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ANIMAL AND IN VITRO MODELS IN HUMAN DISEASES

Morphological signs of cirrhosis regression Experimental observations on carbon tetrachloride-induced liver cirrhosis of rats

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Abstract

Regression of hepatic cirrhosis is a controversial issue. Recently, a list of histopathological features, observed in human material, was suggested as a hallmark of cirrhosis in the process of regression. An investigation for the presence of these morphologic features was performed at monthly intervals in rats with proved carbon tetrachloride (CCl_4)-induced cirrhosis over a period of 9 months following discontinuation of treatment, using sequential liver biopsies. Within the first 4 months, features of the ''hepatic repair complex'' were identified, together with the enlargement of the hepatic nodules and thinning of the fibrous septa. Subsequent to the 4 months, the histological picture, composed of large and inconspicuous nodules and delimited by thin and frequently incomplete fibrous septa ''incomplete septal cirrhosis'', appeared to be stabilized. These fibrous septa, when injected with India ink from the portal trunk, presented blood vessels that were seen to drain directly into the sinusoids. These findings suggested that when the cause of cirrhosis is removed, the liver may adapt itself to a new and permanent structure, probably compatible with normal or near-normal function, which may render hepatic cirrhosis clinically, although not morphologically, reversible.

Keywords: Hepatic fibrosis; Cirrhosis; Cirrhosis reversibility

Introduction

Despite the concept that fibrous connective tissue has a dynamic character, forming and receding under different physiological and pathological conditions [2,13,17], the possibility that extensive cicatricial fibrosis can be reversible is only recently being accepted. Hepatosplenic schistosomiasis was one of the first human diseases where extensive hepatic fibrosis was unequivocally demonstrated to be susceptible to degradation and resorption. Epidemiological [7,12], clinical [6], ultrasonographical [15], and pathological [1] studies have revealed progressive disappearance of portal fibrosis, while the clinical manifestations of portal hypertension (esophageal varices and splenomegaly) subsided following specific treatment of Manson's schistosomiasis. Recently, the subject of hepatic cirrhosis reversibility has come to the foreground [3,9,10,14]. Although cirrhosis means much more than simple fibrosis, evidence of fibrosis resorption has been described side by side with other morphologic hepatic changes. Wanless et al. [21] listed a series of histological findings, collectively named the ''hepatic repair

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complex'', which suggested to represent parameters of cirrhosis in regression. These findings are as follows: delicate perforated septa, isolated thick collagen fibers, delicate periportal fibrous spikes, portal tract remnants, hepatic vein remnants with prolapsed hepatocytes, hepatocytes within portal tracts or splitting septa, minute regenerative nodules, and aberrant parenchymal veins. In addition, it was suggested that many examples of so-called ''incomplete septal cirrhosis'' might represent cirrhosis in the process of regression. Since the putative morphological indicators of cirrhosis regression, described by Wanless et al. [21], could not be adequately followed to the end of the process due to the limitations associated with studies of human material, it appeared that the suggestion presented could be easily tested experimentally. Hence, the carbon tetrachloride (CCl_4)-induced cirrhosis of the rat model seemed adequate, since there are indications that it can be reversed upon discontinuation of CCl_4 treatment [11,17]. The present study was planned to find out whether the changes collectively called the ''hepatic repair complex'' are present in CCl_4-induced rat cirrhosis following discontinuation of treatment and, if so, whether they indeed mean that cirrhosis will regress.

Material and methods

Animals and treatment – Twenty-five young adult Wistar rats of both sexes, weighing 150–200 g, were kept in individual metal cages, with free access to a commercial balanced diet (Nuvital, Curitiba, PR, Brazil) and water. All the animals were treated on the same day with an 8% solution of CCl_4 in mineral oil administered by gavage. Treatment continued twice per week for a total of 16 weeks. The procedure described by Rosa et al. [20], which avoids high CCl_4-associated mortality, was used. It consisted of the administration of an initial dose of 0.04 ml CCl_4 to each animal. Whenever weight loss (5% or more) from previously recorded body weight occurred, the dose was decreased by about one-half. In cases where the animal gained weight (5% or more), the amount of CCl_4 was doubled. Animals were weighed prior to administration of CCl_4. This procedure resulted in very low mortality, (only 2 out of 25 animals), and a high proportion of fully developed cirrhosis (16 out of 23 animals completing the treatment developed cirrhosis, as revealed by the granularity of the liver surface and by histopathology). The animals that did not develop cirrhosis were discarded.

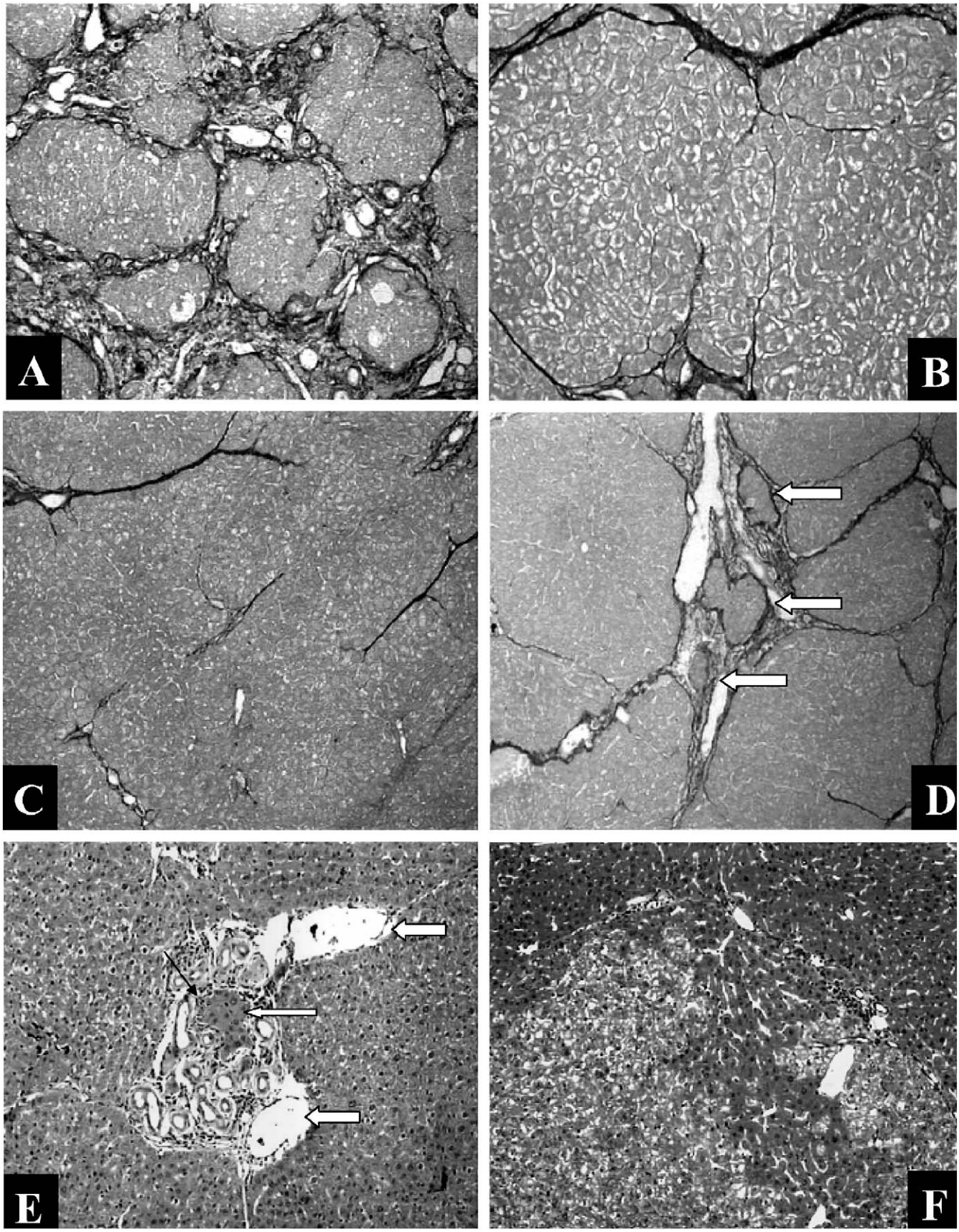
Specimens collected – Two days after the last CCl_4 administration, all the animals were subjected to a liver biopsy. Under sterile conditions and sodium pentobarbital general anesthesia, the skin of the abdomen was shaved and cleaned. A small incision was made at the abdominal middle line and through it part of a liver lobe was exposed, tied, and removed. The majority of the animals presented a small amount of ascitic fluid within the abdominal cavity at the time of the first liver biopsy, but this was not observed during subsequent biopsies. The wound was closed with separate sutures. Subsequently, at monthly intervals and up to 9 months, the liver biopsy was repeated for all the animals, except for two that were randomly selected and killed. Serum samples were collected from blood taken after severing the brachial plexus during necropsy.

Eventually, at the time of sacrifice, the liver was injected through the main portal vein with a mixture of 33% India ink in 10% gelatin. The injection was made with a 20 ml syringe, continuously and carefully until a uniform blackening of the liver surface occurred. The whole organ was placed in 10% formalin for at least 2 days and then sectioned and embedded in paraffin. Sections varying in thickness from 5 to 20 μm were stained with diluted hematoxylin. The livers from normal rats were also similarly injected and examined.

Histology – Pieces of the liver collected at biopsy and autopsy were fixed in 10% buffered formalin (pH 7.2) and embedded in paraffin. Sections were stained with hematoxylin and eosin, the Picro–Sirius method for collagen, PAS–Schiff with and without previous diastase treatment, and the Perl's method for iron.

Immunofluorescence studies – Pieces of fresh liver tissue were immersed in Tissue-Tek (Sakura Finetechnical Co., Tokyo, Japan) and instantly frozen in liquid nitrogen. Blocks were stored at −70 °C in a freezer and cut in a cryostat at −20 °C. Sections were submitted to immunofluorescence for revealing collagens (types I, III and IV), fibronectin and laminin (Institute Merrieux, Lyon, France). These antibodies were raised in rabbits. Primary antisera were diluted 1:40 through 1:100 in PBS. The secondary antibody was a fluoresceinated anti-rabbit IgG (Sigma, St. Louis, MO, USA), diluted 1:50 in a weak solution of Evans blue. Control sections were incubated with saline only or with serum from a normal rat.

Fig. 1. (A) Hepatic nodules and fibrous septa (cirrhosis) soon (2nd day) after discontinuation of carbon tetrachloride (CCl_4) administration. (B) Two months after CCl_4 discontinuation, the hepatic nodules appear enlarged, and the septa are thin and interrupted in certain locations. (C) Presence of incomplete septa within an enlarged hepatic nodule 3 months after treatment. (D) Small islands of hepatic tissue isolated within a portal space (arrows). (E) Hepatic nodule in portal space (thin arrow) and aberrant, dilated, misplaced central veins at its periphery (large arrows). (F) A collection of dark hepatic cells from a nodule seems to invade another nodule composed of clear hepatocytes, through an ''opening'' in the thin fibrous septum separating them. Staining: Sirius-red staining method for collagen (except E and F), stained with Hematoxylin & Eosin. Magnification: × 100 for all.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Hydroxyproline measurements – Biochemical determination of hydroxyproline was made according to the colorimetric method of Bergman and Loxley [5].

Morphometry: The quantitative estimation of fibrous tissue, the measurement of fibrous septa and hepatocellular nodules were done on liver histological sections stained with Sirius-red using the Leica Quantimet Q500MC Image Processing and Analysis System (Leica Cambridge, Cambridge, UK). For morphometric measurements, a total sectional area of $5.0 \times 10\,mm^2$ per case was evaluated. Automatic morphometric analysis was performed in a similar manner to that previously described [4,8]. Briefly, the sectional area of fibrous tissue, red-stained, was directly measured and calculated as a percent of the total area examined. All the fibrous septa present and the sectional area of the parenchymal nodules were evaluated.

Statistical analysis: Variance analysis with one or two factors (ANOVA) and linear regression (Graphpad Prism) were used to test the differences between the means. Results revealing $p<0.05$ were considered significant. For hydoxiproline measurements, the Kruskal–Wallis was used, complemented by Dunn′s post-test.

Controls – Each animal served as its proper control, since material obtained by biopsy was always compared with biopsy and autopsy materials obtained later on. In addition, five healthy intact rats were used as controls for vascular injection procedures and biochemical determinations in liver tissue.

Results

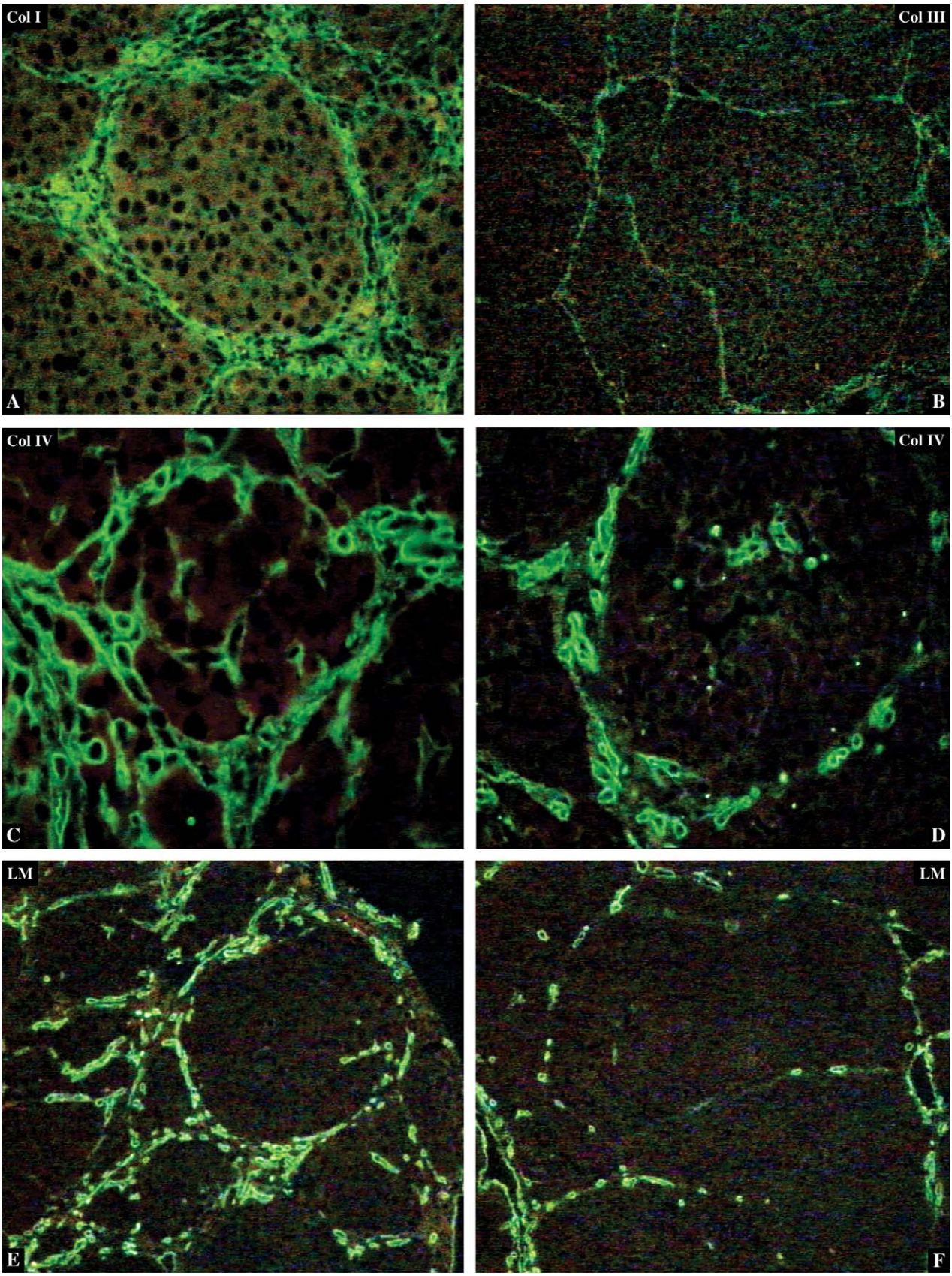
Sixteen animals presented a clear-cut picture of cirrhosis at the end of CCl_4 treatment, when a liver biopsy was examined, as illustrated in Figs. 1A and 2. At that moment, the hepatocytes still presented signs of degenerative changes, especially steatosis, apoptosis, and cytoplasmic swelling. A moderate accumulation of mononuclear leukocytes and edema occurred at the portal spaces and in isolated foci within the parenchyma. Some enlarged macrophages exhibited a brownish PAS positive-diastase resistant pigment, which was also iron-positive. The changes that followed within the first months after the CCl_4 administration was discontinued, consisted of the disappearance of the inflammatory and degenerative changes, the enlargement of the regenerative nodules, and the thinning and fragmentation of fibrous septa. Frequently, the septa appeared incomplete, dissecting the nodular parenchyma and disappearing in the middle of an inconspicuous large nodule (Fig. 1B and C). After the second month and up to the fourth month after the end of CCL_4 treatment, a histological picture of incomplete septal cirrhosis with large inconspicuous nodules, some of them presenting incomplete septa, became dominant. All the other changes depicted in Fig. 1, such as perforated septa (Fig. 1B); fragmented and isolated fibrous septa (Fig. 1C); presence of micro-nodules, isolated nodules inside portal space (Fig. 1D and E, arrows); aberrant central veins at the proximity of portal spaces (Fig. 1E, large arrows) were observed during that period. Sometimes, dark hepatocytes from a regenerative nodule appeared to invade an adjacent nodule formed by cells with a less dark hue (Fig. 1F). From the fourth until the ninth month of observation, there were no further modifications of the histological picture. By looking at a histological section, it was impossible to tell whether it was from any specific time within the 4 months after discontinuation of treatment up to the end of the experiment.

Immunofluorescence studies showed the septa formed predominantly by collagen I and III (Fig. 2A and B). The expression of type IV collagen and laminin, by disclosing basement membranes, helped to reveal the numerous blood vessels within the fibro-cellular septa. A rich vascular network appeared at the time that a full-blown cirrhosis was present (Fig. 2C), and this gradually became less evident after discontinuation of CCl_4 administration. By the end of the period of observation, the presence of blood vessels persisted within the thin fibrous septa ((Fig. 2D–F).

Vascular injections of the portal system showed India ink-filled vessels in portal spaces, septa, and sinusoids in all instances (Fig. 3A). Soon after CCl_4 treatment was discontinued, many sinusoids in the nodules appeared empty, in contrast to the uniformly ink-filled sinusoids observed later on. In particular, the presence of ink-filled vessels was observed, usually a single one, running for a distance within the thin septum delimiting a nodule of residual cirrhosis and opening through several branches into sinusoids (Fig. 3B).

Morphometric measurements showed a significant decrease in the amount of fibrosis (Table 1), appearing

Fig 2. (A) Septa made fluorescent for type I collagen delimiting hepatic nodules in cirrhosis, as seen in the first day after discontinuation of CCl_4 administration. × 200. (B) The presence of enlarged nodules and thin septa can be appreciated in this section stained for type III collagen and taken 5 months after CCl_4 withdrawal. × 100. (C) The vascular basement membranes appear fluorescent for type IV collagen, thus revealing numerous blood vessels within the fibrous septa in hepatic cirrhosis, as seen on the first day after CCl_4 treatment (200 ×), and their persistence in (D), 8 months after discontinuation of the drug (× 200). The same finding is demonstrated with laminin staining in (E), 2 months after cessation of treatment, and (F), which presents 5 months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evident by the early biological point of observation after 1–2 months of the initial biopsy. The other morphometric data showed good correlation with the above-described histological findings, numerically representing the enlargement of the nodules and the thinning of septa after discontinuation of treatment and decrease of hydroxiproline concentration. (Fig. 4). No statistically significant differences were noted between the animals at the 2, 4, and 9 reference points.

Measurements of hydroxyproline concentration disclosed a progressive decrease after discontinuation of CCl_4 administration. However, probably due to the small number of samples tested (2/3 at points), they were not statistically significant ($p > 0.05$).

At the end of the period of observation, large regenerative nodules delimited by thin septa were still present, configuring a histological picture of cirrhosis consistent with a diagnosis of ''incomplete septal cirrhosis''.

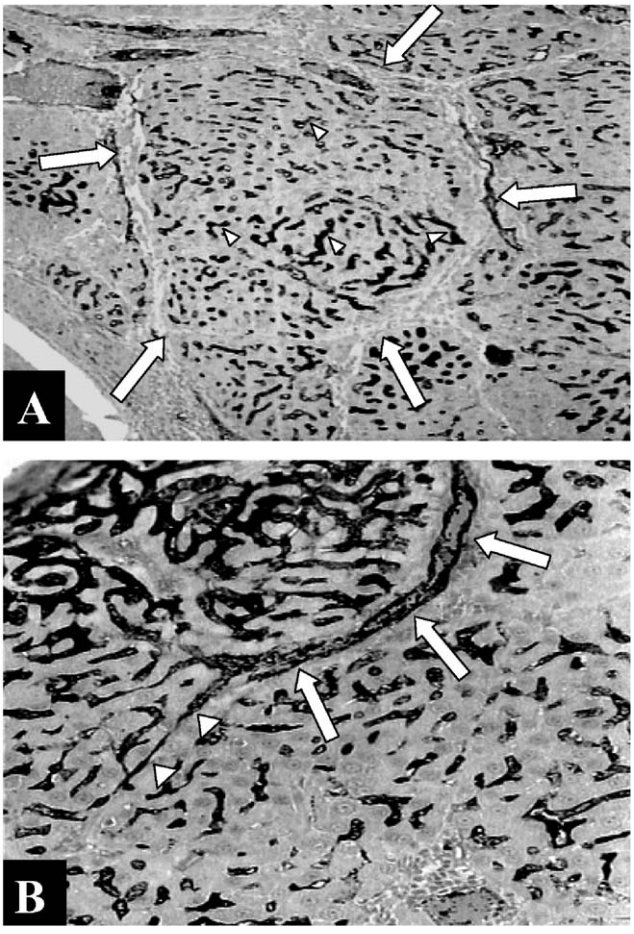

Fig 3. (A) India ink injection into the portal vein system filled blood vessels both within the fibrous septa and hepatic sinusoids. (× 100). (B) An India ink-filled blood vessel is depicted encircling a hepatic nodule (arrows) and draining directly into the hepatic sinusoids (arrow heads). (× 200). Both pictures represent cirrhosis at 9 months after CCl_4 withdrawal.

Discussion

Cirrhosis was considered when the structure of the liver was entirely transformed into regenerative nodules, delimited by fibrous septa and bands. The most prominent and early histological changes seen after discontinuation of CCl_4 administration were represented by expansion of the regenerative nodules and thinning and interruption of the fibrous septa. Soon, several of these septa appeared incomplete, forming projections toward the center of the enlarged and sometimes inconspicuous nodules. Therefore, the findings from the present experimental material are in full agreement with Wanless et al., who reported that regression of cirrhosis mimics the features described as characteristic of so-called incomplete septal cirrhosis [21]. In fact, almost every other change described by Wanless et al. as comprising the *hepatic repair complex* was also registered in the present study, except hepatic vein remnants with prolapsed hepatocytes.

Unexpectedly, all these regressive changes were already established 2–3 months after the administration of CCL_4 was discontinued. From then on, no further modification was apparent; the results persisted as ''cirrhotic'', since the parenchyma retained large nodules delimited by extremely thin and frequently incomplete fibrous septa. This result, observed 2 months after

Table 1. Morphometric evaluation of hepatic fibrosis (%), septa, and nodules in rats treated for 16 weeks with carbon tetrachloride

Parameters	Time point			
	Initial biopsy (control) ($n = 14$)	2 months (after treatment) ($n = 5$)	4 months (after treatment) ($n = 4$)	9 months (after treatment) ($n = 5$)
% Fibrosis	36.3 ± 5.3	16.4 ± 4.3[a]	16.3 ± 7.0[a]	16.5 ± 4.2[a]
Septa width (μm)	13.2 ± 5.8	5.8 ± 2.5[a]	6.1 ± 4.5[a]	7.5 ± 1.2[a]
Nodule size (μm^2)	329,694 ± 147,636	864,540 ± 199,895[a]	706,077 ± 338,940[a]	616,319 ± 177,953[a]

Values are means ± SD.

[a]Differences between the control and the other time points are significant ($p < 0.05$).

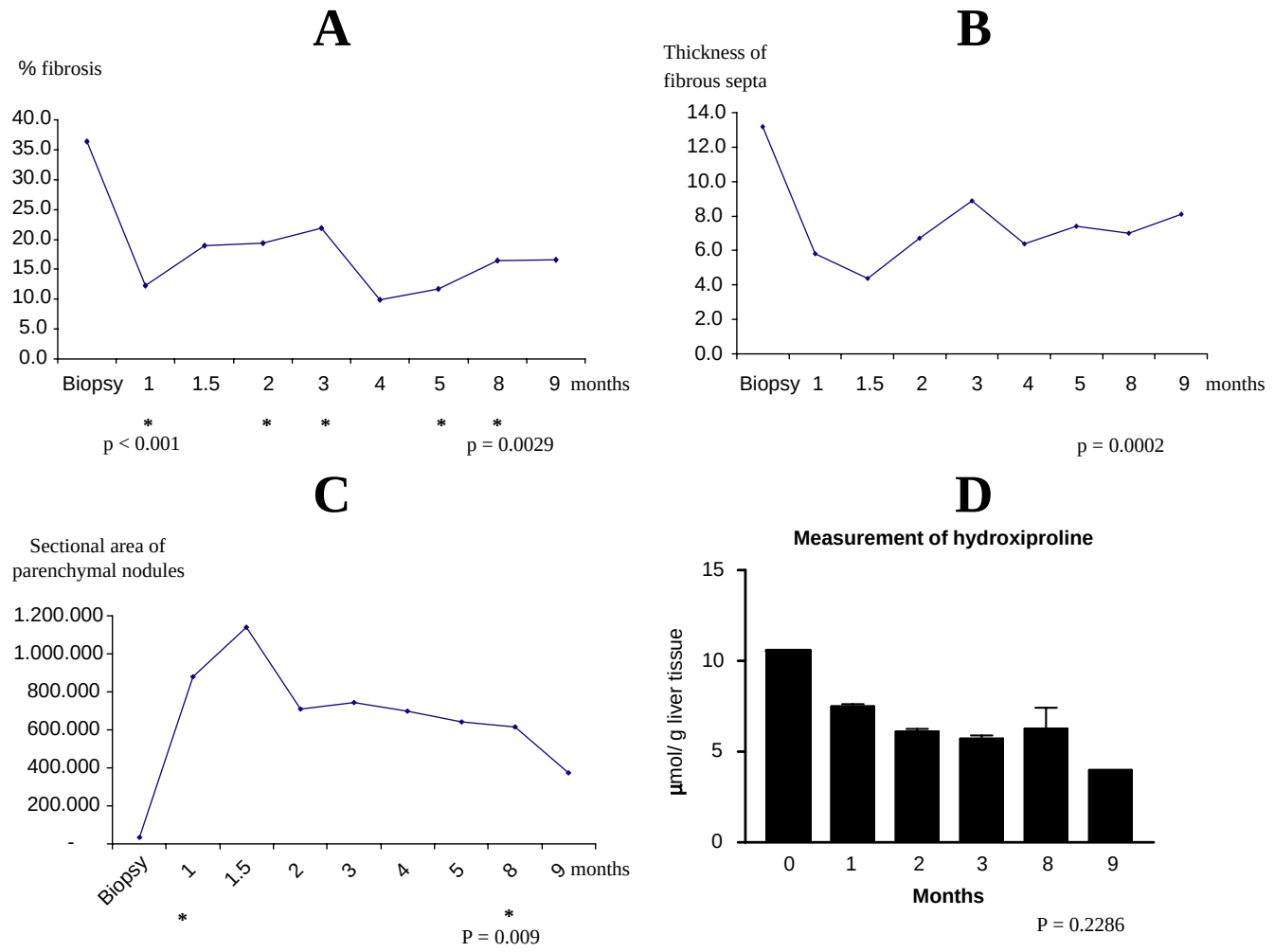

Fig 4. Morphometric data. (A), (B), (C) and (D) represent respectively the behavior of fibrosis, fibrous septa, hepatic-cell nodules, and hydroxiproline concentration along the period after discontinuation of CCl_4 administration.

discontinuation of CCl_4, continued practically unaltered during the subsequent months, up to the end of the experiment. The impression was that of a stable liver morphology, in which no further changes could be expected if the time of observation had been more prolonged. Thus, the presence of the so-called hepatic repair complex indeed refers to changes occurring in cirrhosis when its cause is suppressed or attenuated, but not necessarily indicating that cirrhosis is going to disappear. As observed by Quinn and Higginson [19], every change in experimental CCl_4-induced cirrhosis in mice is reversible except regenerative nodules, which once established, appeared to be entirely independent of the inciting stimulus.

The cirrhosis produced in the animals of the present study could not be considered as being at an extremely advanced stage; however, one cannot exclude the possibility of complete cirrhosis regression in milder cases. Most likely, Perez-Tamayo [16] was right in his statement that reversibility applies to a specific (early) stage of the disease, and there is another (advanced) stage in which the process is apparently not reversible or slower.

Yet, what causes cirrhosis to be irreversible even when there is considerable removal of fibrosis? Present preliminary vascular studies indicate that fibrous septa in old residual cirrhosis contain vessels (portal veins and arterioles) that open freely into the sinusoids. If this vascular structure is functionally adequate, as it seems, it probably represents a successful (and permanent) adaptation of the liver to past damage. There are recent and frequent references in the literature to the reversibility of cirrhosis. These are clinical studies that refer to cases amenable to treatment and cure such as hemochromatosis, alcoholism, virus B and C hepatitis cirrhosis, etc. [2,3,10,14,18]. No doubt, cirrhosis may be clinically reversible, but the reversibility of its morphology in humans still requires a convincing

demonstration. Apparently, only after laparoscopy or necropsy could the gross and histological demonstration of complete cirrhosis regression be made. Present experimental findings suggest that morphological "cirrhotic" features are irreversible but, as Desmet and Roskams [9] rightly stressed, alterations in the hepatic vasculature are a crucial component in the development of the cirrhotic state. Future studies should consider the status of the hepatic vasculature, rather than the simple presence of nodules and fibrous septa, as a demonstration of cirrhosis reversibility.

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