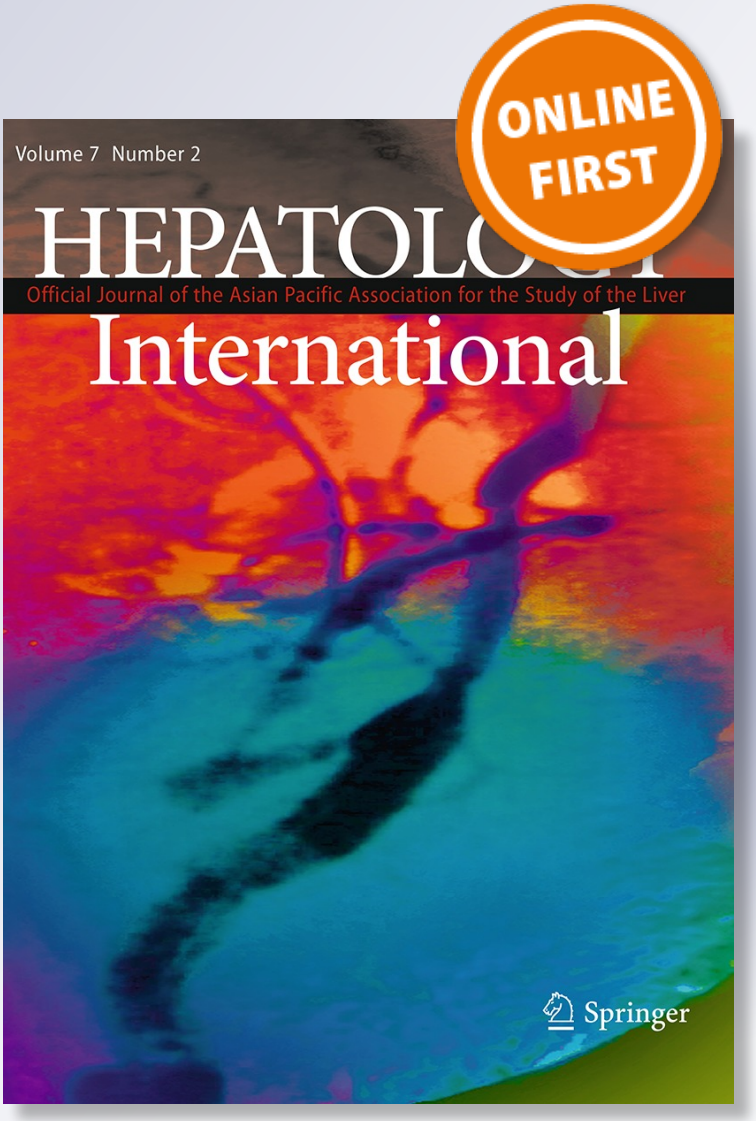
Hepatocytes of cirrhotic rat liver accumulate glycogen more slowly than normal ones

Natalia N. Bezborodkina, Sergey V. Okovity, Anna Yu. Chestnova & Boris N. Kudryavtsev

Hepatology International

ISSN 1936-0533

Hepatol Int
DOI 10.1007/s12072-013-9458-8



Springer

Hepatol Int
DOI 10.1007/s12072-013-9458-8

ORIGINAL ARTICLE

Hepatocytes of cirrhotic rat liver accumulate glycogen more slowly than normal ones

Natalia N. Bezborodkina · Sergey V. Okovity · Anna Yu. Chestnova · Boris N. Kudryavtsev

Received: 28 December 2012 / Accepted: 4 July 2013

Abstract
Purpose To investigate the accumulation of glycogen in cirrhotic rat liver at several time intervals after *per os* administration of glucose to fasted animals.
Methods Liver cirrhosis was produced by inhalation of the hepatotropic poison CCl_4. Glycogen concentration in the liver was determined biochemically. Glycogen content in hepatocytes was measured cytofluorimetrically in the smears stained with a fluorescent PAS reaction. Glycogen content in the hepatocytes of the portal and the central zone of the liver lobule was determined by absorption cytophotometry.
Results Rats poisoned with CCl_4 for 6 months developed typical liver cirrhosis characterized by a fourfold ($p < 0.001$) increase in the proportion of the connective tissue. In the cirrhotic rats fasted for 48 h, glycogen concentration in the liver and glycogen content in hepatocytes were lower as compared with the control by 36 and 27 % ($p < 0.01$), respectively. According to data obtained by different methods, the control animals accumulated glycogen at a high rate. In particular, the glycogen content in hepatocytes increased by 34 % after 10 min ($p < 0.01$). In the cirrhotic rats, glycogen content remained at the same level for 20 min. In both groups of animals, hepatocytes of the portal zone accumulated more glycogen than those of the central zone.
Conclusions Glycogen accumulation in cirrhotic rats starts after a delay and proceeds at a lower rate than in the norm.

Keywords Glycogen · Hepatocytes · Liver cirrhosis · Glucose

N. N. Bezborodkina (✉) · A. Yu. Chestnova · B. N. Kudryavtsev
Laboratory of Cellular Pathology, Institute of Cytology, Russian Academy of Sciences, Tikhoretsky Avenue 4, 194064 Saint Petersburg, Russia
e-mail: natalia_bezborodkina@mail.ru

A. Yu. Chestnova
e-mail: chestnovaanna@mail.ru

B. N. Kudryavtsev
e-mail: bn_kudryavtsev@mail.ru

S. V. Okovity
Department of Pharmacology, Saint-Petersburg State Chemical Pharmaceutical Academy, Professora Popova Street 14, 197376 Saint Petersburg, Russia
e-mail: okovityy@mail.ru

Introduction

Carbohydrate metabolism in cirrhotic patients is impaired [1, 2]. An indication of this impairment is hyperglycemia observed in most such patients after a meal [3–5]. It may be caused by various reasons such as the resistance of skeletal muscles and fat tissue to insulin, the slackened suppression of glucose production and/or the reduction of glucose utilization by the liver [5, 6]. As the liver plays a key role in maintaining the glucose level balance in the blood (by glycogenogenesis and glycogenolysis), the impairment of glucose uptake and its accumulation in hepatocytes as glycogen may be an important factor for the development of hyperglycemia in cirrhotic patients.

Information about glycogen synthesis in the cirrhotic liver is scanty and controversial. On the one hand, it has been established that the synthesis of glycogen in the cirrhotic liver does not differ from that in the normal one [5, 7]. On the other hand, glycogen content in the cirrhotic liver of humans and rats after a meal has been shown to be lower than in normal ones [8, 9].

In this work, we studied, using biochemical, cytofluorimetric and cytophotometric methods, glycogen content in the normal and cirrhotic rat liver at several time intervals after glucose administration to fasted animals.

Published online: 23 July 2013

Materials and methods

Animals and procedures

We used outbred male white rats obtained from "Rappolovo" animal nursery. The rats, weighing ca. 150 g at the start of the experiment, were housed under a 12-h light–dark cycle, with food and water ad libitum.

The animals were separated into two groups. Liver cirrhosis in rats from the first group ($n = 27$) was produced by chronic exposure to carbon tetrachloride (CCl_4). The animals were poisoned for 6 months three times weekly for 15 min, the CCl_4 concentration being 0.05 g/l. The rats from the second group ($n = 27$) were not exposed and served as control.

A week after the termination of poisoning, rats from both groups were fasted for 48 h (water ad libitum) and then given 30 % glucose orally (4 g/kg of body weight). Rats were decapitated immediately after the termination of fasting (0 min) and at 10, 20, 30, 45, 60, 75, 90 and 120 min after the administration of glucose. Three rats were killed at each time point.

Histological methods

Pieces of rat liver were fixed in 10 % neutral formalin and embedded in paraffin blocks. The tissue sections were stained with hematoxylin-eosin and picrofucsin after Van Gieson [10].

The relative volume of the connective tissue and the parenchyma of the rat liver in the sections was determined according to Weibel [11] using the eyepiece reticule (16 × 16), a 25 × 0.50 objective and an 8× eyepiece.

Biochemical methods

The hepatic glycogen concentration was determined according to Lo et al. [12], with some modifications. Liver samples were weighed and digested in 1 ml of hot 30 % KOH (for 60 min at 100 °C), after which 70 % ethanol was added to precipitate the glycogen from the alkaline digests. The samples were centrifuged for 30 min at 1,000 g, washed successively with 80 and 96 % ethanol, and then centrifuged again. The pellets were hydrolyzed in 3 ml of 2 N H_2SO_4 for 2.5 h in a boiling water bath. The results were expressed as micromol glucosyl units per g wet weight of liver.

Cytofluorimetry

Smears of isolated hepatocytes were prepared as previously described and stained with the fluorescent variant of PAS reaction [13]. Glycogen content in hepatocytes (100 cells from each rat) was then measured using a LUMAM IUF-3 cytofluorimeter (LOMO Co., St. Petersburg, Russia). To exclude the effect of the cell size, the glycogen amount in the isolated hepatocytes was related to the total protein content in the cells.

Cells stained with the fluorescent variant of PAS reaction were photographed under an Axiovert 200 M microscope (Carl Zeiss, Jena, Germany) using 20 × 0.50 or 20 × 0.40 objectives.

Absorption cytophotometry

To determine total protein content in isolated hepatocytes, the smears were stained with naphthol yellow S [14] and then measured by scanning absorption cytophotometry ($\lambda = 475$ nm, 25 × 0.50 objective, probe 0.8) using the microscopic complex "Morphoquant" (Carl Zeiss, Jena, Germany).

Glycogen in the hepatocytes of the portal and central liver lobules was revealed using a quantitative variant of the routine PAS reaction on the tissue sections [15]. Glycogen content was determined by the television cytophotometry method, using a Videotest image analyzer (Ista-Videotest Ltd., St. Petersburg, Russia) [15]. One hundred cells in both liver lobule zones were analyzed in each rat with the use of the 40 × 0.6 objective. The obtained data were expressed as the mean optical density units per cell.

The data, analyzed by SigmaPlot 9.0 for MS Windows, were given as mean ± standard error of the mean (SEM) (Systat Software Inc., Chicago, IL, USA). Differences between the mean values were detected using Student's t-criterion.

Results

Structure of the liver parenchyma

Histological examination revealed that rats poisoned with CCl_4 for 6 months developed typical liver cirrhosis. The proportion of connective tissue increased by a factor of 4 ($p < 0.001$) as compared with the normal liver, and the lobular structure of the liver was disturbed (Fig. 1a, b). Large leukocyte infiltrates and numerous Kupffer cells could be seen in the parenchyma (Fig. 1c, d).

Biochemical study of glycogen concentration in the normal and the cirrhotic rat liver

Glycogen concentration in the liver of rats fasted for 48 h was an order of magnitude lower than in fed rats (Fig. 2), in both the experimental and the control group. At the same time,

glycogen concentration in the liver of fasted rats was higher in the normal animals than in cirrhotic ones ($p < 0.01$).

Glucose administration stimulated glycogen synthesis in the liver. Glycogen concentration in the normal liver was higher in fed rats than fasting ones as early as after 10 min (94 %, $p < 0.01$). A noticeable increase in the glycogen concentration in the cirrhotic liver was observed only 20 min after glucose administration.

After that, the glycogen concentration continued to grow in the liver of rats from both groups. However, while the glycogen concentration in the cirrhotic liver increased continuously, in the normal liver periods of rapid increase in glycogen concentration alternated with periods of its decrease (Fig. 2). Nevertheless, at all times after glucose administration except 120 min, the glycogen concentration in the normal liver was higher than in the cirrhotic one. At the end of the experiment, the glycogen concentration in the normal and the cirrhotic liver was increased, respectively, 3.5-fold and 7.4-fold in comparison with the glycogen level in fasted rats ($p < 0.001$).

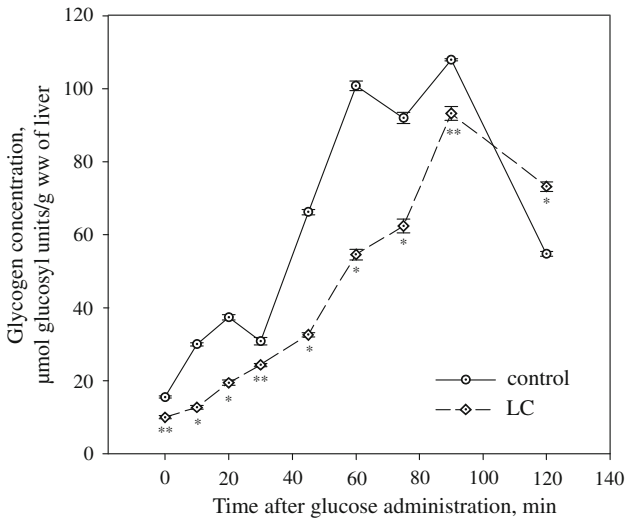


Fig. 2 Glycogen concentration in the normal (control) and cirrhotic (LC) rat liver at different time intervals after glucose administration to fasted animals. $^{*}p < 0.001$, $^{**}p < 0.01$; mean values were significantly different from control

Cytofluorimetric study of glycogen content in hepatocytes of the normal and cirrhotic rat liver

Luminescent microscopic examination of hepatocytes stained by the fluorescent PAS reaction showed that normal hepatocytes had a rather fine structure and distinct contours of the nucleus and cytoplasm (Fig. 3a). On the contrary, hepatocytes in the cirrhotic liver were polymorphic in size as well as shape; many of them were vacuolated, and the contours of the nucleus and the cytoplasm were indistinct (Fig. 3b).

Glycogen content in hepatocytes of the cirrhotic liver after 48 h of fasting was 1.4 times lower than in the hepatocytes of the normal one ($p < 0.01$) (Fig. 4). After administration of glucose, the glycogen content in hepatocytes of the cirrhotic liver remained at the same level for 20 min. On the contrary, the glycogen content in the

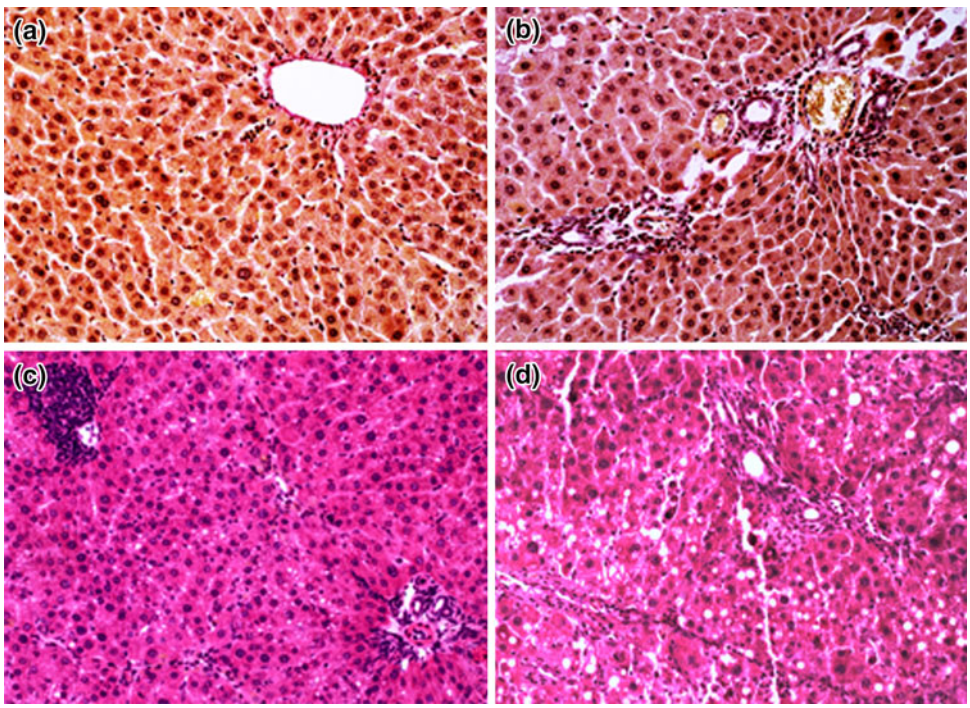


Fig. 1 **a** Normal rat liver samples were stained with VG. No fibrosis (1 × 20). **b** Liver samples from rats with CCl_4-induced cirrhosis were stained with VG. Fibrosis was found (1 × 20). **c**, **d** Liver samples from rats with CCl_4-induced cirrhosis were stained with HE. Inflammatory cell infiltration and steatosis were found (1 × 20)

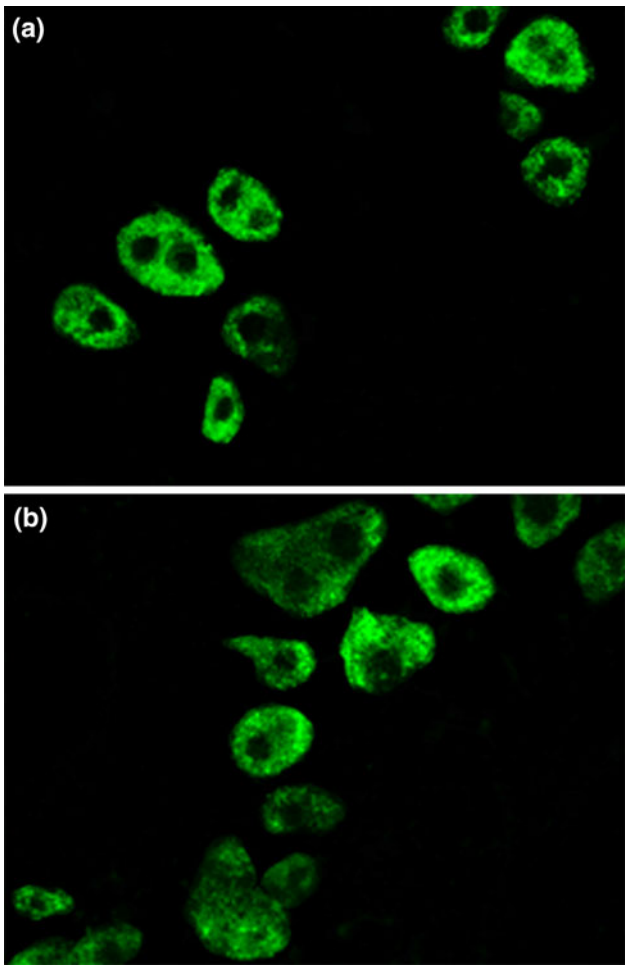


Fig. 3 **a** Hepatocytes from a normal rat were stained by the fluorescent periodic acid-Schiff reaction (1 × 20). **b** Hepatocytes from a rat with CCl_4-induced cirrhosis were stained by the fluorescent periodic acid-Schiff reaction (1 × 20)

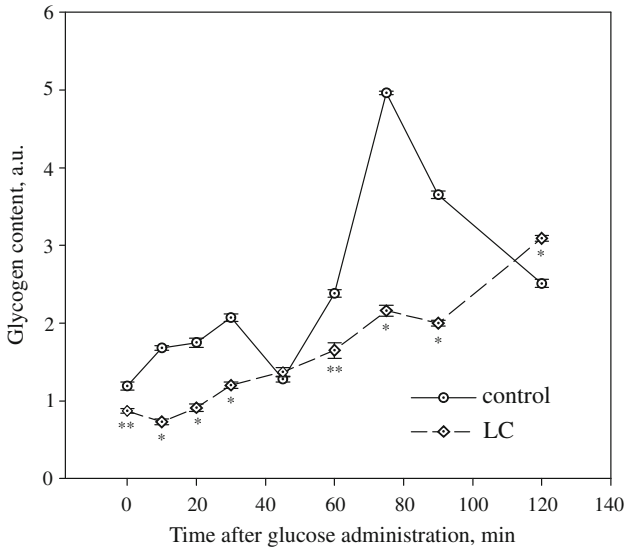


Fig. 4 Glycogen content in hepatocytes of the normal (control) and cirrhotic (LC) rat liver at different time intervals after glucose administration to fasting animals. $^{*}p < 0.001$, $^{**}p < 0.01$; mean values were significantly different from control

hepatocytes of the normal liver was higher ($p < 0.01$) in fed animals than in fasted ones at 10 min after refeeding. At all times of the experiment except 45 and 120 min, the glycogen content in hepatocytes of the normal liver was higher than in the hepatocytes of the cirrhotic one (Fig. 4).

Cytophotometric study of glycogen content in hepatocytes of the portal and central lobule zones of the normal and the cirrhotic rat liver

Analysis of liver sections after PAS reaction showed that all cirrhotic rats had a small number of lobules with an almost normal structure. Glycogen content could be measured in the portal and the central zones of these lobules (Fig. 5).

Glycogen content in hepatocytes of the portal and the central zones of the normal and the cirrhotic rat liver after 48 h of fasting was low (Fig. 5a, b; Table 1). At the same time, it was higher in the cells of the portal zone than in cells of the central zone.

In the normal liver the administration of glucose resulted in a rapid accumulation of glycogen in the cells of both these zones. As a result, after 120 min the hepatocytes of these zones contained approximately three times as much glycogen as hepatocytes of fasted rats (Fig. 5a, c; Table 1).

In the cirrhotic liver glycogen accumulation in the cells of both zones could be discerned only 30 min after glucose administration to fasted animals (Table 1). After that, however, glycogen was accumulating in the hepatocytes of these zones rather intensively, so that at 120 min the cells of the portal and the central zones of the lobule contained 3–3.5 times more glycogen than the cells of the fasting rats ($p < 0.001$).

Discussion

Glycogen content in both the normal and the cirrhotic liver after fasting was at an equally low level, which agrees with the literature data obtained on rats and humans [8, 9].

As shown by different methods, glycogen was accumulated in the liver of the control animals at a high rate. An intensive accumulation of glycogen in the liver after oral administration of similar doses of glucose has been reported before [16]. Whereas in the control rats the glycogen content in the liver increased significantly as early as 10 min after glucose administration, in the cirrhotic rats a 20–30 min delay in glycogen accumulation was recorded (Figs. 2, 4; Table 1). Previously [17], a 40-min lag in the increase of the

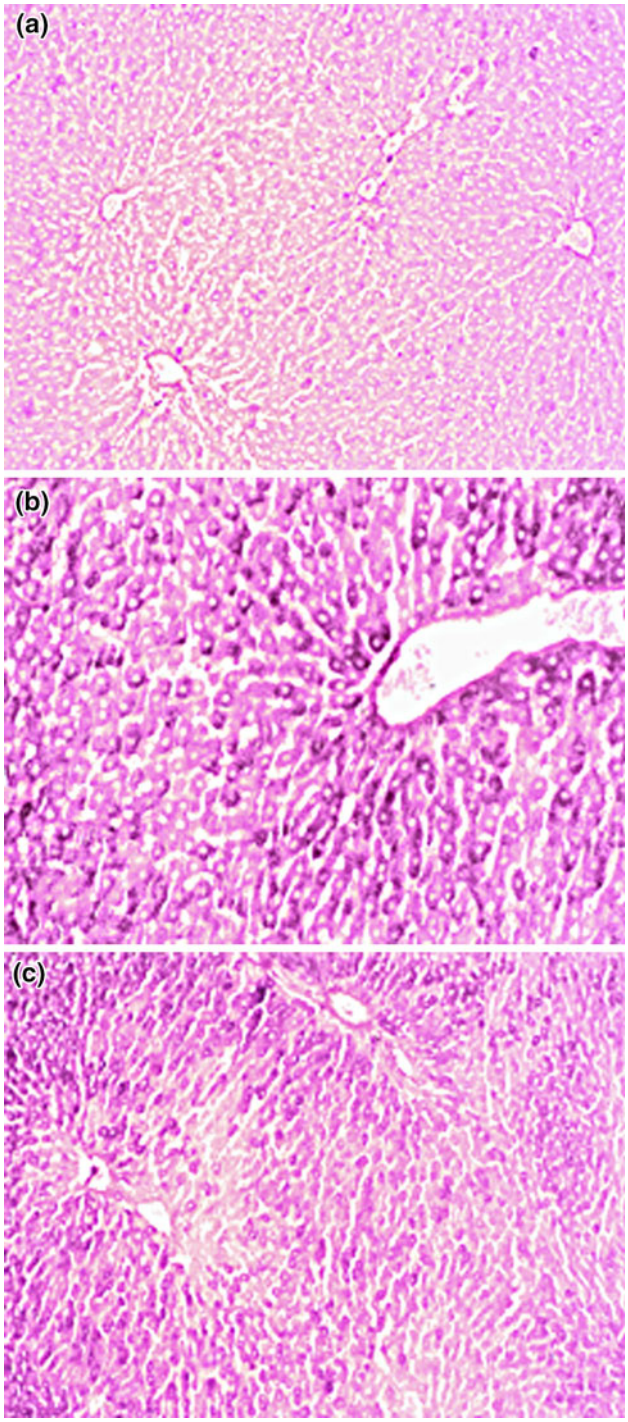


Fig. 5 Glycogen distribution in histological sections of the rat liver shown with the use of PAS reaction. **a** Normal rat liver after 48 hours of fasting (1 × 10). **b** Normal rat liver 120 min after glucose administration (1 × 20). **c** Cirrhotic rat liver after 120 min after glucose administration (the area of an unimpaired lobule) (1 × 10)

glycogen concentration was observed in the liver of the rats with alloxan diabetes after administration of insulin. The authors attributed this lag to the delay in the synthesis of glycogen synthase in the liver of diabetic animals.

According to the data of the biochemical analysis, the intensity of glycogen accumulation in the cirrhotic liver was, on average, 26 % ($p < 0.05$) lower than in the normal one.

As the development of cirrhosis is accompanied by a considerable increase in the proportion of the connective tissue in the liver and, respectively, by the decrease in the proportion of the parenchyma, it is believed that these factors may confound the results of the determination of the glycogen content in this organ [5]. To exclude the possible influence of these factors, we also used quantitative cytochemistry. The results of the cytofluorimetric determination of the glycogen content in the hepatocytes of the normal and the cirrhotic liver confirmed the data obtained with the use of the biochemical method (Fig. 4). Hepatocytes of the cirrhotic liver were shown to accumulate, on average, 21 % ($p < 0.01$) less glycogen than the hepatocytes of the normal one.

In fasted and fed rats glycogen content in the hepatocytes of the portal zone in both the normal and the cirrhotic liver was higher than in the hepatocytes of the central zone of the liver lobule (Table 1). These data confirm our previous results [18].

The available data on the site where glycogen synthesis starts and the order in which it is accumulated are contradictory. It is not entirely clear in which zone of the liver lobule glycogen synthesis starts after feeding of fasted animals [19, 20]. A study of Babcock and Cardell [19] showed that after refeeding glycogen begins to accumulate in the hepatocytes of the central zone of the liver lobule. However, Babcock and Cardell did not measure the glycogen content in the cells, drawing their conclusions from visual observations. They also did not study the early stages of glycogen accumulation in the liver lobule.

We cannot unambiguously answer the question of where exactly the glycogen synthesis starts in the liver. It cannot be ruled out that it begins in the cells of both zones of the liver lobule, although more intensively in the cells of the portal one. The greater intensity of glycogenesis in the portal zone cells (Table 1) may be associated with the fact that in the liver glycogen is mostly produced along an indirect pathway by gluconeogenesis [21], whose rate is considerably higher in cells of the portal zone [22].

Our data demonstrate that glycogen accumulation in the normal liver is a rhythmic process with a period of about 1 h. Such circahoralian rhythms have previously been demonstrated for protein synthesis, enzyme activity and some other processes. They are determined by cell junctions, reflecting the synchronisation of functionally connected cells [23]. In the cirrhotic liver, a distinct lobular structure is almost absent, and cell junctions are impaired. This is possibly the reason why the rhythms of glycogen accumulation in the hepatocytes can no longer be observed.

On the average, according to the data obtained by different methods, at every time interval after glucose

Table 1 Glycogen content (arbitrary units) in hepatocytes of the portal (PZ) and central (CZ) zones of the normal and cirrhotic rat liver lobules at different time intervals after glucose administration to fasting animals (values are means with their standard errors for three rats)

Time after glucose administration (min)									
Lobule zones	0	10	20	30	45	60	75	90	120
Control									
PZ	10.4 ± 0.5	13.4 ± 0.4	16.4 ± 0.4	20.2 ± 0.5	19.1 ± 0.7	28.7 ± 0.5	41.8 ± 0.5	29.3 ± 0.7	30.6 ± 0.7
CZ	8.3 ± 0.4	12.2 ± 0.4	15.8 ± 0.4	18.9 ± 0.5	17.2 ± 0.7	23.4 ± 0.5	35.7 ± 0.7	28.0 ± 0.5	29.7 ± 0.9
Liver cirrhosis									
PZ	11.1 ± 0.4	9.0 ± 0.4*	10.6 ± 0.5*	14.1 ± 0.7#	20.7 ± 0.7	21.4 ± 0.7#	23.4 ± 0.9*	28.5 ± 0.7	33.5 ± 0.7$\surd$
CZ	9.0 ± 0.2	8.9 ± 0.4#	10.4 ± 0.4*	14.4 ± 0.5#	18.5 ± 0.9	20.7 ± 0.9	17.8 ± 0.9*	24.8 ± 0.7$\surd$	32.6 ± 0.5$\surd$

* $p < 0.001$; # $p < 0.01$, $\surd$ $p < 0.05$ compared with control group

administration, the normal liver accumulated ~22 % more glycogen than the cirrhotic one. Glycogen content in the liver is known to be determined by the ratio of synthesis (S) and degradation (D) rates. Regardless of the absolute S and D rates, when S > D, glycogen is accumulating; when S < D, glycogen is being depleted; when S = D, the glycogen content is unchanged. If, following Schneiter [7], one accepts that glycogen synthesis in the cirrhotic liver does not differ from that in the normal one, the reduced rate of glycogen accumulation in the cirrhotic liver should be explained by a more intensive glycogen degradation. However, this is not what we observed.

The available data indicate that the rate of glycogenolysis in the cirrhotic liver is considerably lower than in the normal one [9, 24]. Lower glycogen degradation in the cirrhotic liver as compared to the normal one can also be inferred from the literature data on weak glycogen phosphorylase activity [13, 25].

Glycogen degradation appears to be decreased to a greater extent than glycogen synthesis. It is this decrease in glycogen degradation that results in a slow but sure accumulation of glycogen in the cirrhotic liver, that is, control S/D > cirrhotic S/D > 1.

Thus, the glycogen accumulation in the cirrhotic liver after oral administration of glucose to fasted rats starts after a 20–30-min delay and proceeds at a lower rate than in the normal liver. It is possible that the reduced rate of glycogen accumulation is attributed to a lower rate of the glycogen degradation in the pathologically changed organ. As glycogen levels in the liver are closely associated with the organism's capacity to respond quickly and adequately to external factors, decreased rates of glycogen degradation in cirrhotic patients may indicate their lower resistance to harmful impact.

Acknowledgements The work was supported by the Russian Foundation for Basic Research (RFBR no. 08-04-00971).

Conflict of interest Natalia N. Bezborodkina, Sergey V. Okovity, Anna Y. Chestnova and Boris N. Kudryavtsev declare that they have no conflict of interest.

Compliance with ethical requirements The experiments were performed in accordance with the "Principles of laboratory animal care" (http://grants1.nih.gov/grants/olaw/references/phspol.htm) and national guidelines for the care and use of laboratory animals "On the statement of rules of laboratory practice" (http://www.zdrav.spb.ru/official_documents/MZ/list_mz2003.htm) were followed.

References

1. Petrides AS, DeFronzo RA. Glucose metabolism in cirrhosis: a review with some perspectives for the future. Diabetes Metab Rev 1989;5:691–709
2. Nielsen MF, Caumo A, Aagaard NK, Chandramouli V, Schumann WC, Landau BR, et al. Contribution of defects in glucose uptake to carbohydrate intolerance in LC: assessment during physiological glucose and insulin concentrations. Am J Physiol Gastrointest Liver Physiol. 2005;288:G1135–G1143
3. Kruszynska YT, Meyer-Alber A, Darakhshan F, Home PD, McIntyre N. Metabolic handling of orally administered glucose in cirrhosis. J Clin Invest 1993;91:1057–1066
4. Hickman IJ, Macdonald GA. Impact of diabetes on the severity of liver disease. Am J Med 2007;120:829–834
5. Kruszynska YT. Carbohydrate Metabolism: Oxford Textbook of Clinical Hepatology. Oxford: Oxford University Press; 1999. 257–286
6. Postic C, Dentin R, Girard J. Role of the liver in the control of carbohydrate and lipid homeostasis. Deabetes Metab 2004;30:398–408
7. Schneiter P, Gillet M, Chiolero R, Jequier E, Tappy L. Hepatic nonoxidative disposal of an oral glucose meal in patients with LC. Metabolism 1999;48:1260–1266
8. Giardina MG, Matarazzo M, Sacca L. Kinetic analysis of glycogen synthase and PDC in cirrhotic rat liver and skeletal muscle. Am J Physiol 1994;267:E900–E906
9. Petersen KF, Krssak M, Navarro V, Chandramouli V, Hundal R, Schumann WC, et al. Contributions of net hepatic glycogenolysis and gluconeogenesis to glucose production in cirrhosis. Am J Physiol 1999;276:E529–E535
10. Pearse AG. Cytology and cytochemistry of adenomas of the human hypophysis. Acta Unio Int Contra Cancrum 1962;18:302–304

11. Weibel ER, Staubli W, Gnagi HR. Correlated morphometric and biochemical studies on the cell. I. Morphometric model, stereologic methods and normal morphometric data for rat liver. J Cell Biol 1969;42:68–91
12. Lo S, Russel JC, Taylor AW. Determination of glycogen in small tissue samples. J Appl Physiol 1970;28:234–236
13. Kudryavtseva MV, Bezborodkina NN, Kudryavtsev BN. Restoration of the glycogen-forming function of hepatocytes in rats with LC is facilitated by a high-carbohydrate diet. Brit J Nutr 1999;81:473–480
14. Deitch AD. Microspectrophotometric study of the binding of the anionic dye Naphthol Yellow S, by tissue sections and by purified proteins. Lab Invest 1955;4:324–351
15. Kudryavtseva MV, Bezborodkina NN, Radchenko VG, Okovity SV, Kudryavtsev BN. Metabolic heterogeneity of glycogen in hepatocytes of patients with LC: the glycogen content of the liver lobule zones in cirrhosis. Eur J Gastroenterol Hepatol 2001;13:693–697
16. Ercan N, Gannon MC, Nuttall FQ. Liver glycogen synthase, phosphorylase, and the glycogen concentration in rats given a glucose load orally: a 24-hour study. Archiv Biochem Biophys 1994;315:35–40
17. Steiner DF, King J. Induced synthesis of hepatic uridine diphosphate glucose-glycogen glucosyltransferase after administration of insulin to alloxan-diabetic rats. J Biol Chem 1964;239:1292–1299
18. Kudryavtseva MV, Bezborodkina NN, Okovity SV, Kudryavtsev BN. Effects of the 2-ethylthiobenzimidazole hydrobromide (bemithyl) on carbohydrate metabolism in cirrhotic rat liver. Exp Toxic Pathol 2003;54:339–347
19. Babcock MB, Cardell RR. Hepatic glycogen patterns in fasted and fed rats. Am J Anat 1974;140:299–338
20. Chen KS, Katz J. Zonation of glycogen and glucose synthesis, but not glycolysis in rat liver. Biochem J 1988;255:99–104
21. Katz J, Kuwajima M, Foster DW, McGarry JD. The glucose paradox: new perspectives on hepatic carbohydrate metabolism. Trends Biochem Sci 1986;11:136–140
22. Gebhard R. Metabolic zonation of the liver: regulation and implications for liver function. Pharmacol Ther 1992;53:275–354
23. Brodsky VYa. Direct cell–cell communication: a new approach derived from recent data on the nature and self-organisation of ultradian (circahoralian) intracellular rhythms. Biol Rev 2006;81:143–162
24. Krahenbuhl S, Reichen J. Decreased hepatic glucose production in rats with carbon tetrachloride-induced cirrhosis. J Hepatol 1993;19:64–70
25. Krahenbuhl L, Talos C, Reichen J, Krahenbuhl S. Progressive decrease in tissue glycogen content in rats with long-term cholestasis. Hepatology 1996;24:902–907