# An ultrasound and histomorphological analysis of experimental liver cirrhosis in rats

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We investigated whether liver injury by dual exposure to ethanol and carbon tetrachloride (EtOH + $CCl_4$) for 15 weeks would persist after hepatotoxic agents were removed (EtOH + $CCl_4$/8wR). After 15 weeks of hepatic injury with ethanol (5.5%, m/v) and carbon tetrachloride (0.05, mL/kg, *ip*), 5 of 11 female Wistar rats were sacrificed. The other 6 rats were maintained for an additional 8 weeks without hepatotoxic agents. Ultrasonography showed increased liver echogenicity and dilation of portal vein caliber in both groups (EtOH + $CCl_4$: 0.22 ± 0.01 cm, P < 0.001; EtOH + $CCl_4$/8wR: 0.21 ± 0.02 cm, P < 0.01) *vs* control (0.16 ± 0.02 cm). Histopathology showed regenerative nodules in both experimental groups. Histomorphometry revealed increased fibrosis content in both groups (EtOH + $CCl_4$: 12.6 ± 2.64%, P < 0.001; EtOH + $CCl_4$/8wR: 10.4 ± 1.36%, P < 0.05) *vs* control (2.2 ± 1.21%). Collagen types I and III were increased in groups EtOH + $CCl_4$ (collagen I: 2.5 ± 1.3%, P < 0.01; collagen III: 1.3 ± 0.2%, P < 0.05) and EtOH + $CCl_4$/8wR (collagen I: 1.8 ± 0.06%, P < 0.05; collagen III: 1.5 ± 0.8%, P < 0.01) *vs* control (collagen I: 0.38 ± 0.11%; collagen III: 0.25 ± 0.06%). Tissue transglutaminase increased in both groups (EtOH + $CCl_4$: 66.4 ± 8%, P < 0.01; EtOH + $CCl_4$/8wR: 58.8 ± 21%, P < 0.01) *vs* control (7.9 ± 0.8%). Cirrhosis caused by the association of $CCl_4$-EtOH remained for at least 8 weeks after removal of these hepatotoxic agents. Ultrasound images can be a useful tool to evaluate advanced hepatic alterations.

## Introduction

Hepatic fibrosis is an early finding in chronic liver injury characterized by failure of degradation and excessive synthesis of extracellular matrix components, mostly collagen types I and III. Persistence of the insult disrupts normal hepatic architecture, leading to development of regenerative nodules and vascular dysfunction, configuring the cirrhosis stage (1,2). Hepatic cirrhosis was described as an irreversible disease, which results from the incapacity of the wounded liver to remodel fibrosis (3,4). However, experimental studies with animals (5,6) and humans (7-9) have recently refuted this concept. Whether advanced cirrhosis undergoes remodeling to a near nor-

mal liver architecture is controversial (10).

Animal models of hepatic cirrhosis use carbon tetra-chloride ($CCl_4$) extensively as a hepatotoxic agent to evaluate liver injury and recovery (10-15). Spontaneous remodeling of extracellular matrix has been reported after cessation of $CCl_4$ intoxication leading to macronodular cirrhosis (14-16). This spontaneous recovery might limit application of these experimental models in chronic fibrosis studies and make long-term investigations of liver cirrhosis difficult when the hepatotoxic agent is interrupted.

The association of $CCl_4$ with ethanol potentiates toxicity of the former, since it increases activation of cytochrome P450, the enzymatic complex responsible for $CCl_4$ metabolism (17). This reaction leads to production of oxidative radicals that cause lipid peroxidation and, consequently, hepatotoxic injury (7). Ethanol also induces synthesis of type I collagen fibrils (18,19), inhibition of hepatocyte proliferation (20-22), exacerbation of activity in hepatic stellate cells (20,23), and increased expression of tissue transglutaminase (tTG) (24). Since a mechanism that renders matrix resistant to degradation results from the formation of $\varepsilon$-($\gamma$-glutamyl) lysine cross-links by tissue transglutaminase (5,25,26), use of the hepatotoxic agents in combination could be more effective in maintaining the fibrotic pattern.

The purpose of the present research was to determine if liver injury by exposure to both ethanol and $CCl_4$ for 15 weeks would persist after the hepatotoxic agents were interrupted. Using a non-invasive diagnostic method, the ultrasound, and comparing it with the "gold standard" histological analysis for diagnosis of hepatic cirrhosis, we show that ultrasonographic images can be used to follow-up the morphological changes associated with liver injury in an experimental model of cirrhosis in rats. Furthermore, our results demonstrate that the association of ethanol and $CCl_4$ resulted in cirrhosis, which was sustained for at least 8 weeks after insult withdrawal.

## Material and Methods

### Animals

This investigation conforms to "The Guide for Care and Use of Laboratory Animals" [DHHS Publication No. (NIH) 85-23, revised 1996, Office of Science and Health Reports, Bethesda, MD] and was approved by the Ethics Committee of Instituto de Biofísica Carlos Chagas Filho (protocol #021).

Female Wistar rats were obtained from the Instituto de Biofísica Carlos Chagas Filho (IBCCF, Rio de Janeiro, RJ, Brazil). Animals were housed at controlled temperature (23°C) with daily exposure to a 12:12-light-dark cycle.

### Chronic liver injury model

Cirrhosis was induced in 11 female Wistar rats (3 to 4 months of age, weighing 150-200 g) with injections of a 20% solution of $CCl_4$ (1:5 in olive oil, dose of 0.05 mL/kg) intraperitoneally (*ip*) three times a week on alternate days for 15 weeks. In response to the need to develop an animal model with an alcohol consumption of clinical relevance, while maintaining dietary control, an alcohol liquid diet in accordance with the AIN-93 guidelines (27) was administered for 15 weeks. Prior to $CCl_4$ administration, an adaptation phase was carried out with a non-alcoholic liquid diet (control diet) administered for 1 week followed by a second week of the ethanol diet (EtOH + $CCl_4$). Control diet ingredients were identical to those used in the alcoholic diet, except that ethanol was replaced by water in the same volume. Rats were given *ad libitum* access to liquid diets.

After 15 weeks, 5 animals were sacrificed and the remaining 6 animals were returned to the standard chow and water diet. They were sacrificed 8 weeks after removal (8wR) of the hepatotoxic agents (EtOH + $CCl_4$/8wR).

A control group consisting of 7 age- and sex-matched animals was fed the liquid diet without alcohol. Three of these animals were sacrificed at the end of the 15th week (Ctrl) and the remaining 4 animals were sacrificed 8 weeks after discontinuation of hepatotoxic agents (Ctrl/8wR).

### Ultrasound examination

Animals were anesthetized and placed in the supine position breathing spontaneously. Trichotomy was performed and a 10-MHz linear transducer of a Caris Plus® ultrasound equipment (Esaote, Italy) was used to analyze the portal vein caliber and liver parenchyma echogenicity.

### Histology

Animals were sacrificed after either 15 weeks of chronic liver injury or 8 weeks after toxic induction interruption. Liver tissue slices were fixed for 5 h in Bouin's solution followed by overnight exposure to 10% buffered formalin solution, pH 7.2, and then embedded in paraffin. Liver samples were sectioned (5 µm) and stained with hematoxylin and eosin (H&E) and picrosirius according to standard protocols (28).

### Collagen quantification

Quantification of fibrillar collagen content was performed by histomorphometric analysis. Using a digital Q-color 5 camera (Olympus, Japan) coupled to an epifluorescence microscope (Axiovert 100, Zeiss, Germany), ten randomly picked fields (10X magnification) of picrosirius-stained liver slides (5-µm sections) were scored for each animal at a final magnification of 100X. Images were

analyzed using the Image Pro Plus 5.0 software (Media Cybernetics, USA). Using the "measurement" tool window, images were prepared for analysis using "erode" filter tool for best determination of each pixel to analyze. Red areas were selected using the following settings: "count/size" option was set to manual and "sensitivity" was set to 5. The same settings were used to select the total field area. Collagen quantification was reported as % of red-stained area divided by the total area times 100.

The determination of liver collagen content was performed by measuring hydroxyproline (29). In short, 2 g fresh hepatic tissue from each animal was dehydrated in acetone for 2 days and dried for 24 h at 60°C. Dry tissue samples were mashed, homogenized and stored at 4°C. Ten milligrams of the dry samples was hydrolyzed in 6 M HCl for 18 h at 107°C. Samples were suspended in 200 µL buffered solution (5% citric acid, 1.2% acetic acid, 12% sodium acetate, and 3.4% sodium hydroxide, pH 6.0, 1:10 dilution in distilled water) and incubated with 1 mL chloramine-T (60 mM in 50% n-propanol, pH 6.0) for 20 min at 38°C. Finally, samples were incubated with 1 mL aldehyde/perchloric acid solution (1 M 4-(dimethylamino)-benzaldehyde in 16% perchloric acid and 60% n-propanol) for 20 min at 60°C. Absorbance was read at 570 nm. Concentration was determined by the linear regression equation of a standard calibration curve (range: 0–20 µg) of 4-hydroxyproline (Sigma-Aldrich, USA).

### Immunofluorescence

Liver tissue was embedded in Tissue-Tek™ OCT compound (Sakura, Japan) and preserved at -70°C. Six-micrometer sections of the liver samples were obtained with a cryostat at -20°C and fixed in acetone. Indirect immunofluorescence technique was performed using anti-tTG (Stratech Scientific, England) at 1:40 and anti-collagen types I and III antibodies (Chemicon International, USA) at 1:30. Secondary antibodies were FITC goat anti-mouse IgG (H+L) (at 1:50; Zymed Laboratories, USA) for tTG and FITC goat anti-rabbit IgG (H+L) (at 1:50; Zymed) for collagen types I and III (28). As control, liver sections were incubated with non-immune mouse or rabbit serum instead of antibodies, followed by incubation with secondary antibodies. Quantification of tTG and collagen types I and III by immunofluorescence was performed as described above in histomorphometric analysis.

### Statistical analysis

Statistical differences among means were assessed using ANOVA with the Tukey post-test for multiple comparisons. $P < 0.05$ was considered to be statistically significant. Data are reported as mean ± SD.

## Results

### Control groups

There were no statistical differences between the control group sacrificed at 15 weeks (Ctrl, N = 3) and the control group sacrificed at 23 weeks (Ctrl/8wR, N = 4) for any of the parameters analyzed (data not shown). Thus, data for both control groups were combined into one group named control group (N = 7).

### Ultrasound assessment

Ultrasound analysis showed increased liver echogenicity characterized by an extensive coarsened and heterogeneous parenchyma with disorganized hepatic surface after 15 weeks of injury induction. This same pattern was still observed 8 weeks after intoxication was interrupted, which is consistent with the diagnosis of cirrhosis (compare control to other groups in Figure 1A, E, and I). There was an increase in portal vein caliber in both groups (EtOH + CCl$_4$: 0.22 ± 0.01 cm, $P < 0.001$; EtOH + CCl$_4$/8wR: 0.21 ± 0.02 cm, $P < 0.01$) compared with the control group (0.16 ± 0.02 cm).

### Histology

Macroscopically, the liver of the control group had a brownish-red color, a smooth surface and elastic consistency (Figure 1B). Microscopically, hepatocytes were radially arranged in plates aligned to sinusoids converging to centrolobular veins (Figure 1C). Moreover, picrosirius only stained portal spaces and centrolobular vessels (Figure 1D).

At the 15th week of induction, there was clear evidence of macronodular cirrhosis. The liver surface was extremely irregular with multiple nodules and had a rigid consistency (Figure 1F). Histological analysis showed liver cirrhosis, characterized by global loss of normal parenchymal architecture, and formation of nodules separated by collagen septa containing some inflammatory infiltrate (Figure 1G). Collagen deposition was intense, forming thick septa interconnecting regenerative nodules (Figure 1H).

After 8 weeks of recovery, the EtOH + CCl$_4$/8wR group presented the same macroscopic and microscopic patterns found in the EtOH + CCl$_4$ group (Figure 1J, K, and L) indicating that cirrhosis was sustained after aggression was interrupted.

### Collagen quantification

Morphometric analysis of picrosirius-stained areas showed an increase in fibrillar collagen content in both groups (EtOH + CCl$_4$: 12.6 ± 2.64%, $P < 0.001$; EtOH + CCl$_4$/8wR: 10.4 ± 1.36%, $P < 0.05$) compared with the
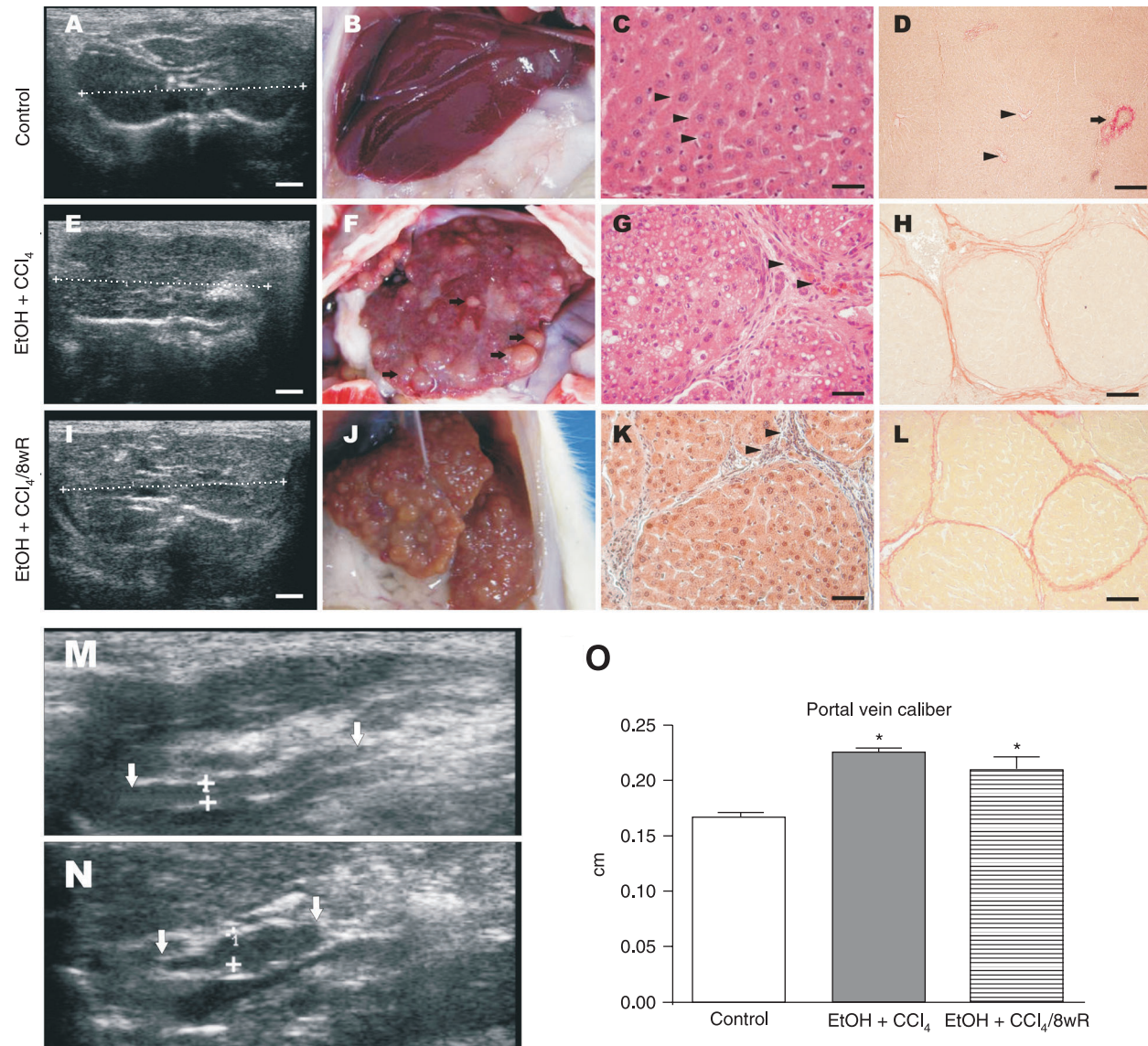
**Figure 1.** Ultrasonographic images, histology and measurement of portal vein caliber. Normal liver (N = 5): *A*, ultrasonographic image illustrates homogeneous liver echotexture with regular contours. *B*, Macroscopy of normal liver. Note smooth surface and brownish-red color. *C*, Microscopy of normal liver (H&E). The parenchyma is homogeneous showing hepatocytes (arrowheads) with round nuclei and sinusoids with radial arrangement. *D*, Picrosirius staining indicates collagen in red. Deposition occurs only in vessels (portal triad - arrow; central vein - arrowheads). After 15 weeks of induction (N = 5): *E*, ultrasonographic image illustrates coarse liver echotexture; *F*, liver cirrhosis: irregular surface and multiple nodules (arrows). *G*, Microscopy shows modification of liver normal structure, with inflammatory infiltrate (arrowheads) and tissue necrosis (H&E). *H*, Picrosirius staining indicates deposition of collagen in thick septa, forming regeneration nodules. Eight weeks after chronic injury interruption (N = 6): *I*, ultrasonographic image illustrates the same pattern as observed after 15 weeks of induction with a diffuse coarse liver echotexture. *J*, Macroscopy shows nodules on liver surface. *K*, Microscopy shows thick fibrous septa (arrowheads), and *L*, regenerative nodules as seen at 15 weeks. Portal vein caliber (PVC): *M*, ultrasonographic image shows normal PVC in control group (arrows); *N*, ultrasonographic image shows dilated and tortuous portal vein representative of both intoxicated groups (arrows), and *O*, quantification of PVC shows increase in portal vein caliber (0.24 cm) in both groups intoxicated with ethanol and CCl$_4$ compared with control group (0.14 cm). Data are reported as means ± SD. *P < 0.01 compared to control group (ANOVA with the Tukey post-test for multiple comparisons). Calibration bars for ultrasonographic liver images: 5 mm; for H&E: 50 μm; for picrosirius: 100 μm, except for Figure 1D (40 μm).

control group (2.2 ± 1.21%; Figure 2A).

Hydroxyproline (HYP) is an amino acid found almost exclusively in collagen proteins. Sample concentration of HYP has a direct correlation with the amount of fibrosis in the liver. There was an increase in HYP concentration (µg/g tissue) in both groups (EtOH + CCl$_4$: 1.9 ± 0.035, P < 0.001; EtOH + CCl$_4$/8wR: 1.6 ± 0.18, P < 0.05) compared

with the control group (1.2 ± 0.17) (Figure 2B).

### Immunofluorescence

We performed immunofluorescence assays to evaluate the presence of tTG in cirrhotic liver. In Figure 3A, there is deposition of tTG only in perivascular regions in a normal liver. The EtOH + CCl$_4$ group, as shown in Figure 3B,
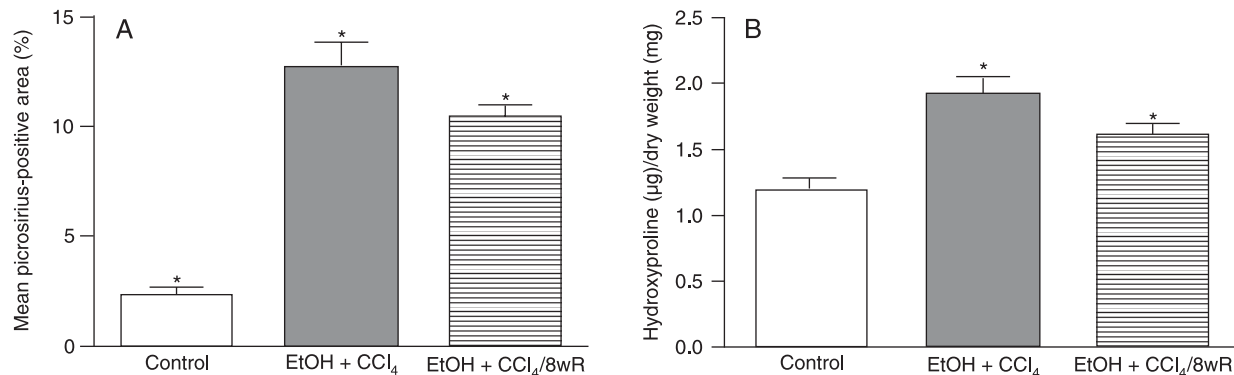


**Figure 2**. Picrosirius morphometric analysis (A) shows that chronic cirrhosis induction for 15 weeks leads to an increase in the amount of fibrosis compared with the control group (N = 5). The fibrillar collagen content was sustained 8 weeks after interruption of induction (N = 6). Hydroxyproline assay (B) showed an increase in liver collagen content after 15 weeks of cirrhosis induction and 8 weeks after interruption. Data are reported as means ± SD. *P < 0.01 compared to control group (N = 7; ANOVA with the Tukey post-test for multiple comparisons).
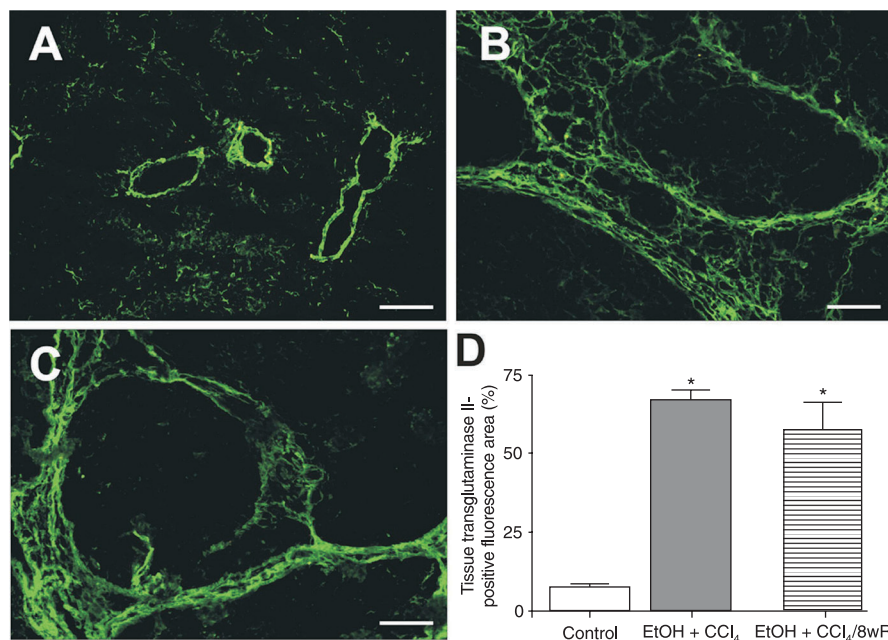


**Figure 3**. Immunofluorescence for tissue transglutaminase (tTG). *A*, In the control group tTG is present only around vessels (N = 3). *B*, Septal stain indicates an increase of this enzyme for 15 weeks (EtOH + CCl$_4$; N = 4). *C*, Hepatic nodules are still stained 8 weeks after interruption of ethanol and CCl$_4$, indicating that tTG is still present after this period (N = 5.) *D*, Morphometric analysis shows an increase in tTG enzyme in all groups compared with the control group. Data are reported as means ± SD. *P < 0.01 compared to control group (ANOVA with the Tukey post-test for multiple comparisons). Calibration bar: 100 µm for panels A-C.

presented intense deposition of this enzyme. This same pattern was also observed in the EtOH + CCl$_4$/8wR group (Figure 3C).

Quantification of the area stained with tTG showed that there was an increase in the deposition of this enzyme in both groups (EtOH + CCl$_4$: 66.4 ± 8%, P < 0.01; EtOH + CCl$_4$/8wR: 58.8 ± 21%, P < 0.01) compared with the control group (7.9 ± 0.8%; Figure 3D).

In order to determine collagen isotypes, collagen types I and III were detected by immunofluorescence. In normal rats, both collagens were detected in portal spaces and as tiny fibrils in liver parenchyma (Figure 4A and B). After 15 weeks of induction, both types of collagen were deposited in hepatic septa (Figure 4C and D). A similar pattern was still observed at the 8th week after aggression was interrupted (Figure 4E and F).

Collagen quantification showed an increase in both collagen I and II in groups EtOH + CCl$_4$ (collagen I: 2.5 ±

1.3%, P < 0.01; collagen III: 1.3 ± 0.2%, P < 0.05) and EtOH + CCl$_4$/8wR (collagen I: 1.8 ± 0.06%, P < 0.05; collagen III: 1.5 ± 0.8%, P < 0.01) compared with the control group (collagen I: 0.38 ± 0.11%; collagen III: 0.25 ± 0.06%) (Figure 4G and F).

## Discussion

We have established and characterized an experimental model of cirrhosis in rats by inducing liver aggression for 15 weeks with a combination of CCl$_4$ and alcohol. In this model, cirrhosis was sustained for at least 8 weeks after insult withdrawal.

Hepatic cirrhosis is characterized by failure of degradation and excessive synthesis of the extracellular matrix (1). One major limitation of experimental cirrhosis models is the lack of tools that allow longitudinal studies of fibrotic change in the liver of individual rats (5). The "gold stand-
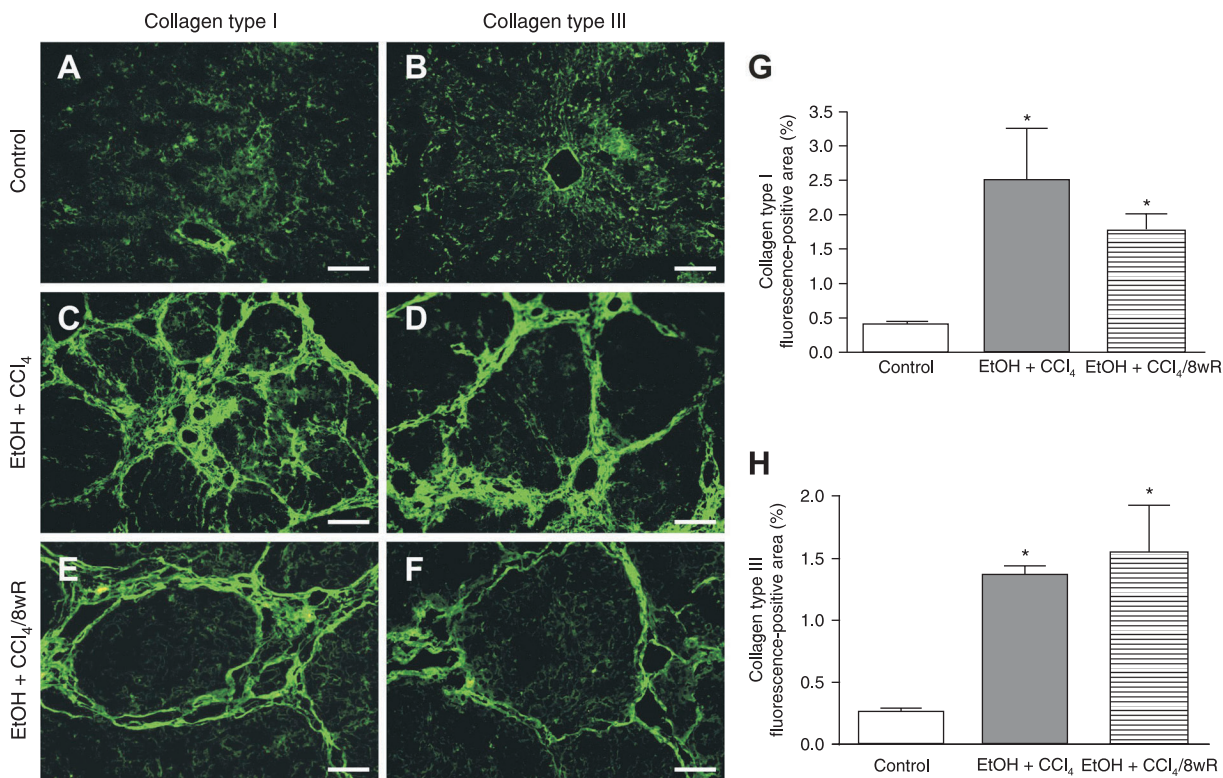


**Figure 4.** Immunofluorescence of collagen types I and III. Collagen type I (A) and type III (B) detection in normal hepatic parenchyma (N = 3). After intoxication for 15 weeks (EtOH + CCl$_4$; N = 3), both collagen types (C and D) have increased expression. This same pattern of staining was observed in the group with interrupted chronic liver injury (EtOH + CCl$_4$/8wR; N = 5) (E and F). Morphometric analysis of collagen type I (G) and type III (H) shows an increase of both collagen types at 15 weeks of intoxication and 8 weeks after interruption of chronic liver injury. Data are reported as means ± SD. *P < 0.01 compared to control group (ANOVA with the Tukey post-test for multiple comparisons). Calibration bar: 100 µm for panels A-F.

ard" for diagnosis of liver cirrhosis has been histopathological examination (1), which demands invasive procedures such as biopsy or whole organ removal after animal sacrifice. The present study shows a correlation between ultrasound parameters and histological analysis. Ultrasound images demonstrated increased liver echogenicity with a coarse pattern and nodular surface indicating a severe tissue alteration over time, which was confirmed by histological results. In addition, dilation of the portal vein was also sustained after induction was interrupted. Portal vein remodeling is directly related to portal hypertension, a severe complication of liver cirrhosis (30). We suggest the use of ultrasound analysis as a useful tool to follow up chronic hepatic injury and to diagnose cirrhosis.

Studies using chronic injection of $CCl_4$ have been performed to determine spontaneous reversal of hepatic fibrosis/cirrhosis (5,6,14,31). Following chronic induction for 12 weeks, micronodular cirrhosis developed and underwent remodeling resulting in macronodular cirrhosis 28 days after withdrawal of intoxication (5). In another study, hepatic cirrhosis was established after 16 weeks of $CCl_4$ administration, but fibrous septa presented thinning and disruption after one-month recovery, remodeling to enlarged regenerative nodules (6). The reversal seen in these models of chronic liver injury may reflect the well-known liver regenerative capability. An important issue concerning the reversal process is the presence of mature fibrosis areas with broad collagen septa and extensively cross-linked by tTG, an enzyme that stabilizes the extracellular matrix, rendering the collagen fibers less sensitive to proteolytic degrading (32,33). These areas do not undergo remodeling and can represent the limit of matrix reversal (5). In our model, the combined effect of two hepatotoxic agents (ethanol and $CCl_4$) seems to surpass endogenous spontaneous regeneration, leading to a minimum reversal process over 8 weeks. Although we did not study animals for longer periods of recovery, eight weeks is a useful time window that can be used to study drug- or cell-based therapies.

Collagen types I and III show a three- to eight-fold increase in hepatic injury (34). These proteins are mainly synthesized by activated hepatic stellate cells. Type I procollagen expression in activated hepatic stellate cells is stimulated when these cells are exposed to ethanol in culture (18,35-37). Both collagen types are substrates for tTG (38).

We have identified histological and biochemical features associated with irreversibility of fibrosis. The association of ethanol and $CCl_4$ in the present study resulted in a large amount of tTG localized mainly at fibrous septa at the end of 15 weeks of intoxication, a period when chronic cellular injuries are at the highest level and tTG is linked to extracellular matrix. We suggest that the presence of tTG 8 weeks after chronic liver injury discontinuation can contribute to the elevated amounts of collagen types I and III observed at this time in the present study. Collagen cross-linking makes this molecule more resistant to collagenase degradation, therefore inhibiting fibrosis remodeling. In agreement with other reports (5,20), our results showed that the persistent presence of tTG after withdrawal of toxic agents might determine a point of "no return" for hepatic cirrhosis.

Our observations show that ultrasound images can be used as a useful tool to evaluate advanced hepatic fibrotic change. Moreover, we suggest that the presence of tTG, after discontinuation of toxic agents, might contribute to collagen resistance to degradation, leading to irreversible hepatic fibrosis.

## Acknowledgments

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