

Rat liver carbohydrate alterations in streptozotocin-induced diabetic rats

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SUMMARY

Diabetes mellitus (DM) currently belongs to the most widespread human pathologies, affecting about 4% of the world adult population. Despite the pivotal role of the liver in the development of metabolic disorders, the influence of DM on hepatic glycoconjugates remains obscure. The aim of the present investigation was to use a set of lectins with different carbohydrate affinities to investigate impairment in rat liver glycoconjugates influenced by streptozotocin-induced diabetes mellitus. The lectin panel included 7 conventional lectins – Con A, SNA, RCA, WGA, PNA, SBA, and HPA, supplemented with the original fucose-specific lectin preparation from *Laburnum anagyroides* bark (LABA). Tissue samples were fixed in 4% neutral formalin, embedded in paraffin, and subjected to lectin-peroxidase-diaminobenzidine staining.

In control rats a strong reactivity against Con A, LABA, SBA and SNA with cytoplasmic granularities of hepatocytes was detected, while RCA, WGA and HPA showed a strong reactivity with vascular endothelium, and WGA and HPA with bile capillaries. Experimental diabetes was associated with a redistribution of Con A and LABA receptor sites from centrolobular hepatocytes to hepa-

toocytes with peripheral localization. Among the most remarkable observations was DM-induced exposure of lectin reactivity with hepatocyte and endothelial cell nuclei. The endothelial lining of sinusoidal hemocapillaries, of central veins, and portal tract vessels also displayed a significant and differential rearrangement of carbohydrate determinants when influenced by DM. Diabetes-induced activation of Kupffer cells was accompanied by the expression of SNA, PNA and SBA receptor sites within the cytoplasm of these cells, which was lectin-negative in control specimens. The results reported provide a new insight into the pathogenesis of DM-induced impairment of hepatic carbohydrates, and demonstrate the applicability of the original fucose-specific lectin preparation to experimental histopathology.

Key words: Streptozotocin – *Diabetes mellitus* – Rat liver – Lectin histochemistry – *Laburnum anagyroides* bark agglutinin (LABA)

INTRODUCTION

Diabetes mellitus (DM) currently belongs to the most widespread of all human pathologies, affecting about 4% of the world adult popula-

tion, with a foreseen rise to 5.4% by 2025. In developed countries, DM morbidity affects 8-10% of the adult population, being the leading cause for death and disability, and contributing to 1.6-6.6% of total health-care costs (Dunger and Todd, 2008).

In Ukrainian official statistics 2,043 cases of DM are reported per 100,000 population, with the total number of diabetic patients exceeding 969,000 cases. However, due to its hidden forms, the real prevalence of DM could be 3-4 times higher (Tronko and Tchernobrov, 2005). The high rates of mortality, as well as life-threatening and disabling complications, make DM an urgent problem for health services. Despite the pivotal role of the liver in the development of metabolic disorders (Sherlock and Dooley, 1997; Eckel et al., 2005), the influence of DM upon hepatic glycoconjugates remains obscure.

The aim of the present investigation was to use a set of lectins with different carbohydrate affinities to examine the impairment of rat liver glycoconjugates affected by streptozotocin-induced *Diabetes mellitus*, and to test the applicability of original fucose-specific lectin preparation from *Laburnum anagyroides* bark (LABA) for experimental histopathology.

MATERIALS AND METHODS

Animals

The study was performed on 55 male Wistar rats with a weight of 110-120 g, subdivided into two groups: a control (n=10) and an experimental one (n=45). Experimental DM was induced by a single intra-abdominal injection of 70 mg/kg streptozotocin (Sigma, St. Louis, MI, USA) dissolved in 0.1 M citrate buffer, pH 4.2 (Wada and Yagihashi, 2004; Abdollahi et al., 2011). Development of DM was monitored through glucose levels, measured with the gluco-oxidase test (LaChema, Prague, Czech Republic), in accordance with the manufacturer's instructions. DM was verified by blood glucose levels reaching 10-18 mMol/l. Control group rats were given a single intra-abdominal injection of 1 ml citrate buffer at pH 4.2.

The investigation was carried out according to the ethical criteria for the use and handling of laboratory animals established by the Lviv National Medical University, and in accor-

dance with "General ethical principles on experiments with animals", conferred by the 1st National Congress on Bioethics (Kyiv, 2001), and in compliance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (1996).

Tissue specimens

Fourteen days after streptozotocin injection the animals whose blood glucose levels had reached 10-18 mMol/l were sacrificed by diethyl narcosis overdose. Samples of liver were fixed in 4% neutral formalin overnight and embedded in paraffin according to the standard protocol. For general morphology studies, 5-7 μ m cross-sections were stained with hematoxylin and eosin.

Lectin histochemistry

The lectin panel is shown in Table 1. All lectins and their peroxidase conjugates were prepared by Dr. V. Antonyuk Pharm. Sci. (Lectinotest, Lviv, Ukraine). Carbohydrate determinants were visualized according to the lectin-peroxidase-diaminobenzidine staining protocol.

In detail, deparaffinized sections were incubated for 30 min in methanol containing 0.3% H₂O₂ to block the activity of endogenous peroxidase, dehydrated through ethanol grades, rinsed in three portions of phosphate buffered saline (PBS) pH 7.4 (5 min each), and incubated in a humid chamber for 60 min with lectin-peroxidase conjugate (for each lectin preparation, dilution was selected individually, ranging between 10-25 μ g/ml); lectin receptor sites were visualized in PBS, containing 0.05% diaminobenzidine \times 4HCl (Sigma) and 0.015% H₂O₂. Then, the slides were washed twice in distilled water and, after dehydration, mounted in balsam.

The specificity of the histochemical reactions was controlled at 3 levels by: (1) omitting lectin-peroxidase from the staining protocol; (2) pre-incubation of tissue sections prior to lectin labelling for 60 min in 1% HIO₄ (Reanal, Budapest, Hungary) for oxidative damage of carbohydrate determinants; and (3) the addition of 0.5 M complementary monosaccharide to the lectin-peroxidase incubation medium. In the first case, the staining results were completely negative; in the second, significantly reduced; in the third case a decrease was detected, but not the complete elimination of LABA, WGA, PNA, SBA

binding, apparently due to the higher affinity of these lectins to tissue glycoconjugates than to inhibitory monosaccharides. Additionally, as an *in situ* control for the specificity of lectin

labelling, we treated negative images of cell nuclei on the background of lectin-reactive cytoplasm, as shown in Figs. 2C, 3A-D.

Table 1. Lectins and their respective carbohydrate specificities.

N°	Lectin designation, abbreviation	Specific monosaccharide	Complementary oligosaccharide / polysaccharide*
1.	<i>Canavalia ensiformis</i> agglutinin, ConA	α DMan/ α DGlc	Glycogen**
2.	<i>Laburnum anagyroides</i> bark agglutinin, LABA***	LFuc	Gal(β 1-4)Fuc(β 1-3)Glc
3.	<i>Sambucus nigra</i> agglutinin, SNA	NeuNAc(α 2-6)DGal	NeuNAc(α 2-6)Gal(β 1-4)GlcNAc(β 1-2)
4.	<i>Ricinus communis</i> agglutinin, RCA	β DGal>NeuNAc	NeuNAc(2-6)Gal(β 1-4)GlcNAc
5.	Wheat germ agglutinin, WGA	DGlcNAc>NeuNAc	NeuNAc(α 2-6)Gal(β 1-4)GlcNAc, Man(β 1-4)GlcNAc(β 1-4)GlcNAc
6.	Peanut agglutinin, PNA	DGal	DGal(β 1-3)GalNAc
7.	Soybean agglutinin, SBA	α DGalNAc> β DGalNAc	GalNAc(α 1-3)Gal(β 1-3)GalNAc
8.	<i>Helix pomatia</i> agglutinin, HPA	α DGalNAc	GalNAc(α 1-3)GalNAc

* More detailed information about the carbohydrate affinities of the lectins used in this study can be found in a handbook by Brooks et al. (1997).

** According to Zlotowski et al. (2006), Con A strongly reacts with glycogen deposits.

*** Preparation and characteristics of original fucose-specific lectin from *Laburnum anagyroides* bark was described by Lutsyk and Antonyuk (1982).

Table 2. Lectin binding characteristics of rat liver: control vs. diabetes mellitus samples.

	Lectin; carbohydrate specificity	Experimental group of animals	Hepatic lobule				Portal tracts	Stromal elements
			Hepatocytes	Sinusoidal capillaries	Bile canaliculi	Central venules		
1.	ConA, α DMan/ α DGlc	Control	+++	-	-	++	-	+
		DM	++	-	-	+++	+++	+
2.	LABA, LFuc	Control	++	-	-	-	-	+
		DM	++	-	-	+	+++	+
3.	SNA, NeuNAc(α 2- 6)DGal	Control	+++	-	-	++	-	+
		DM	+	++	-	+++	-	+
4.	RCA, β DGal> NeuNAc	Control	+	++	-	++	+++	+
		DM	+	-	-	-	-	+
5.	WGA, DGlcNAc> NeuNAc	Control	+	+++	+	+++	+++	+
		DM	+	+	++	+++	+++	+
6.	PNA, DGal	Control	+	-	-	-	-	+
		DM	+	++	-	-	-	+
7.	SBA, α DGalNAc> β DGalNAc	Control	++	++	-	+	+++	+
		DM	++	++	-	-	+++	+
8.	HPA, α DGalNAc	Control	++	++	++	++	-	+
		DM	+	+++	+++	+++	+++	+

Microscopic investigation was performed using a Carl Zeiss Ng (Jena, Germany) microscope equipped with a Canon IXUS 700 digital camera (Canon, Tokyo, Japan). For semiquantitative evaluation of lectin binding, two investigators performed the analysis independently, blinded to lectin type. Binding intensity was represented on a semiquantitative scale as follows: +++ strong, ++ moderate, + faint, - negative labelling (Table 2).

RESULTS

General morphology

Fourteen days after the streptozotocin injection a heavy lymphocyte infiltration of hepatic lobules and connective tissue was observed around the portal tracts, as well as dilatation of sinusoidal capillaries and central venules (Figs. 1A, B). In some cases, lipid dystrophy of hepatocytes, morphological signs of their necrosis, and apoptosis were also visible.

Lectin histochemistry revealed the specificity of labelling, whose most characteristic features are shown in Table 2 and in Figures 2-5.

Concanavalin A (Con A)

In control rats, the highest Con A reactivity was detected in the cytoplasm of hepatocytes with centrolobular localization, and intensity of binding was gradually reduced towards the periphery of the lobules (Fig. 2A). DM induced the decrease in Con A binding with

centrolobular hepatocytes simultaneously with an enhanced reactivity of nuclear and cytoplasmic glycoconjugates of the central venule endothelium, the nuclei of adjacent hepatocytes (Fig. 2B), and enhancement of lectin binding to the hepatocytes at the periphery of lobules and to portal tract endothelium.

Laburnum anagyroides bark agglutinin (LABA)

The distribution of fucosoglycans as detected by LABA reactivity was similar to the distribution of Con A receptor sites in both the control and experimental rats, with minor differences in the binding intensity (Figs. 2C, D).

Sambucus nigra agglutinin (SNA)

In the liver of intact rats SNA was bound predominantly to cytoplasmic granularities of hepatocytes, and, to a lesser extent, central venules endothelial cells (Fig. 3A). DM was accompanied with the reduced lectin binding to hepatocytes, and enhanced reactivity of central venules endothelium. The expression of SNA receptor sites in Kupffer cells was revealed as specifically interesting (Fig. 3B, C, D), since in control specimens these were completely negative.

Ricinus communis agglutinin (RCA)

In the livers of control rats, RCA reacted strongly with the endothelial lining of sinusoidal capillaries, of central venules, and portal tract vessels. Influenced by DM, lectin binding was greatly reduced.

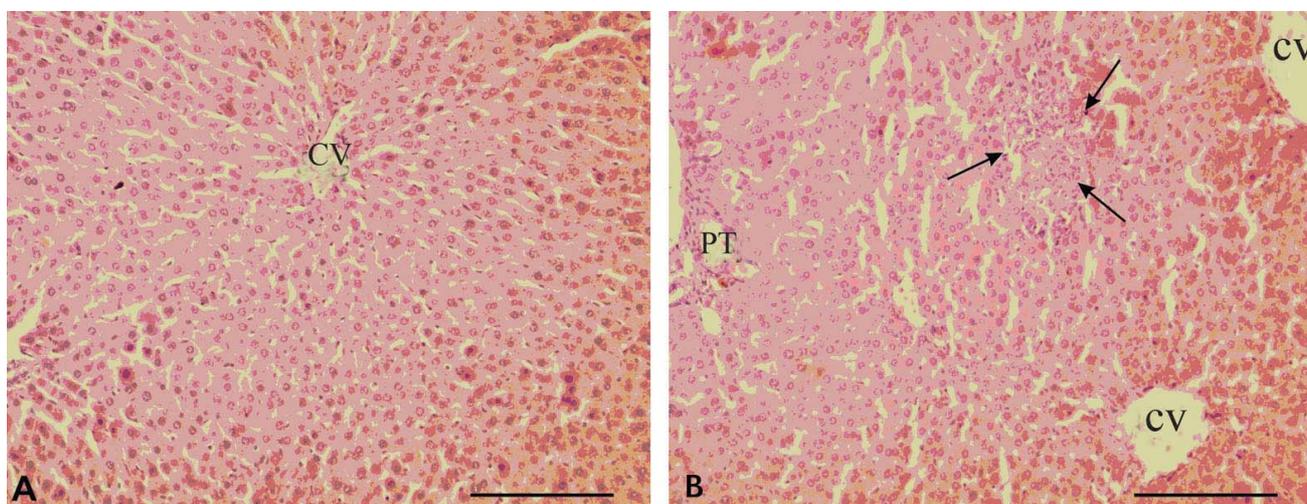


Figure 1. Hepatic lobule of control (A) and DM-affected rat (B). Diabetic lesions characteristic of intralobular (arrows) and periportal (PT) lymphocyte infiltration due to dilatation of the central veins (CV) and of sinusoidal capillaries. Hematoxylin and eosin. $\times 150$. Bar = 30 μm .

Wheat germ agglutinin (WGA)

In the liver of control rats, WGA was predominantly attached to the basement membranes of sinusoidal capillaries and to endothelial lining of central venules and portal tracts (Fig. 4A). Under DM conditions, lectin reactivity with sinusoidal capillaries was reduced, but persisted within the endothelium of central venules and portal tract vessels (Fig. 4B). It should be noted that, both in control and in diabetic specimens, the bile canaliculi were WGA-positive.

Peanut agglutinin (PNA)

In control rats, PNA exhibited a moderate affinity towards the cytoplasmic glycoconjugates of hepatocytes and collagen fibres in the perisinusoidal space of Disse. Additionally, under diabetic conditions PNA strongly

bound to Kupffer cells, which in the control samples lacked these lectin receptor sites.

Soybean agglutinin (SBA)

SBA binding in control rats was localized mainly in the cytoplasm of hepatocytes, the endothelial lining of sinusoidal capillaries, and of the central venules and portal tracts. Under experimental diabetes conditions a gradual reduction of hepatocytes reactivity was detected in the direction from central venules to the periphery of the lobules, accompanied by the expression of SBA receptor sites in the cytoplasm of Kupffer cells.

Helix pomatia agglutinin (HPA)

Although the specificity of this lectin carbohydrate was similar to that of SBA, we observed considerable differences in these lectin-binding affinities towards hepatic tissues. In particular, in control rats the HPA

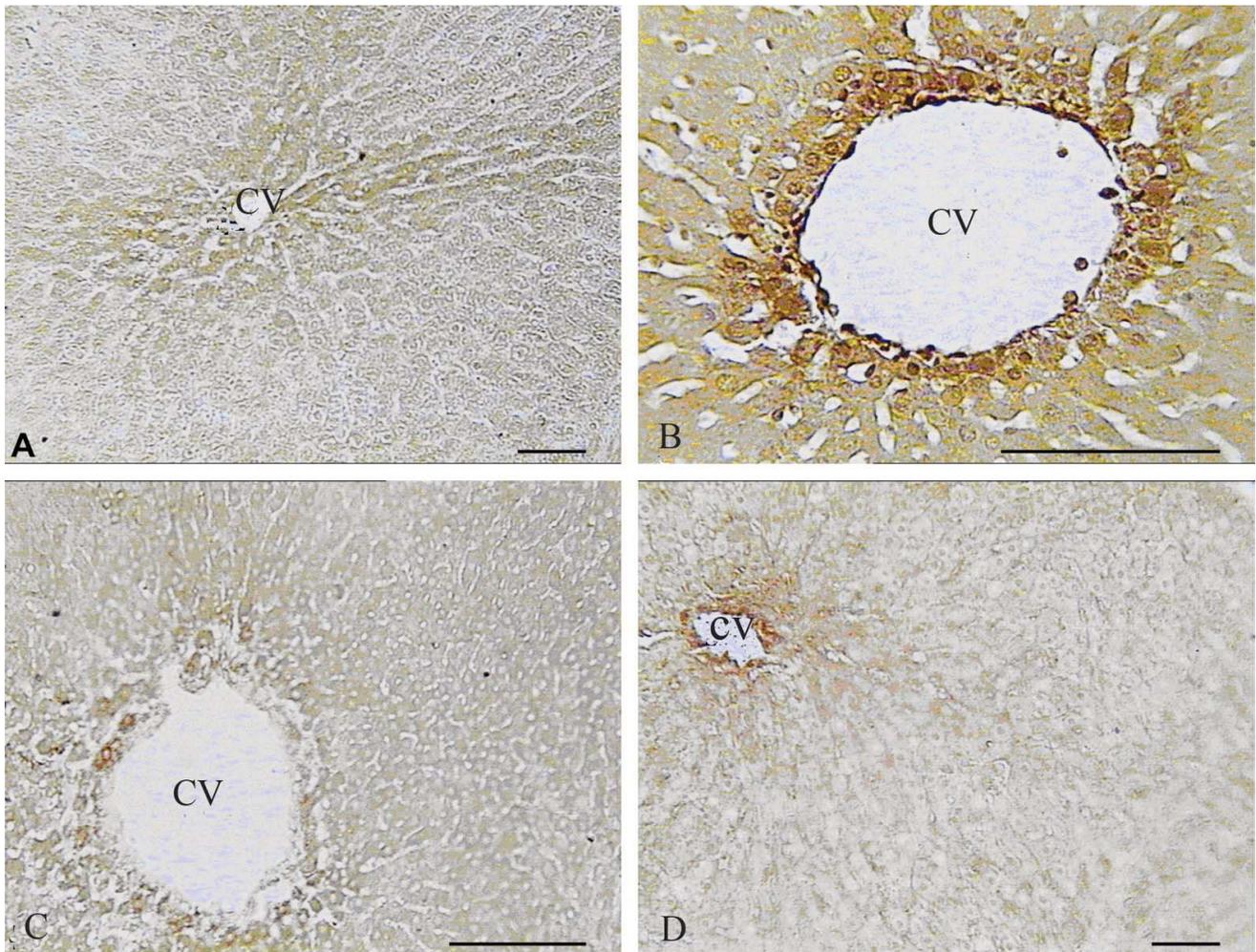


Figure 2. Comparative lectin histochemistry of control (A, C) and diabetic (B, D) rat liver: Con A (A, B) and LABA (C, D) labelling. In control specimens, both lectins demonstrated the highest reactivity with the cytoplasmic glycoconjugates of the centrolobular hepatocytes, with a gradual reduction in lectin binding towards the periphery of the lobule. Diabetic hepatopathy was accompanied by the accumulation of mannos- and fucosoglycans within the nuclei of hepatocytes adjacent to the central vein (CV), as well as in the nuclei and cytoplasm of its endothelial lining. $\times 300$ (A, D) and $\times 600$ (B, C). Bar = 30 μm .

receptor sites were localized in the cytoplasm of hepatocytes, collagen fibres within the space of Disse, and in the endothelial lining of

the central venules (Figs. 5A, B). Under DM conditions, a decreased reactivity of hepatocytes was detected, with a simultaneous

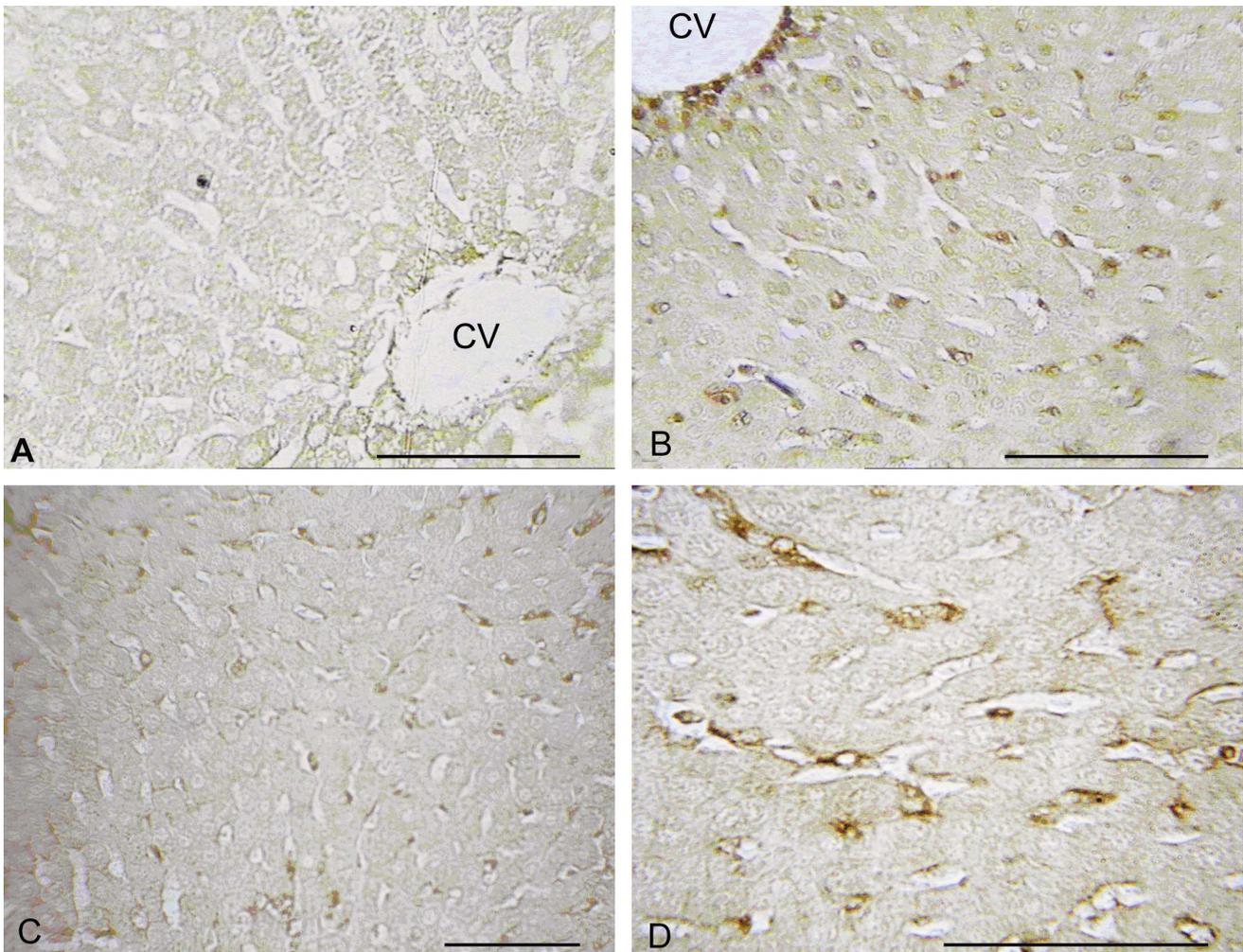


Figure 3. Histotopography of sialoglycans (SNA receptor sites) in the livers of control (A) and diabetic rats (B, C, D). In control rats, the lectin label is restricted to the cytoplasmic granularities of hepatocytes. DM induced strong lectin labelling of Kupffer cells and of the central vein (CV) endothelium. $\times 600$ (A), $\times 400$ (B, C), $\times 750$ (D). Bar = 30 μm .

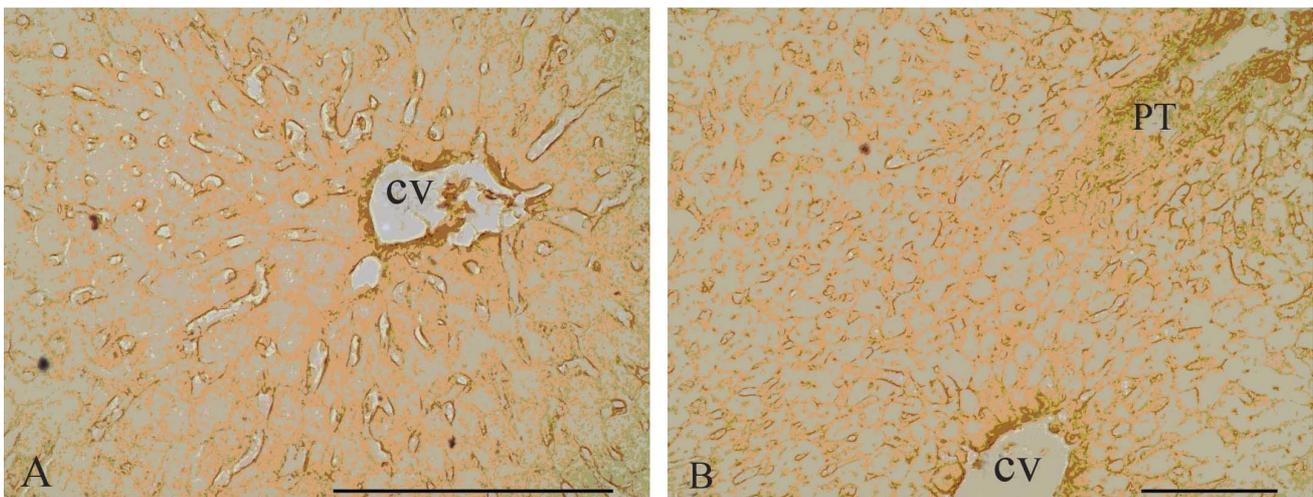


Figure 4. Distribution of WGA receptor sites in control (A) and diabetic (B) rat liver. Diabetic impairment was accompanied by a decreased reactivity of the sinusoidal capillary bed, of the central vein (CV) and of the portal tract (PT) endothelium. $\times 400$ (A), $\times 200$ (B). Bar = 30 μm .

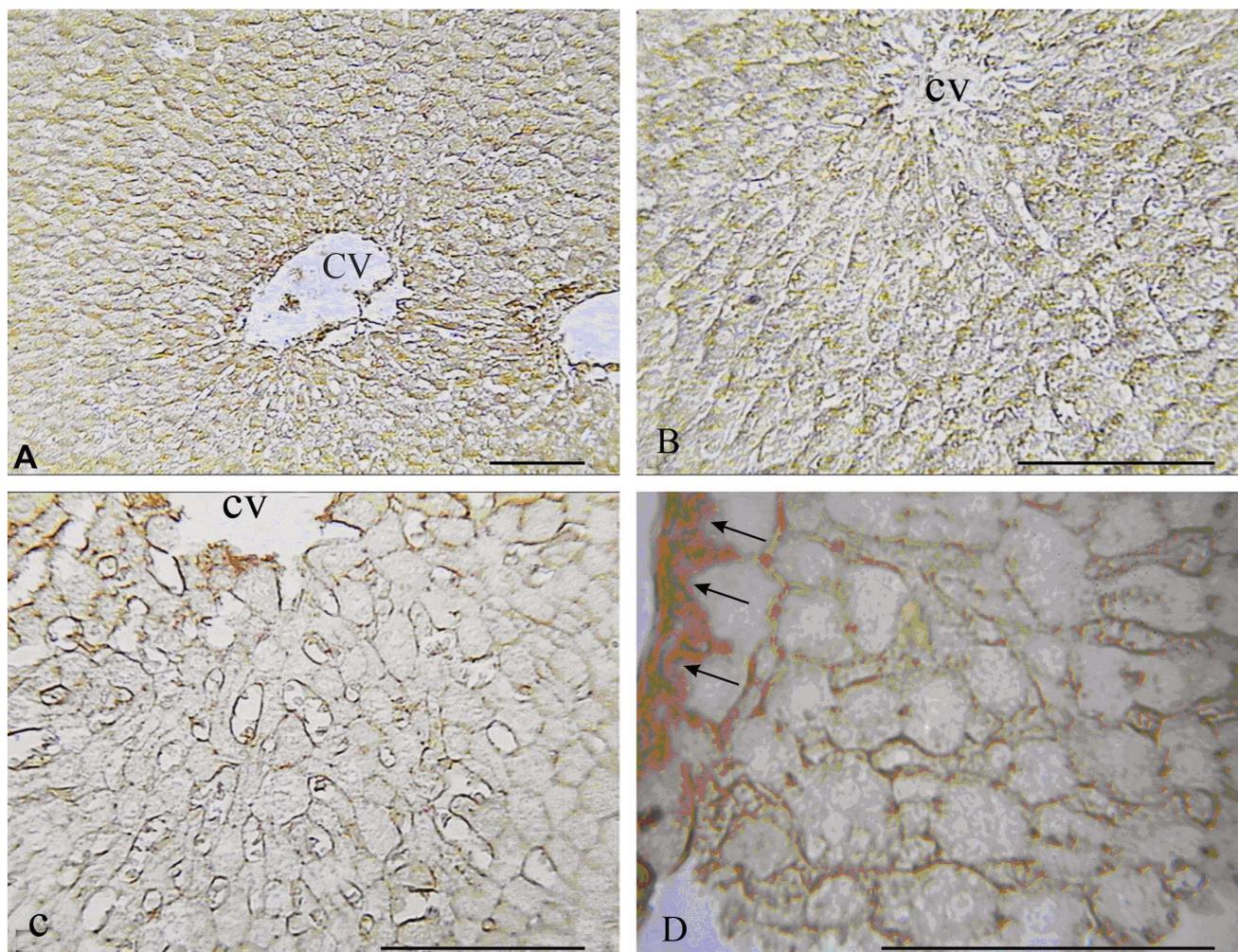


Figure 5. HPA labelling of control (A, B) and diabetes-affected (C, D) rat liver. In control rats HPA receptor sites associated with the cytoplasmic granularities of hepatocytes. Diabetic lesions characteristic of reduced hepatocyte reactivity and enhanced lectin binding to reticular fibres surrounding sinusoidal capillaries and basement membranes adjacent to portal tracts (arrows). CV – central vein. $\times 300$ (A, B), $\times 600$ (C), $\times 750$ (D). Bar = 30 μm .

enhancement of lectin binding to perisinusoidal collagen fibres and to basement membranes of the portal tract vessels (Figs. 5C, D). The bile canaliculi showed HPA receptor sites in both normal and diabetic conditions.

DISCUSSION

According to the literature (Sherlock and Dooley, 1997), prominent morphological manifestations of diabetic hepatopathy include accumulation of glycogen deposits in nuclei and cytoplasm of hepatocytes, their lipid and hydropic dystrophia with subsequent development of hepatomegaly, accumulation of collagen fibers in perisinusoidal space of Disse. Our general morphology data additionally revealed marked lymphocytic infiltration of hepatic lobules, dilatation of sinusoidal capillaries and central venules in association

with necrotic and necrobiotic changes of hepatocytes. However, it should be considered that under experimental conditions at least two mechanisms were involved in formation of pathological lesions: (1) indirect metabolic action of hyperglycemia; (2) direct cytotoxic effect of streptozotocin. Both mechanisms apparently play a pivotal role in hepatotoxic effect of experimental DM.

Using lectin histochemistry methods, we detected a significant redistribution of lectin receptor sites in hepatocytes influenced by streptozotocin-induced DM. Among the most remarkable signs accumulation of Con A-reactive glycoconjugates was noticed inside the nuclei of the hepatocytes surrounding central venule. A similar accumulation of Con A receptor sites was also characteristic of the nuclei of endothelial cells, lining the central venules and portal tract vessels.

Combining the reported Con A reactivity to glycogen (Zlotowski et al., 2006) with data on the accumulation of glycogen deposits within the nuclei of DM-affected hepatocytes (Sherlock and Dooley, 1997), we assume that, as detected in our study on the redistribution of Con A receptor sites reflects DM-induced alterations of glycogen synthesis, with the subsequent overloading of hepatocyte and endothelial cell nuclei with glycogen deposits.

In this context it should be noted that by means of electron microscopy it has been demonstrated affinity of Con A, besides glycogen, with plasma membrane, rough and smooth endoplasmic reticulum, ribosomes and mitochondria, condensed and nucleolar-associated chromatin of rat hepatocytes (Roth, 1983). Apparently these elements in our investigation also added to total hepatocyte reactivity, though it is hard to determine which pool of Con A receptor sites was lost during formalin fixation and paraffin embedment protocol.

Hepatocytes of the control rats showed high amounts and a vast diversity of cytoplasmic glycoconjugates, as revealed by their reactivity with lectins of different carbohydrate affinities (Table 2). These data are consistent with results of others (Roth, 1983; Kaneko et al., 1995), as well as with our earlier observations (Smolkova et al., 2001) despite the different fixation and tissue processing protocols used. We detected highest reactivity of control rat hepatocytes with Con A and SNA. Streptozotocin treatment induced dramatic losses of Con A and SNA binding was revealed as specifically interesting apparently due to the impairment of glycogen and sialoglycan synthesis. The redistribution of glycogen deposits from the cytoplasmic to nuclear compartments of hepatocytes and from centrilobular to periportal hepatocytes, as well as the accumulation of glycogen deposits in the vascular endothelium of portal tracts and central venules were among the most characteristic signs of DM-induced hepatopathy.

Earlier, we reported the heterogeneity of the rat vascular endothelium as detected with HPA and GS-I lectin-gold probes (Smolkova et al., 2001). Our recent findings confirmed and extended these observations with respect to the differential lectin binding of hepatic sinusoid capillaries, central venules and portal tract endothelium both in control and diabetic rats. Under the influence of DM, increased

Con A, LABA and HPA reactivity to endothelium of central venules and portal tract vessels, but not that of sinusoidal capillaries, was observed; at the same time, RCA binding to these same vessels was reduced. It can be speculated that the detected changes in glycoconjugate terminal sugar residues involve alterations in the permeability and adhesive properties of the vascular endothelium under the influence of DM.

Remodelling of the vascular wall under DM conditions, among others, is accompanied by a thickening of the microcirculatory bed basement membranes and modification of carbohydrate determinants on the surface of endothelial cells (Pickup and Williams, 2002). The latter phenomenon contributes to enhanced leukocyte adhesion and facilitates their subsequent penetration into the inflammatory site (Kierszenbaum, 2007). The characteristic changes in the sinusoidal capillary endothelium, including lectin receptor sites, have been described in chronic hepatitis, cirrhosis of the liver and hepatocellular carcinoma (Terada et al., 1991). Our study extends these observations to diabetic hepatopathy.

It is generally accepted, that Kupffer cells are the first line of protective cells of the liver, mediating the pathological influence on the sinusoidal endothelial cells and perisinusoidal cells of Ito (Kierszenbaum, 2007). The latter, beside other responses, increase the secretion of collagen into the perisinusoidal space of Disse, initiating cyrrhotic transformation. When activated, Kupffer cells release an excess of tissue-toxic mediators, leading to damage of sinusoidal endothelial cells, hypercoagulability and increased leucocyte adherence (Arii and Imamura, 2000). Blockade or selective destruction of Kupffer cells prevents further hepatic tissue damage (Arii and Imamura, 2000; Rivera et al., 2007).

We detected DM-induced expression of SNA, PNA and SBA receptor sites in Kupffer cells, normally non-reactive with the same lectins. We assume that the diabetic activation of these phagocytic cells is associated with extra sialization and simultaneous unmasking of their DGal/ DGalNAc sugar determinants. Our findings point to the high sensitivity of Kupffer cells and, apparently, their important role in diabetic liver impairment.

Bile canaliculi in our hands were negative with most of the lectins used, except of WGA and HPA, which therefore can serve as histo-

chemical markers of these hepatic structures. It is noteworthy that, under DM conditions, intensity of lectin binding to bile canaliculi was enhanced most likely due to the increased bile flow.

To conclude, streptozotocin-induced diabetic hepatopathy was characteristic with marked redistribution of lectin reactive glycoconjugates in rat liver. We detected shift in Con A reactive glycoconjugates from cytoplasmic to nuclear compartments of hepatocytes and from centrolobular to periportal hepatocytes, as well as the accumulation of Con A-reactive deposits in vascular endothelium of central venules and portal tracts. Diabetes-activated Kupffer cells exposed receptors for SNA, PNA and SBA, not detected in control specimens. Differential lectin reactivity and DM-induced redistribution of lectin receptor sites revealed heterogeneity of rat vascular endothelium, lining sinusoidal capillaries, central venules and portal tracts. It was demonstrated applicability of WGA and HPA as alternative histochemical markers of bile canaliculi. Reported observations give a new insight into pathogenic mechanisms of diabetic hepatopathy with regards to liver carbohydrate determinants remodeling, proving the applicability of original fucose-specific lectin preparation from *Laburnum anagyroides* bark (LABA) for experimental histopathology.

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