; Levine and Payne 1960; Scoazec et al. 1988). A direct hepatotoxic effect of opiates as part of the mechanism of hepatic injury has rarely been suggested. In addicts, the demonstration of constant sinusoidal dilatation and a terminal hepatic vein inflammatory reaction replaced by a mural venular fibrosis after drug withdrawal (Trigueiro de Araujo et al. 1990), without hepatocyte lesions (Trigueiro de Araujo et al. 1992), led us to investigate, at an ultrastructural level, the liver target of heroin and/or its adulterants. We suggest that the lobular vascular system is undoubtedly injured by the toxic action of drug, on the basis of our findings of hyperactivation and hypertrophy of endothelial and Ito cells, confirmed by ultrastructural morphometric analysis, sinusoidal lumen and space of Disse inflammation and fibrosis of the space of Disse, frequently associated with continuous or discontinuous basement-membrane-like material. There is flattening of hepatocyte microvilli and interhepatocytic disjunction.

Way et al. (1965) showed that the pharmacological

action of heroin was closely related to its deacetylation into monoacetylmorphine and morphine. The liver is particularly efficient in this enzymatic biotransformation, which also takes place in the blood, kidney and brain. A high level of morphine accumulation after incubation of mouse liver homogenates with heroin for 20 min at 37° C was demonstrated by Way et al. (1960). Given that, in humans, 43% of intravenously injected heroin (Elliott et al. 1971) and 77% of orally administered heroin (Twycross et al. 1974) is recovered as morphine in urine and taking into account the morphological liver alterations in addicts, we postulate two hypotheses on the physiopathogenesis of heroin vascular hepatotoxicity. The first is that sinusoidal changes may be related to initial terminal hepatic vein damage. In addicts, the CSD of endothelial and Ito cells were generally not modified; findings which can be explained by a proportional increase of cellular surfaces and sinusoidal wall thickness in relation to the sinusoidal dilatation described previously (Trigueiro de Araujo et al. 1990). Moreover, oedema of interhepatocyte space without collagenous matrix deposit may demonstrate regional haemodynamic disturbance. The centrilobular change associated with the terminal hepatic vein thickening (Trigueiro de Araujo et al. 1990) evokes certain similarities with veno-occlusive hepatic disease. This obliterative vascular lesion, frequently drug-induced, has been implicated as a cause of lobular congestion and perivenular fibrosis (Higginson 1965). It has been also related to alcoholic intoxication by Goodmann and Ishak (1982). These authors have pointed out the physiopathological role of the phlebosclerosis in the genesis of the terminal hepatic vein obstruction. In addicts, terminal hepatic vein lesions could induce an increase of the small hepatic vein pressure and consequently a centrilobular congestion and sinusoidal dilatation. Our second hypothesis in that after heroin conversion in the hepatocyte, part of the morphine may return to the sinusoidal blood flow from hepatocyte microvilli, through the space of Disse. Metabolites of morphine produced in the blood might act in the sinusoids against Ito and endothelial cells, stimulating sinusoidal barrier thickening. These pathways might be responsible for accumulation of the drug in the sinusoidal barrier, causing cell alteration and a fibrotic reaction.

Interaction between hepatocyte, Ito, endothelial and Kupffer cells and extracellular matrix may occur during morphine intoxication. This cellular stimulation could trigger the fibrogenesis and the basement-membrane-like material production in the space of Disse. All these vascular lobular changes associated with a decrease of endothelial cell fenestrations may create a mechanical barrier hindering free transport and exchange between sinusoidal blood and parenchyma (Schaffner and Popper 1963). In addicts, sinusoidal wall alterations are probably responsible for a disturbance in the haemodynamic equilibrium between the sinusoidal lumen and the hepatocytes and thus affect liver function. Possibly the sinusoidal capillarization and fibrosis of the space of Disse constitute a barrier which might protect the liver parenchyma from the diffusion of morphine.

Heroin can be added to the list of hepatotoxic drugs, such as vitamin A, methotrexate, mercaptopurine and azathioprine, which induce cell sinusoidal lesions and perisinusoidal fibrosis (Zafrani and Feldmann 1988; Zafrani et al. 1983). Several papers have suggested that hepatocytes are involved in the genesis of the connective tissue biomatrix (Bianchi et al. 1984; Chojkier et al. 1988; Clément et al. 1984, 1986; Rescan et al. 1989), although non-parenchymal cells have been primarily incriminated in hepatic fibrosis (Ballardini et al. 1983; Friedman et al. 1985; McGee and Patrick 1972; Milani et al. 1989; Okanoue et al. 1983; Rieder et al. 1987).

In drug addicts, ultrastructural signs of Ito cell myofibroblastic differentiation were rarely found and morphometric data showed Ito cell activation to be represented only by thickening of the cytoplasmic processes. In this study, we have been unable to confirm which type of sinusoidal cell was principally responsible for the vascular fibrotic reaction in the space of Disse. Independently of collagenogenesis, fibrosis represents a common morphological finding in the evolution of all these hepatic diseases, contributing to their severity and persistance.

In conclusion, the ultrastructural liver target of heroin is represented by endothelial and Ito cells. The changes seen may be the result of either sinusoidal dilatation secondary to terminal hepatic vein damage or to a direct cellular toxic effect of the drug, but the changes in them, mainly related to the cellular size, triggered perisinusoidal fibrosis. Thickening of the space of Disse may present a mechanical barrier against the diffusion of heroin metabolites. It will also alter the quality of exchange through the sinusoidal barrier, leading to impairment of the liver's capacity for drug detoxification.

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