Restoration of the glycogen-forming function of hepatocytes in rats with liver cirrhosis is facilitated by a high-carbohydrate diet

Margarita V. Kudryavtseva*, Natalia N. Besborodkina and Boris N. Kudryavtsev
Institute of Cytology, Russian Academy of Sciences, St Petersburg, Russia

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Using cytofluorimetric and biochemical methods, the content of glycogen and its labile and stable fractions, as well as activities of glucose-6-phosphatase (EC 3.1.3.9), glycogen phosphorylase (EC 2.4.1.1) and glycogen synthase (EC 2.4.1.11) were determined in the rat liver for 6 months after chronic poisoning of the animals with CCl₄ and then at 1, 3, and 6 months after the end of the poisoning. One group of rats was given a standard diet, the other, a high-carbohydrate diet. The 6-month long chronic intoxication with CCl₄ was shown to produce development of typical liver cirrhosis characterized by a 2×8-fold increase in the total glycogen content in hepatocytes as compared with normal cells, by a fall in the glycogen labile fraction (from 85 to 53 % of the total glycogen) as well as by decreases in the activities of glycogen phosphorylase and glucose-6-phosphatase by 25 and 82 % respectively. The structural rehabilitation occurred faster and more completely at the cellular level than at the tissue level. Functional variables of the cirrhotic liver tissue also recovered, after cessation of poisoning, faster and more completely than the liver structure at the tissue level: glycogen levels in hepatocytes fell dramatically, the labile : stable glycogen fraction ratio recovered completely, and the activity of glycogen phosphorylase rose to the level characteristic of the normal liver. Use of the high-carbohydrate diet promoted a somewhat faster and more complete recovery of hepatic structure and function.

Liver cirrhosis: Glycogen: High-carbohydrate diet

Liver cirrhosis is a most common and severe human pathology. It causes different disturbances in various liver functions and specifically the glycogen-forming function. To achieve rehabilitation of numerous impaired functions of the cirrhotic liver is a task of great practical importance. There are encouraging data in the literature about the reversibility of cirrhotic changes in the hepatic parenchyma (Solopaev, 1985; Surkisov, 1987). One of the approaches in therapy of liver cirrhosis is to give a special diet with a high carbohydrate content. Such a diet has been shown to increase resistance of hepatocytes to the effects of damaging factors (Jennische, 1983; Frederiks et al. 1985) and to promote the functional efficiency of the liver (Hinson et al. 1983; Kabadi et al. 1985), whereas fasting, in contrast, is known to enhance impairment of this organ (Diaz-Gomes et al. 1975; Pushpendran et al. 1977). Meanwhile, liver cirrhosis is accompanied by a progressive starvation (Anthonius et al. 1991) which is probably due to impairment of glucose utilization and activation of energy metabolism (Proietto et al. 1980). In this connection, the use of glucose is highly recommended in the treatment of liver cirrhosis in clinical practice (Kabadi et al. 1985). The liver plays a major role in the regulation of blood glucose levels because it is capable of synthesizing and degrading glycogen in its parenchymal cells, i.e. owing to its glycogen-forming functions.

The aim of the present work was to study the recovery of glycogen-forming function of rat hepatocytes following chemically-induced damage. We determined levels of glycogen and its fractions in hepatocytes, and the activities of some enzymes of glycogen metabolism, in the cirrhotic rat liver at various time intervals after termination of CCl₄ poisoning and addition to the food of a glucose–sucrose mixture.

Materials and methods

Animals, biopsies, and diets

The study was performed on adult white breedless male rats weighing initially 120–150 g. The liver cirrhosis in the animals was produced by inhalation of the hepatotropic poison CCl₄. The animals were poisoned for 6 months in a special closed chamber of 40 litres volume three times weekly.

Abbreviations: HC, high-carbohydrate diet; PAS, periodic acid–Schiff; SD, standard diet.

* Corresponding author: Dr Margarita Kudryavtseva, fax +7 812 247 0341, email cellpath@link.cytspb.rssi.ru
for 15 min (the concentration of CCl₄ was 0·05 g/l; Kudrya-
tseva et al. 1994). The degree of cirrhosis development was
evaluated by histological examination of haematoxylin–
eosin- and van Gieson-stained liver sections.

Under light diethyl ether anaesthesia, always at 10.00–
12.00 hours, punctate needle biopsies of the liver were
obtained from each animal several times: before poisoning,
at the time of cirrhosis development (6 months after the
start of the poisoning with CCl₄) then 1, 3, and 6 months after the
start of recovery (on termination of the 6-month-long
poisoning). On termination of the poisoning, one group of
rats was kept throughout the recovery period on a standard
diet (SD group); a second group received, in addition to the
standard diet, a high-carbohydrate mixture: 50 g/l glucose–
150 g/l sucrose solutions (1:1, v/v) (HC group). As a
control, a third group of rats of the same age was used,
with no poisoning. Each group comprised five rats.

The liver tissue material from each animal was used, (1)
for sections to be examined histologically, (2) for smears
of isolated hepatocytes on object glasses by our own method
(Kudryavtseva et al. 1983) for quantitative cytochemistry,
(3) for microchemical determinations of activities of some
enzymes of glycogen metabolism.

**Cytofluorimetry of total glycogen and its fractions**

The contents of total glycogen and its labile fraction and
stable fraction in hepatocytes were measured cytofluorime-
trically in the smears stained with a fluorescent periodic
acid–Schiff (PAS)-reaction. The smears of isolated hepatocytes were obtained from the supravital punctate biopsy of the liver, using two phosphate buffers: (1) pH 8·0 and (2) pH
7·4 into which the punctate pieces were placed consecutively for 10 and 15 min respectively. We developed a
method of detecting glycogen in hepatocytes, using the
fluorescent dye auramine OO-SO₂ (Schiff-type reagent),
as a modification of the routine PAS-reaction. The fluo-
rescent PAS-reaction was performed as follows: the prepara-
tions, smears of isolated hepatocytes, were oxidized for
90 min in KIO₄ solution (8 g/l) 0·01 M-HNO₃ at pH 2·2.
After rinsing in tap and distilled waters, the preparations
were placed into a SO₂-saturated (0·2 ml thionyl chloride)
4·5 g/l auramine solution for 40 min (for determination of the labile glycogen fraction) or for 90 min (for total glyco-
gen); the stable glycogen fraction was calculated as the
difference between these two glycogen fractions. The prepara-
tions were dehydrated using the standard procedure:
SO₂-waters, tap water, alcohols of increasing concentra-
tions. Specificity of the fluorescent staining for glycogen
was confirmed by a control treatment of the preparations
with α-amylase (EC 3.2.1.1) (Sigma, St Louis, MO, USA)
by the standard procedure (Rosa & Johnson, 1967): 60 min,
at 37°, with a 10 g/l solution of the enzyme.

Several controls confirmed a substantial specificity of this
reagent. Using auramine OO-SO₂, the microfluorimetric
method was shown to be suitable for the quantitative
determination of glycogen in hepatocytes. The data
obtained agreed well with the results of glycogen mea-
surements by routine absorptiional cytophotometry, using
the standard Schiff reagent. Effects of the duration of periodeate oxidation and of staining with auramine-SO₂ on
the intensity of the fluorescent PAS-reaction were studied.
As a result, the optimal time for oxidation and staining was
established. Thus, the fluorescent PAS-reaction was shown
to be highly specific and to depend on glycogen alone, and
the possibility was confirmed of revealing and measuring
quantitatively the amounts of total glycogen and its labile
stable fractions in individual cells (Kudryavtseva et al.
1974).

The measurements were performed in isolated hepatop-
cytes, using a cytofluorimeter designed at our laboratory
In each preparation, 500–600 cells were measured.

**Microchemical determination of enzyme activities**

The enzyme activities in the rat liver biopsies were deter-
mined using standard methods specially modified for small
amounts of the material (10–30 mg). The biopsy pieces
were homogenized at 2–4°C in the diluted buffer (1:6, v/v)
that contained (mmol/l): 50 Tris-HCl (pH 7·4), 250 sucrose,
60 NaF, 5 EDTA. The homogenate was centrifuged for
10 min at 12 000 g, and the supernatant fraction was used for
the spectrophotometric determination of the enzyme activi-
ties. Activities of glucose-6-phosphatase (EC 3.1.3.9) and
glycogen phosphorylase (EC 2.4.1.1) were determined by
the methods of Swanson (1950) and Hers (1964) respec-
tively, by measuring changes in the content of inorganic
phosphate formed during the course of the reaction (Nordlie,
1979). The activity of glycogen synthase (EC 2.4.1.11) was
measured in a coupled system of pyruvate kinase (EC
2.7.1.40) and lactate dehydrogenase (EC 1.1.1.2) (Barber
et al. 1967) using a Specoll 11 spectrophotometer (Carl
Zeiss Jena GmbH, Jena, Germany). In all cases, the enzyme
activity depended on the protein content in the sample and
was proportional to the incubation time. The protein content
was determined by the method of Lowry et al. (1951). The
protein content calculations were made using a calibration
curve. Bovine serum albumin was used as the standard.

**Microscopy of liver sections**

Morphological examination and photography of the liver
sections stained with haematoxylin–eosin and van Gieson
stains were performed under an Amplival microscope (Carl
Zeiss Jena GmbH) with a 40× objective lens and 4× eye-
piece. The examination and photography of hepatocytes
stained with the fluorescent PAS-reaction for glycogen were
done using a LUMAM-1 luminescent microscope (LOMO,
St Petersburg, Russia) with objective lenses 90× and 20×.

All the data obtained were treated statistically using
Student’s t test, and when appropriate, the χ² criterion,
using Statgraphics 5·0 (Manugistics Inc., Rockville, MD,
USA) and SigmaPlot for Windows 1·0 (Jandel Corporation,
Jandel Scientific GmbH, Erkrath, Germany).

**Results**

**Structure of the liver parenchyma**

Morphological analysis of the liver showed (Fig. 1(a)) that in
control animals the hepatic parenchyma was characterized by
a distinct radial distribution of hepatocyte cords around
central veins, by thin walls of the portal tract vessels, and by other features typical of the normal liver. In cirrhosis, the lobular liver structure disappeared (Fig. 1(b, c)), the connective tissue bands grew into the liver parenchyma and divided it into the pseudolobules whose centres often failed to contain the central vein lumen. In the parenchyma, large leucocyte infiltrates and numerous Kupffer cells appeared, which is characteristic of this pathology. In 3–6 months (depending on individual peculiarities of the animals) after termination of the CCl₄ poisoning, the connective tissue bands that disturbed the normal lobular structure became thinner (Fig. 1(d, e, f)). The leucocyte infiltrates disappeared,
and the number of Kupffer cells decreased, which indicated a tendency for recovery of the normal liver structure.

Examination of hepatocytes by luminescent microscopy, on performance of the fluorescent PAS-reaction, showed that whereas normal hepatocytes were characterized by a fine structure and distinct contours of the nucleus and cytoplasm (Figs. 2(a), 3(a)), in cirrhosis, a pronounced polymorphism of the cells was revealed with respect to both their size and shape and to their structure (Figs. 2(b), 3(b)). In the cirrhotic liver, apart from the hepatocytes rather close morphologically to normal cells, there were quite a few cells with a blurred clumped cytoplasm and indistinct contours of the nucleus and cytoplasm. On termination of the poisoning, in rats of the HC group, the structure of hepatocytes had improved by as early as 1 month and had become close to normal in 6 months (Fig. 3(c)). In animals of the SD group, these changes in the hepatocyte structure tended to be similar, but they were less pronounced. Thus, on termination of the hepatotoxic effect, changes in the liver structure at the cytological level were characterized by a more substantial tendency for normalization than changes at the tissue level.

**Glycogen content**

In control rats that had not undergone poisoning or surgery, the amounts of total glycogen and its labile and stable fractions per cell remained practically constant throughout the entire duration of the experiment.

As seen from Table 1, the total glycogen content in hepatocytes from cirrhotic liver was 2.8-fold greater than in normal hepatocytes. With regard to the glycogen fraction composition, the liver cirrhosis was characterized by a significant reduction (down to 53 %) of the labile fraction and a corresponding rise in the stable fraction in hepatocytes (Table 1).

After the end of the hepatotropic poisoning period, the total glycogen content in hepatocytes was decreasing, the reduction being in 1 month approximately the same in the SD and HC groups of rats: by 1.92 and 1.86 times respectively. However, the subsequent changes in cell glycogen level were somewhat different in these two groups of animals. The glycogen concentration continued to decrease slowly in the SD group, while it remained stable in the HC group (Table 1, Fig. 4(a, b)). As a result, 6 months after the termination of the CCl4 poisoning, the total glycogen content in hepatocytes of the SD group reached the level characteristic of the normal liver, whereas in rats of the HC group it exceeded the normal level by about 35 %. At all

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**Table 1. Content of total glycogen (TG), its labile fraction (LF) and its stable fraction (SF) (arbitrary units) in the livers of rats before poisoning, during liver cirrhosis caused by 6 months treatment with carbon tetrachloride, and at various time intervals during rehabilitation on a standard diet (SD) or a high-carbohydrate diet (HC)†.**

(Values are means with their standard errors for five rats)

<table>
<thead>
<tr>
<th>Form of glycogen</th>
<th>Before poisoning</th>
<th>Liver cirrhosis</th>
<th>Rehabilitation strategy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean SE</td>
<td>Mean SE</td>
<td>Mean SE</td>
</tr>
<tr>
<td>TG</td>
<td>37.6 1.1</td>
<td>82.1†††</td>
<td>1.7</td>
</tr>
<tr>
<td>LF</td>
<td>36.8 1.0</td>
<td>82.1†††</td>
<td>1.7</td>
</tr>
<tr>
<td>SF</td>
<td>30.9 1.0</td>
<td>53.2†††</td>
<td>1.4</td>
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<tr>
<td></td>
<td>5.9 0.2</td>
<td>28.9†††</td>
<td>0.4</td>
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<tr>
<td>Length of rehabilitation (months)</td>
<td></td>
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</tr>
<tr>
<td>1</td>
<td>Mean SE</td>
<td>Mean SE</td>
<td>Mean SE</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG</td>
<td>42.7†††</td>
<td>1.2</td>
<td>33.7†††</td>
</tr>
<tr>
<td>LF</td>
<td>44.0***†††</td>
<td>0.8</td>
<td>45.0***†††</td>
</tr>
<tr>
<td>SF</td>
<td>39.3***†††</td>
<td>0.8</td>
<td>40.6***†††</td>
</tr>
<tr>
<td></td>
<td>9.7†††</td>
<td>0.7</td>
<td>6.6†††</td>
</tr>
<tr>
<td></td>
<td>4.7***†††</td>
<td>0.4</td>
<td>4.4***†††</td>
</tr>
</tbody>
</table>

Mean values were significantly different from those for the SD group: * P < 0.05, ** P < 0.01, *** P < 0.001.

Mean values were significantly different from the corresponding values before poisoning: † P < 0.05, †† P < 0.01, ††† P < 0.001.

†For details of procedures, see pp. 473–474.
stages of the experiment, differences in the hepatic glycogen content between individual animals were less than 20%.

Unlike the total glycogen content the fractional composition returned to normal, after the termination of the CCl\textsubscript{4} poisoning, much faster in the HC group than in the SD group (Table 1). The percentage labile fraction in hepatocytes of the HC group reached normal values as early as 1 month after the end of poisoning, whereas in hepatocytes of the SD group it remained somewhat lower than normal even after 6 months of rehabilitation (Table 1).

Table 2. Activities of glucose-6-phosphatase (G6P), glycogen phosphorylase (GP) and glycogen synthase (GS) (nmol/min per mg protein) in the livers of rats before poisoning, during liver cirrhosis caused by 6 months treatment with carbon tetrachloride, and at various time intervals during rehabilitation on a standard diet (SD) or a high-carbohydrate diet (HC)‡

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Before poisoning</th>
<th>Liver cirrhosis</th>
<th>Rehabilitation strategy</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>Mean</td>
<td>SE</td>
<td>Mean</td>
</tr>
<tr>
<td>G6P</td>
<td>79.2</td>
<td>0.7</td>
<td>182</td>
</tr>
<tr>
<td>GP: a form</td>
<td>169</td>
<td>0.7</td>
<td>463</td>
</tr>
<tr>
<td>total</td>
<td>228</td>
<td>0.8</td>
<td>126.8</td>
</tr>
<tr>
<td>a form</td>
<td>169</td>
<td>0.7</td>
<td>463</td>
</tr>
<tr>
<td>total</td>
<td>228</td>
<td>0.8</td>
<td>126.8</td>
</tr>
<tr>
<td>GS</td>
<td>0.50</td>
<td>0.02</td>
<td>0.50</td>
</tr>
</tbody>
</table>

Mean values were significantly different from those for the SD group: *P < 0.05, **P < 0.01, ***P < 0.001.

Mean values were significantly different from the corresponding values before poisoning: †P < 0.05, ††P < 0.01, †††P < 0.001.

‡ For details of procedures, see pp. 473–474.

Fig. 3. Hepatocytes from (a) a normal rat; (b) a rat with liver cirrhosis induced by chronic poisoning with carbon tetrachloride; and (c, d) rats with induced liver cirrhosis after rehabilitation for 1 and 6 months respectively on a high-carbohydrate diet. Hepatocytes were stained by the fluorescent periodic acid–Schiff reaction. Objective 90×, eye piece 3×.
Enzyme activities

As seen from Table 2, activities of glycogen phosphorylase and glucose-6-phosphatase fell after the 6-month-long CCl₄ poisoning, to 75 and 18% respectively of the normal levels of these enzymes and returned to normal somewhat faster in rats of the HC group.

Discussion

Liver cirrhosis, apart from producing essential changes in the hepatic structure, is also known to cause disturbances in various liver functions (Marchesini et al. 1984; Murata et al. 1985). Specifically, impairment of the glycogen and glucose metabolism in the cirrhotic liver has been shown to accompany the morphological alterations (Owen et al. 1981; Marchesini et al. 1982; Petrides & de Fronzo, 1989).

Literature data on carbohydrate metabolism in the cirrhotic liver are not numerous and are rather controversial. Most authors report glucose intolerance and hyperinsulinaemia in liver cirrhosis (Pelkonen et al. 1981; Harewood et al. 1982; Keller et al. 1982; Riggio et al. 1982; Fernandez et al. 1983a,b). In addition, some studies suggest that production of glucose by the cirrhotic liver is impaired (Owen et al. 1981). At the same time, the blood glucose level in patients with chronic liver diseases is usually within normal levels or even somewhat higher (Kruszynska & McIntyre, 1991). This is partly explained by a substantial reserve capability of the liver for glucose production. As little as 20% of the entire functional mass of hepatocytes is believed to be necessary to maintain the glucose production by the liver in normal circumstances (Kruszynska & McIntyre, 1991).

We have shown previously that the total glycogen content of hepatocytes in the cirrhotic liver increases three or four times compared with the norm (Kudryavtseva et al. 1992, 1994, 1996). There were also changes in the fractional composition of glycogen, especially with respect to the stable fraction, whereas the labile fraction changed mostly in resynthesis of the glycogen and glycogenolysis under different physiological conditions. The stable fraction changed markedly under pathological conditions and appeared to be an indicator of severe pathological alterations in the liver.

Information on activities of enzymes of carbohydrate metabolism in the cirrhotic liver is also rather scanty. Previously we have shown a decrease in activities of the key enzymes of carbohydrate metabolism, glycogen phosphorylase and glucose-6-phosphatase, in the cirrhotic liver, while the activity of glycogen synthase remained within normal limits (Kudryavtseva et al. 1992, 1994, 1996). Determination of glycogen synthase in the present study revealed an astonishing stability of activity throughout the experiment (Table 2), in spite of tremendous structural and functional changes that occurred in the cirrhotic liver.

In the course of post-poisoning rehabilitation, the activity of glycogen phosphorylase in rats of the SD and HC groups gradually reached the level characteristic of that in the normal liver (Table 2). It is to be noted that the activities of both total glycogen phosphorylase and its active form (glycogen phosphorylase a) returned to normal somewhat faster in rats of the HC group. At the same time, the proportion of phosphorylase a throughout the rehabilitation remained higher than in the normal liver in animals of both the SD and HC groups.

Unlike glycogen phosphorylase, the activity of hepatic glucose-6-phosphatase in rats of both groups, although it rose severalfold as compared with the initial cirrhotic level, still did not reach the normal level even as long as 6 months.
after the end of the CCl₄ poisoning (Table 2). The glucose-6-phosphatase activity in both groups increased by approximately 3.5 times as early as 1 month after the termination of poisoning (Table 2). Subsequently, activity of this enzyme in the HC group remained at the same level, whereas in rats of the SD group it fell again within 6 months down to the level that was about half the normal level (Table 2).

Thus, after a period of hepatotoxicity, the use of a food with a high carbohydrate content promotes a somewhat faster and more complete recovery of the structure and functions of the liver.

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