



Original Article

Combined therapy with danazol, pegylated interferon, and ribavirin improves thrombocytopenia and liver injury in rats with fibrosis

Guillermo Cabrera Álvarez;¹ Vicente Madrid-Marina;² Ricardo Jiménez-Mendez;³ Angel Leon Buitimea;⁴ Margarita Bahena Román;² Rudyard Cortez-Gomez;⁵ Jorge Reyes Esparza;⁴ Lourdes Rodríguez-Fragoso⁴

Abstract

The aim of this study was to investigate the effects of combinations of pegylated-interferon (PEG-IFN), ribavirin, and danazol on thrombocytopenia and liver injury in rats with fibrosis. Male adult Wistar rats were treated with either mineral oil, danazol (0.83 mg/kg per day), PEG-interferon α -2a (PEG-IFN, 0.3 μ g/week) + ribavirin (12 mg/kg per day), PEG-IFN + ribavirin + danazol, CCl₄ (4 g/kg for eight weeks), CCl₄ + PEG-IFN + ribavirin, or CCl₄ + PEG-IFN + ribavirin + danazol. The following assays were conducted: hematology, clinical chemistry, liver function, liver fibrosis, lymphocyte cytokine mRNA expression, and bone-marrow DNA content. Platelet counts were low in sham-treated animals and animals treated with PEG-IFN + ribavirin (30% and 25% respectively; $P < 0.05$). PEG-IFN + ribavirin + danazol reduced platelet counts of fibrotic animals by only 9% ($P < 0.05$). PEG-IFN + ribavirin reduced hepatic collagen content by 50%, whereas danazol + PEG-IFN + ribavirin reduced hepatic collagen content by 60% ($P < 0.05$). PEG-IFN

+ ribavirin reduced the total bilirubin concentration by 27%, alanine amino transferase (ALT) activity by 75% and γ -glutamyl transpeptidase (γ -GTP) activity by 74% ($P < 0.05$). In contrast, danazol + PEG-IFN + ribavirin reduced total bilirubin levels by 61%, alkaline phosphatase activity by 45%, ALT activity by 76%, and γ -GTP activity by 74% ($P < 0.05$). The only treatment that increased interleukin 10 (IL-10) mRNA in fibrotic rats was PEG-IFN + ribavirin. However, danazol + PEG-IFN + ribavirin reduced the expression of IL-6, IL-10, tumor necrosis factor α and transforming growth factor β . Bone-marrow DNA content was not altered by any treatment. In conclusion, PEG-IFN + ribavirin + danazol could be a new therapeutic option for patients with liver injury, fibrosis, and thrombocytopenia.

Key words: Danazol, thrombocytopenia, fibrosis, collagen, PEG-interferon, ribavirin.

Hepatitis C virus (HCV) infection affects about 170 million people worldwide. Chronic hepatitis develops in up to 80% of individuals who contract acute infections and may cause liver fibrosis and cirrhosis.^{1,2}

HCV infection is an inflammatory disease characterized by the enhanced expression of various pro- and anti-inflammatory cytokines in the liver. The initiation of several intracellular signal pathways that involve apoptosis, proliferation, and extracellular matrix synthesis constitutes the major impetus for the development of hepatic injury, fibrosis, and cirrhosis.³⁻⁵ During the past decade, HCV infection has also been associated with many extrahepatic manifestations. In large studies that assessed the prevalence of extrahepatic manifestations, at least one clinical manifestation was exhibited by 38% of patients,⁶ and up to 74% of patients exhibited at least one serological manifestation.⁷ Autoimmune thrombocytopenia may be an extrahepatic manifestation of HCV infection.⁸⁻¹⁰ Several theories have been proposed to explain the presence of thrombocytopenia in chronic liver injury.¹¹

Pegylated-interferon α -2a (PEG-IFN) and ribavirin combination therapy is the gold standard in the treatment

¹ Internal Medicine Division, Gastroenterology and Hepatology Department, Hospital General Regional con UMF # 1, Instituto Mexicano del Seguro Social, Cuernavaca, Morelos, México

² Chronic Infections and Cancer Division, Center for Research on Infectious Diseases, Cuernavaca, Morelos, México

³ External Section of Pharmacology, Center for Research and Advanced Studies, IPN, México City, México

⁴ Faculty of Pharmacy, Universidad Autónoma del Estado de Morelos, Cuernavaca, Morelos, México.

⁵ Clinical Laboratory, Hospital General Regional con UMF # 1, Instituto Mexicano del Seguro Social, Cuernavaca, Morelos, México

Address for correspondence:
Lourdes Rodríguez-Fragoso Ph.D
Flavio García Núm. 32
Presidentes Ejidales 04470
México D.F.
Tel/fax 777 329-708
E-mail: mlrodrig1@yahoo.com.mx

of chronic hepatitis C and is associated with a high rate of sustained virological response. However, a high incidence of adverse hematological side effects is associated with this therapeutic regimen. Adverse hematological effects are particularly common, and the bone-marrow suppression caused by interferon may result in neutropenia and thrombocytopenia.¹²⁻¹⁴ Previous studies have suggested that HCV induces immune thrombocytopenia, and that interferon itself induces autoimmune thrombocytopenia.¹⁵⁻¹⁷

Danazol is a synthetic attenuated androgen and has been used for the treatment of several unrelated immune-mediated diseases.¹⁸⁻²⁰ Danazol has also been used successfully to treat various diseases associated with autoimmune thrombocytopenia. This drug has a corticosteroid-sparing effect and increases platelet counts, even in patients who are refractory to other therapeutic approaches. Recent studies have indicated that danazol modifies the level of antiplatelet antibodies, inhibits the mononuclear phagocyte system, and reduces the GPIIb-IIIa complex in patients with refractory autoimmune thrombocytopenia.²¹⁻²³

The aim of this study was to investigate the effects of combinations of danazol, PEG-IFN α -2a, and ribavirin on hematological, biochemical, and functional liver indices in rats with fibrosis.

Research methods and procedures

Animal model and experimental protocol

Seventy male Wistar rats weighing 200 g each were used. The animals were housed in a temperature- and humidity-controlled environment and were given food (Standard Purina Chow Diet, Purina, St Louis, MO, USA) and water *ad libitum*. The body weights and health of the rats were monitored throughout the study. This investigation was conducted in accordance with the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals.²⁴ The rats were randomly divided into seven groups (10 rats per group). The animals were treated as follows: group 1, animals received only mineral oil (control); group 2, animals received danazol at a dose of 0.83 mg/kg for 5 d per week for four weeks; group 3, animals received PEG-IFN at a dose of 0.3 μ g/week by intraperitoneal injection plus ribavirin at a dose of 12 mg/kg for 5 d per week for four weeks; group 4, animals received danazol, PEG-IFN, and ribavirin for four weeks; group 5, animals were treated with CCl₄ to induce liver injury and fibrosis; group 6, animals received CCl₄ alone for four weeks, after which they received CCl₄ plus PEG-IFN and ribavirin for four weeks; group 7, animals were treated with CCl₄ plus PEG-IFN, ribavirin, and danazol at the doses described for the other treatments. Ribavirin and danazol were administered by gavage. Liver injury and fibrosis were induced with intraperitoneal injections of 0.15 mL of a 1:7 (v/v) solution of CCl₄ (4 g/

kg) in mineral oil, three times a week for eight weeks. Two days after they had received the last treatment dose, the animals were deprived of food but not water for 12 h, and were killed under light ether anesthesia. Serum and liver tissue samples were collected from each animal and stored at -4 °C and -20 °C, respectively, until analysis.

Collagen analysis

Collagen concentrations were determined by measuring the hydroxyproline content of fresh liver samples after their digestion with acid.²⁵ The procedure was as follows: fresh liver samples (100 mg) were placed in ampoules, 2 mL of 6 N HCl was added, the ampoules were sealed, and the samples were hydrolyzed at 100 °C for 48 h. The water in the samples was then evaporated at 50 °C for 24 h and the samples were resuspended in 3 mL of sodium acetate-citric acid buffer (pH 6.0); 0.5 g of activated charcoal was added, and the mixture was stirred vigorously and then centrifuged at 5000 \times g for 10 min. The mixture was kept for 20 min at room temperature, and the reaction was stopped by the addition of 2 M sodium thio-sulfate and 1 N sodium hydroxide. The aqueous layer was transferred to test tubes. The oxidation product of hydroxyproline was converted to pyrrole by boiling. The pyrrole-containing samples were incubated with Ehrlich's reagent for 30 min, and their absorbance was measured at 560 nm. The recovery of known amounts of standards from similar liver samples was used to calibrate the assay.

Hematological and biochemical analyses

Blood samples were collected from each animal for the quantification of white cells, red blood cells, platelets, hemoglobin concentrations, and hematocrit. The plasma was separated and used for biochemical assays of hepatic function. Serum alkaline phosphatase (AP) activity, alanine amino transferase (ALT) activity, γ -glutamyl transpeptidase (γ -GTP) activity, and bilirubin content were evaluated using a kit (Merck Naucalpan, Estado de Mexico, Mexico). Biochemical evaluation of the serum samples was conducted using an automated system (Cell Dyn 3700, Abbot Laboratories, USA; Synchron CX7, Beckman Coulter, USA).

Lymphocyte isolation

Lymphocytes were isolated from heparinized whole blood by density gradient centrifugation using Lymphoprep (Axis-shield, Oslo, Norway).

RNA extraction

Total cellular RNA was isolated using TRIzol® Reagent (Invitrogen Life Technologies) according to the

method of Chomzynski and Sacchi.²⁶ Briefly, cells were homogenized in 1 mL of TRIzol Reagent and incubated for 5 min at room temperature. Then, 200 μ L of chloroform was added to the mixture. After vigorous mixing and centrifugation at $10,000 \times g$ for 15 min, the aqueous phase was harvested and the RNA was precipitated with an equal volume of isopropanol. The RNA was washed in 70% ethanol and dissolved in DEPC-treated water. The RNA was quantified using a DU-40 spectrophotometer; 3 μ g of the sample was used to analyze the integrity of the RNA on a 1% agarose gel.

cDNA synthesis

First-strand cDNA was synthesized using 1 μ g of total RNA obtained from a suspension of lymphocytes in DEPC-treated water. Briefly, 1 μ L of primer dT₁₂₋₁₈ (Invitrogen Life Technologies) was added, and the volume was made up to 11 μ L with DEPC-treated H₂O. Each sample was incubated at 70 °C for 10 min and was then incubated on ice for 1 min. To each RNA primer mixture were added 9 μ L of reaction mixture, containing 4 μ L of 5 ¢ RT buffer (250 mM Tris-HCl [pH 8.3], 375 mM KCl, 50 mM dithiothreitol, 15 mM MgCl₂), 40 U of RNasin (Invitrogen Life Technologies), 4 μ L of dNTP mix (10 mM each of dATP, dCTP, dGTP, and dTTP), and 200 U of Moloney Murine Leukemia Virus reverse transcriptase (Invitrogen Life Technologies). The mixture was mixed gently, incubated at 37 °C for 50 min, and then quickly chilled on ice.

RT-PCR for cytokines

PCR amplification was carried out in 25 μ L of amplification buffer containing 200 mM Tris-HCl (pH 8.4), 500 mM KCl, 1.5 mM MgCl₂, 200 μ M dNTPs, 10 pmol each of the 5' and 3' primers, 2.0 U of *Taq* DNA polymerase (Invitrogen Life Technologies, Brazil), and 2.0 μ L of cDNA. The samples were then amplified in a PCR System 2700 (Applied Biosystems) as follows: denaturation at 94 °C for 5 min and 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, followed by 5 min at 72 °C. A 10 μ L aliquot of the PCR product was then separated electrophoretically on a 6% polyacrylamide gel and visualized under UV by ethidium bromide staining. The mRNA bands were quantified using Bioimaging Systems software (LabWorks 4.0) and normalized to glyceraldehyde 3-phosphate dehydrogenase (G3PDH) bands. The sequences of the primer pairs used to amplify specific cytokines of the rat were as follows. G3PDH: sense 5'-AC-CACAGTCCATGCCATCAC-3' and antisense 5'-TC-CACCACCCTGTTGCTGTA-3', amplifying a fragment of 452 bp (39); transforming growth factor β (TGF- β 1): sense 5'-GGCTTCTAGTGCTGACG-3' and antisense 5'-GGGTGCTGTTGTACAAAG-3', 203 bp;²⁷ tumor necrosis factor α (TNF- α): sense 5'-CACCACGCTCTTCT-

GTCTACTGAAC-3' and antisense 5'-CCGGACTCCGT-GATGTCTAAGTACT-3', 545 bp;²⁸ interleukin 2 (IL-2): sense 5'-CATGTACAGCATGCAGCTCGCATCC-3' and antisense 5'-CCACCACAGTTGCTGGCTCATCATC-3', 410 bp; IL-6: sense 5'-GACTGATGTTGTTGACAGC-CACTGC-3' and antisense 5'-TAGCCACTCCTTCTGT-GACTCTAACT-3', 509 bp;²⁸ IL-10: sense 5'-ACCTGG-TAGAAGTGATGCCCCAGGCA-3' and antisense 5'-CTATGCAGTTGATGAAGATGTCAAA-3', 237 bp.²⁹

The sequences of the primer pairs used to amplify specific cytokines for humans were as follows. TGF- β 1: sense 5'-GCCCTGGACACCAACTATTGCT-3' and antisense 5'-GGGTGCTGTTGTACAAAG-3', amplifying a fragment of 161 bp; IL-6: sense 5'-ATG-TAGCCGCCCCACACAGA-3' and antisense 5'-CATC-CATCTTTTTTCAGCCAT-3', 190 bp; TNF- α sense 5'-GAGTGACAAGCCTGTAGCCCATGTTGTAGCA-3' and antisense 5'-GCAATGATCCCAAAGTAGACCT-GCCCAGACT-3', 444 bp; IL-2 sense 5'-CATTGCAC-TAAGTCTTGCACTTGTCA-3' and antisense 5'-CGT-TGATATTGCTGATTAAGTCCCTG-3', 305 bp; IL-10 sense 5'-ATGCCCAAGCTGAGAACCAAGACCCA-3' and antisense 5'-TCTCAAGGGGCTGGGGCTGGGT-CAGCTATCCCA-3', 351 bp. The PCR conditions were: denaturation at 95 °C for 5 min followed by 35 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min, and a final extension at 72 °C for 5 min. For IL-2, the PCR conditions were: denaturation at 95 °C for 5 min followed by 35 cycles of 94 °C for 1 min, 68 °C for 1 min, and 72 °C for 1 min, with a final extension at 72 °C for 10 min.

The amplification of G3PDH was performed separately to compare the expression of a constitutively expressed gene and to normalize the cDNA input in each cytokine mRNA-cDNA amplification. Repeated PCR analyses of the samples yielded reproducible results and indicated that no inhibitory factors were present in the samples. Several precautions were taken to avoid PCR artifacts. Negative controls, consisting of buffer alone or nonreverse-transcribed sample RNA, were included in each experiment.

Cell-cycle analysis of bone-marrow cells

Propidium iodide (PI) and flow cytometry were used for the analysis of the DNA contents of bone-marrow samples. Briefly, the cells (10^5) were fixed in 80% ethanol for 24 h, washed in phosphate-buffered saline, and resuspended in 0.1% Nonidet P40 (Biochemica Fluka) and DNase-free RNase (10 μ g/mL) for 20 min at room temperature (30). PI was then added (final concentration, 5 μ g/mL), and the samples were incubated for 12 h at 4 °C in the dark. The samples were analyzed using a FACSCalibur flow cytometer (Becton Dickinson). For each sample, 10,000 cells were analyzed using four replicates. The results were analyzed using the CELLQuest program.

Other assays

Small liver sections fixed in Bouin's medium were used for trichromic staining and histological examination under light microscopy.

Statistical analysis

Data are reported as the means \pm standard deviations of three independent experiments conducted in quadruplicate. Statistical analysis was performed using parametric ANOVA. Individual differences between treatments were analyzed using Tukey's test. Significant differences were established at $P < 0.05$.

Results

Effect of CCl₄ treatment

The onset of fibrosis was determined by measuring changes in the collagen content of the livers of CCl₄-treated rats. Liver injury was characterized by an increase in bilirubin content and serum AP, ALT, and γ -GTP activities relative to those of untreated rats ($P < 0.05$; Table I). Histological analysis of the liver samples from the animals treated with CCl₄ revealed an increase in the amount of collagen fibers and changes in the liver architecture compared with those of liver samples from untreated animals (Figure 1).

Hepatic collagen content

The collagen content of the liver samples was estimated from the hydroxyproline content. CCl₄ treatment induced a fivefold increase in liver collagen content (Figure 2). Animals with fibrosis that were treated with PEG-IFN + ribavirin had lower liver collagen contents (50%) than those of CCl₄-treated rats ($P < 0.05$). However, animals with fibrosis that were treated with danazol plus PEG-IFN and ribavirin had a 60% reduction in liver col-

lagen content ($P < 0.05$) compared with that of rats with fibrosis. Treatment with PEG-IFN, ribavirin, or danazol alone or ribavirin plus danazol produced no change in liver collagen contents.

Analysis of liver architecture

Figure 1 shows the histology of liver samples from all treatment groups. Animals with CCl₄-induced fibrosis exhibited the degeneration of hepatocytes and increased inflammatory infiltrate in the necrotic areas. These animals also developed severe fibrosis, with complete distortion of the lobular architecture (Figure 1B). Histological sections of livers from animals with fibrosis that were treated with PEG-IFN plus ribavirin showed a significant improvement in the liver architecture, as well as reductions in the amount of collagen fibers and the extent of the necrotic area (Figure 1C). A significant reduction in the amount of collagen fibers and a reduction in liver damage were also observed in animals with fibrosis that were treated with danazol or PEG-IFN plus ribavirin (Figure 1D).

Hematological and biochemical analyses

Hematological analysis showed that animals with liver fibrosis had a 30% reduction in platelet counts ($P < 0.05$), a 30% reduction in albumin levels, and a twofold increase in cholesterol concentrations ($P < 0.05$) compared with those of the control group. Animals with fibrosis that were treated with PEG-IFN and ribavirin also had reductions in their platelet numbers (by 25%; $P < 0.05$) and albumin levels (by 23%; $P < 0.05$), and a 1.2-fold increase in their cholesterol levels ($P < 0.05$). However, animals with fibrosis that were treated with danazol plus PEG-IFN and ribavirin had a reduction of 9% in their platelet counts ($P < 0.05$) compared with those of untreated animals with fibrosis. Although platelet counts did not differ between groups, animals with fibrosis that were treated with danazol plus PEG-IFN and

Table I. Liver function analysis.^a

Treatment	Total bilirubin ($\mu\text{mol/L}$)	AP (IU)	ALT (IU)	γ -GTP (IU)
Control	2.5 \pm 0.8	204 \pm 24	55.7 \pm 5.1	101 \pm 13
Da	3.9 \pm 0.9	201 \pm 59	52.2 \pm 2.2	135 \pm 20
PEG-IFN + Ri	5.1 \pm 0.3	229 \pm 37	65.7 \pm 19	158 \pm 44
PEG-IFN + Ri + Da	1.9 \pm 1.0	184 \pm 51	60.2 \pm 21	149 \pm 66
Fibrosis	65 \pm 26 ^b	617 \pm 13 ^b	3264 \pm 161 ^b	5913 \pm 112 ^b
Fibrosis + PEG-IFN + Ri	47 \pm 19 ^b	608 \pm 57 ^b	798 \pm 237 ^{b c}	1494 \pm 263 ^{b c}
Fibrosis + PEG-IFN + Ri + Da	25 \pm 12 ^{b c}	338 \pm 56 ^{b c}	759 \pm 254 ^{b c}	1487 \pm 462 ^{b c}

^a Results are expressed as the means \pm Standard Deviation of experiments performed with duplicate assays of samples from six animals. Alkaline phosphatase (AP), alanine amino transferase (ALT), γ -glutamyl transpeptidase (γ -GTP), international units (IU), PEG-interferon (PEG-IFN), ribavirin (Ri), danazol (Da).

^b $P < 0.05$ vs the control group.

^c $P < 0.05$ vs the fibrosis group.

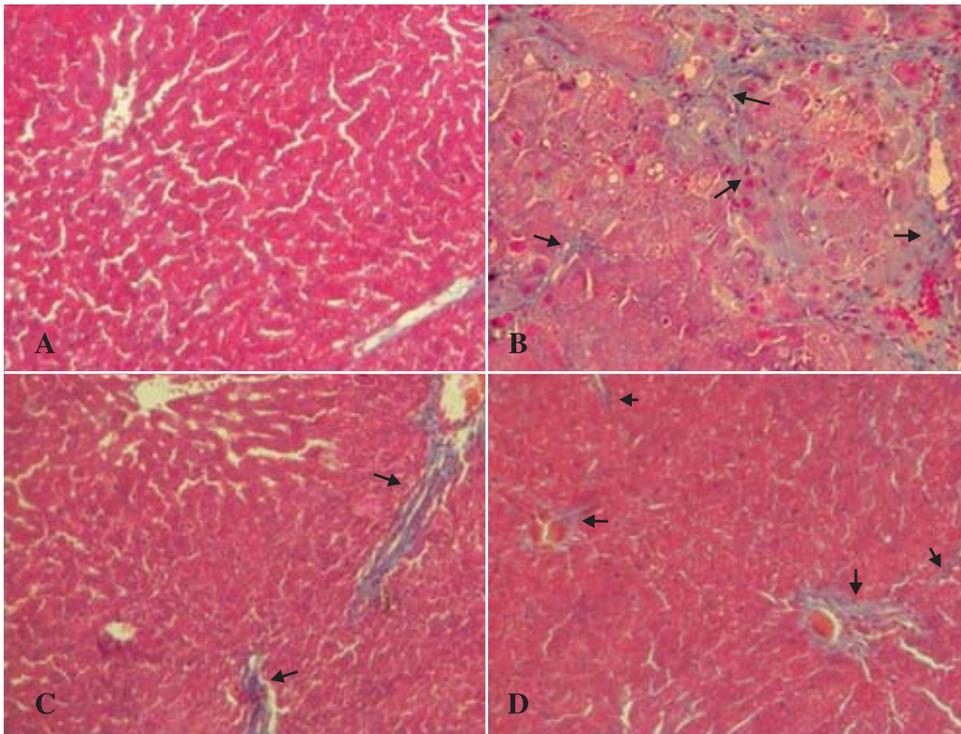


Figure 1. Histological study of liver sections from rats treated with danazol and the conventional therapy. (A) Control (untreated rats); (B) CCl_4 -induced liver fibrosis; (C) animals with fibrosis treated with PEG-interferon α -2a and ribavirin; (D) animals with fibrosis treated with danazol plus PEG-interferon α -2a and ribavirin. Liver tissues were stained with Masson trichrome. Collagen is indicated by blue staining (arrows; 100x). All treatments were administered for eight weeks.

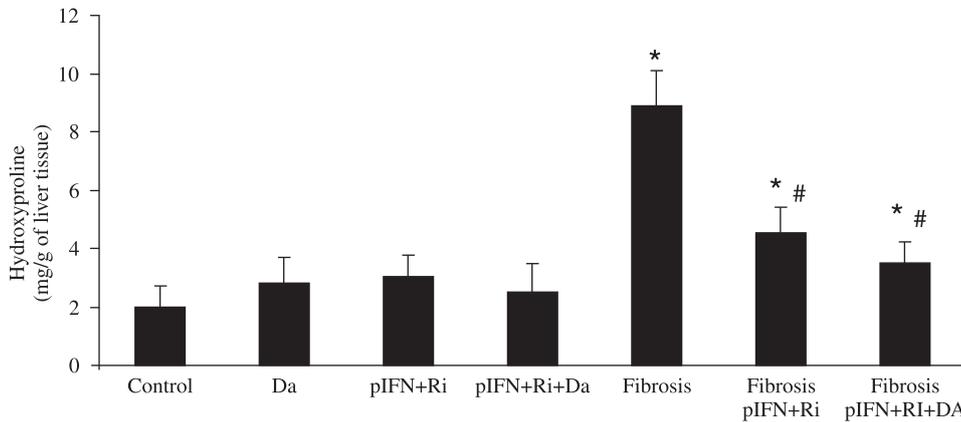


Figure 2. Hepatic collagen content. Collagen was measured as the hydroxyproline content of liver slices. Each bar represents the mean \pm SD of experiments performed in duplicate. All groups consisted of 10 animals. PEG-interferon α -2a (PEG-IFN), ribavirin (Ri), danazol (Da). * different from that of the control group ($P < 0.001$). # different from that of the fibrosis group ($P < 0.001$).

ribavirin had lower levels of albumin (40%) than the other groups (Tables I and II).

Analysis of liver function

Animals with fibrosis had a significant increase in total bilirubin levels (26-fold) and threefold, 5.8-fold, and 58.5-fold increases in the serum activities of AP, ALT, and γ -GTP, respectively ($P < 0.05$). Animals with fibrosis that received PEG-IFN and ribavirin had lower levels of total bilirubin (27%) and lower serum activities of ALT (by 75%) and γ -GTP (by 74%) than those of animals with fibrosis ($P < 0.05$). In contrast, significant reductions relative to those of fibrotic rats were observed in the level of total bilirubin (61%) and the activities of AP (45%), ALT (76%), and γ -GTP (74%) in animals with fibrosis that

were treated with danazol plus PEG-IFN and ribavirin ($P < 0.05$; Table III).

Expression of IL and growth factor mRNAs in lymphocytes

There is increasing evidence that several cytokines play major roles in various aspects of inflammatory liver diseases and liver tissue repair. Therefore, in this study, we evaluated the expression of IL-6, IL-10, TNF- α , and TGF- β in blood lymphocytes (Figure 3). The expression of cytokines in lymphocytes was modified by the treatments. Animals with fibrosis had a reduction in the mRNA levels of all cytokines compared with those of the control animals. Animals with fibrosis that were treated with PEG-IFN and ribavirin only showed an increase in

the mRNA levels of the anti-inflammatory cytokine, IL-10, relative to that of animals with fibrosis. However, animals with fibrosis that were treated with danazol plus PEG-IFN and ribavirin had reductions in the blood levels of all cytokines (IL-6, IL-10, TNF- α , and TGF- β) compared with those of untreated animals with fibrosis. It is noteworthy that treatment with PEG-IFN, ribavirin, or danazol alone reduced the mRNA levels of IL-6, IL-10, and TNF- α . Ribavirin plus danazol increased TGF- β mRNA levels, whereas PEG-IFN, ribavirin, or danazol alone reduced TGF- β mRNA levels.

Cell-cycle analysis of bone-marrow cells

Cell-cycle analysis was performed to determine whether the treatments affected bone-marrow cells and, as a consequence, the levels of blood cells. The DNA of bone-marrow cells was not altered by any of the treatments (Figure 4). Therefore, there was no correlation between the changes in blood platelet levels and the cell cycle in bone-marrow cells.

Discussion

HCV infection is a frequent cause of chronic hepatitis. Persistent HCV infection can produce disorders not only

of the liver, but also of other organs.³¹⁻³³ Recently, HCV has been implicated in the development of many extra-hepatic manifestations.^{6,7,34}

Thrombocytopenia is a common finding in advanced liver cirrhosis and is usually related to the congestive splenomegaly of portal hypertension and possibly to inadequate thrombopoietin synthesis by the failing liver.^{35,36} In adult patients, IFN- α and pegylated interferons are effective in decreasing abnormal levels of transaminases and levels of HCV viremia, but are associated with many adverse effects. IFN- α induces and exacerbates several autoimmune abnormalities, including thrombocytopenia. The presence of severe thrombocytopenia can prompt dose reduction and treatment discontinuation.³⁷ HCV-associated thrombocytopenia is an important and unresolved problem, particularly because the mechanism responsible for the occurrence of thrombocytopenia in these individuals is unclear. This study shows that therapy with PEG-IFN plus ribavirin and danazol increases platelet counts and ameliorates liver injury and fibrosis in rats.

Danzol has been used successfully for the treatment of various diseases associated with autoimmune thrombocytopenia. The main mechanism responsible for the thrombocytopenia of immune diseases involves an increase in platelet destruction by platelet-bound antibody

Table II. Hematological analysis.^a

Treatment	White blood cells (x 10 ³ / μ L)	Red blood cells (x 10 ⁶ / μ L)	Platelets (x 10 ³ / μ L)	Hemoglobin (g/dL)	Hematocrit (%)
Control	5.45 \pm 0.2	8.8 \pm 1.1	961 \pm 25	15.1 \pm 0.9	44.4 \pm 8
Da	4.7 \pm 0.4	7.9 \pm 0.8	998 \pm 19	14.8 \pm 0.7	46.1 \pm 7
PEG-IFN + Ri	5.1 \pm 0.2	8.1 \pm 0.7	897 \pm 32	15.7 \pm 0.3	44.5 \pm 8
PEG-IFN + Ri + Da	5.7 \pm 0.3	7.5 \pm 1.0	967 \pm 19	15.3 \pm 0.7	42.6 \pm 7
Fibrosis	4.8 \pm 0.6	7.0 \pm 2.5	673 \pm 21 ^b	14.5 \pm 0.5	38.2 \pm 9
Fibrosis + PEG-IFN + Ri	4.5 \pm 0.7	7.3 \pm 1.4	713 \pm 18 ^b	14.6 \pm 0.3	40.1 \pm 5
Fibrosis + PEG-IFN + Ri + Da	5.0 \pm 0.4	7.8 \pm 1.8	878 \pm 23 ^{b,c}	14.8 \pm 0.7	41.3 \pm 3

^a Results are expressed as the means \pm SD of experiments performed with duplicate assays of samples from six animals. PEG-interferon (PEG-IFN), ribavirin (Ri), danazol (Da).

^b $P < 0.05$ vs the control group.

^c $P < 0.05$ vs the fibrosis group.

Table III. Biochemical analyses.^a

Treatment	Glucose (mg/dL)	Total protein (g/dL)	Albumin (g/dL)	Cholesterol (IU)
Control	99 \pm 31	5.82 \pm 1.25	3.32 \pm 0.05	62 \pm 7
Da	104 \pm 37	5.60 \pm 0.22	3.17 \pm 0.22	61 \pm 2.1
PEG-IFN + Ri	94 \pm 29	6.10 \pm 0.14	3.40 \pm 0.16	60 \pm 4.2
Fibrosis	96 \pm 24	5.70 \pm 0.92	3.17 \pm 0.43	63 \pm 5.6
PEG-IFN + Ri + Da	136 \pm 27	4.32 \pm 0.78	2.32 \pm 0.61 ^b	135 \pm 12 ^b
Fibrosis + PEG-IFN + Ri	142 \pm 35	4.62 \pm 0.35	2.55 \pm 0.55 ^b	78 \pm 17 ^c
Fibrosis + PEG-IFN + Ri + Da	128 \pm 28	3.47 \pm 0.85	1.97 \pm 0.53 ^b	82 \pm 15 ^c

^a Results are expressed as the means \pm SD of experiments performed with duplicate assays of samples from six animals. International units (IU), PEG-interferon (PEG-IFN), ribavirin (Ri), Danazol (DA).

^b $P < 0.05$ vs the control group.

^c $P < 0.05$ vs the fibrosis group.

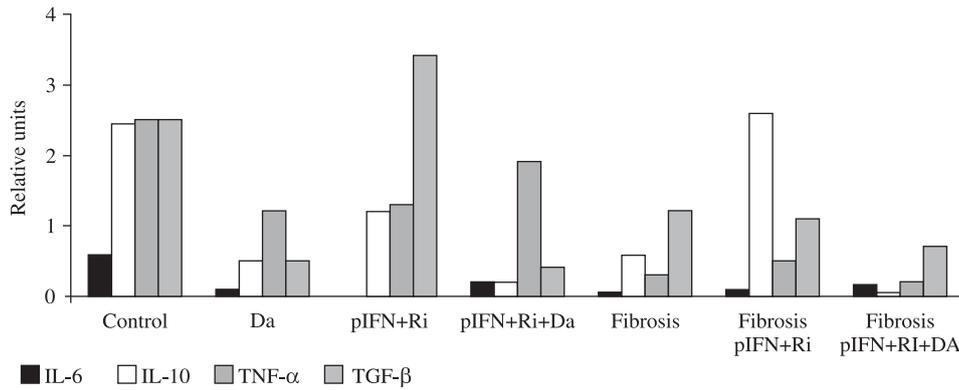


Figure 3. Cytokine mRNA expression in blood lymphocytes. Expression of cytokine mRNA was measured by RT-PCR. Densitometric analysis of PCR products on Southern blots was used for the semiquantitative analysis of cytokine mRNA expression. Relative mRNA levels are expressed as a percentage of the control value. Tumor necrosis factor α (TNF- α), transforming growth factor β (TGF- β), PEG-interferon α -2a (PEG-IFN), ribavirin (Ri), danazol (Da). All groups consisted of six animals.

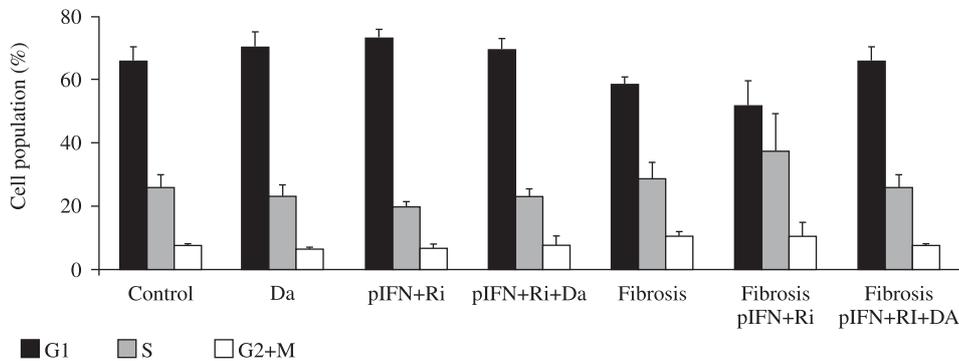


Figure 4. Cell-cycle analysis of bone-marrow cells. Each bar represents the mean \pm SD of experiments performed in duplicate. All groups consisted of 10 animals. PEG-interferon α -2a (PEG-IFN), ribavirin (Ri), danazol (Da).

ies and/or the altered function of splenic macrophage Fc (IgG) receptors. Recently, it was demonstrated that danazol increases thrombopoietin production.³⁸ However, it is possible that, in some cases, the predominant cause of thrombocytopenia is ineffective bone-marrow platelet production rather than accelerated platelet removal. In our study, danazol did not change the phase of the cell cycle in the bone marrow of normal or fibrotic animals, indicating that danazol does not have any effect on hematopoiesis in bone-marrow cells and that another mechanism is responsible for CCl₄-induced liver fibrosis.

It has been known for more than two decades that the use of danazol for the treatment of hematological diseases, cystic disease of the breast, endometriosis, and hereditary angioneurotic edema causes hepatic damage.³⁹⁻⁴¹ Recently, danazol was implicated in the development of cholestatic jaundice, hepatic peliosis, and liver tumor.⁴²⁻⁴⁴ The mechanism of danazol-induced liver injury is unclear. However, massive zone 3 necrosis of the liver has been demonstrated. This striking zonal liver necrosis is consistent with liver damage caused by other drugs or toxins.^{41,44} Three aspects of the use of danazol in these studies are worthy of mention: (1) in most reports, the schedule of danazol therapy varied between 3.0 mg/kg per day and 8.5 mg/kg per day; (2) danazol was administered for long periods of time (months to years); and (3) most authors indicated that the cholestasis and liver injury resolved completely after the withdrawal of danazol therapy.⁴⁵⁻⁴⁷ Taken together, these facts suggest that

hepatotoxicity may develop at various times during therapy with high doses of danazol.

This study demonstrates that animals with fibrosis that received conventional therapy and danazol had increased platelet counts, improved liver function, and ameliorated fibrosis. We treated the animals with 0.83 mg/kg per day of danazol for four weeks and did not observe any changes in liver function or liver morphology. In contrast, animals with fibrosis that were treated with danazol plus PEG-IFN and ribavirin showed an improvement in liver function and a reduction in liver fibrosis. Cicardi et al. previously reported that long-term treatment¹⁵⁻⁴⁷ (months) with low doses of danazol did not induce significant hepatic damage detectable by laboratory tests or liver biopsies in 13 patients with hereditary angioedema.⁴⁸ Conversely, it has recently been shown that there is an association between danazol therapy and hepatocellular carcinoma and hepatitis, but only in patients with a functional deficiency of C1 inhibitor protein.^{43,49} These data suggest that low doses of danazol could be used to treat liver injury and fibrosis without adverse effects.

Hepatic fibrosis is a pathological condition characterized by a marked deposition of collagen and other components of the extracellular matrix in the liver. This eventually results in cirrhosis, because the excessive deposition of extracellular matrix proteins causes hepatic failure resulting from the malfunction of hepatocytes and hemodynamic changes that induce portal hyperten-

sion.^{50,51} It has been reported that therapy with PEG-IFN and ribavirin results in the improvement of serum levels of fibrotic markers both in patients who respond to therapy and in those who do not.^{52,53} Moreover, quantitative histopathological analyses of paired liver biopsy specimens showed some improvement in the degree of fibrosis after therapy, irrespective of the initial virological response.^{12,13} Our results are consistent with those of previous reports.

Most acute and chronic liver diseases are characterized by inflammatory processes and the enhanced expression of various pro- and anti-inflammatory cytokines in the liver. These cytokines are the driving forces behind many inflammatory liver disorders and often induce fibrosis and cirrhosis⁴ because they have synergistic and sometimes antagonistic effects on the immunological and inflammatory processes in the liver.⁵ The combined administration of PEG-IFN and ribavirin leads to HCV elimination and the inhibition of the inflammatory reaction and liver fibrosis in some patients.⁵⁴ Low levels of TNF- α and TGF- β are predictors of sustained responses to therapy with IFN α -2a, alone or in combination with ribavirin.⁵⁵ In contrast, the level of IL-6, which is a marker for reparative liver processes, increases as the inflammatory process abates. Thus, a gradual increase in IL-6 levels is accompanied by lower ALT activity during sustained responses to therapy with PEG-IFN and ribavirin.⁵⁶ The treatment of chronic hepatitis also modulates cytokine levels, inducing an increase in the anti-inflammatory cytokine, IL-10.⁵⁷

In this context, our results contrast with those of previous reports. We observed an increase in IL-10 mRNA levels, but no significant changes in the mRNA levels of IL-6, TNF- α , or TGF- β in the lymphocytes of fibrotic rats that were treated with PEG-IFN plus ribavirin. This disparity may have been caused by the induction of liver injury and fibrosis with a toxic agent in our model. However, an important finding of our study is that combined therapy with danazol plus PEG-IFN and ribavirin altered the pattern of cytokine mRNA expression in animals with fibrosis. There was a substantial reduction in IL-10 mRNA expression and smaller reductions in TNF- α and TGF- β mRNA expression. Previous reports have suggested that danazol can improve some diseases by influencing the function of the immune system.^{18-20,58} Danazol suppresses both spontaneous and activated human lymphocyte-mediated cytotoxicity and decreases the levels of TNF- α and IL-6 in the peritoneal macrophages of infertile patients with endometriosis.^{59,60}

There have been no reports of the use of danazol plus conventional therapy for the treatment of chronic HCV infection or other liver diseases. Our results show that danazol in association with PEG-IFN and ribavirin improves thrombocytopenia and liver injury in rats with fibrosis. According to previous reports, danazol modulates the production and secretion of cytokines in hepatic and

blood cells during liver injury and increases platelet levels by modulating the immune system. In conclusion, the combination of PEG-IFN plus ribavirin and danazol appears to be a new therapeutic option for the treatment of patients with liver injury and fibrosis who display thrombocytopenia.

References

1. Wong JB. Hepatitis C: cost of illness and considerations for the economic evaluation of antiviral therapies. *Pharmacoeconomics* 2006; 24: 661-72.
2. Wong T, Lee SS. Hepatitis C: a review for primary care physicians. *CMAJ* 2006; 174: 649-59.
3. Rockey DC. The cell and molecular biology of hepatic fibrogenesis. Clinical and therapeutic implications. *Clin Liver Dis* 2000; 4: 319-55.
4. Marra F. Chemokines in liver inflammation and fibrosis. *Front Biosci* 2002; 7: 1899-1914.
5. Tilg H, Kaser A, Moschen AR. How to modulate inflammatory cytokines in liver diseases. *Liver Int* 2006; 26: 1029-39.
6. Cacoub P, Renou C, Rosenthal E, Cohen P, Louri I, Loustaud-Ratti Yamamoto, A, et al. Extrahepatic manifestations associated with hepatitis C virus infection. *Medicine* 2000; 79: 47-56.
7. Sène D, Limal N, Cacoub P. Hepatitis C virus-associated extrahepatic manifestations: a review. *Metab Brain Dis* 2004; 19: 357-80.
8. Wang C-S, Yao W-J, Wang S-T, Chang T-T, Chou P. Strong association of hepatitis C virus (HCV) infection and thrombocytopenia: implications from a survey of a community with hyperendemic HCV infection. *Clin Infect Dis* 2004; 39: 790-6.
9. Rajan SK, Espina BM, Liebman HA. Hepatitis C virus-related thrombocytopenia: clinical and laboratory characteristics compared with chronic immune thrombocytopenic purpura. *Br J Hematol* 2005; 129: 818-24.
10. Kajihara M, Kato S, Okazaki Y, Kawakami Y, Ishii H, Ikeda Y, Kuwana M. A role of autoantibody-mediated platelet destruction in thrombocytopenia in patients with cirrhosis. *Hepatology* 2003; 37: 1267-76.
11. Vyzantiadis T, Theodoridou S, Giouleme O, Evgenidis N, Vyzantiadis A, Garipidou V. Serum thrombopoietin levels in thrombocytopenic patients with liver cirrhosis. *Haematologica* 2002; 87: 890-1.
12. Mangia A, Santoro R, Minerva N, Ricci GL, Carretta V, Persico M, Vinelli F, et al. Peginterferon alfa-2b and ribavirin for 12 vs 24 weeks in HCV genotype 2 or 3. *N Engl J Med* 2005; 352: 2609-17.
13. Anatol P, Robert F, Danuta P. Effect of interferon alpha2b plus ribavirin treatment on selected growth factors in respect to inflammation and fibrosis in chronic hepatitis C. *World J Gastroenterol* 2005; 11: 1854-8.
14. Sporea I, Popescu A, Irlu R, Golea O, Totolici C, Dănilă M, Vernic C. Pegylated-interferon alpha 2a treatment for chronic hepatitis C in patients on chronic haemodialysis. *World J Gastroenterol* 2006; 12: 4191-94.
15. Garcia-Suarez J, Burgaleta C, Hernanz N, Albarran F, Tobaruela P, varez-Mon M. HCV-associated thrombocytopenia: clinical characteristics and platelet response after recombinant α 2b-interferon therapy. *Br J Haematol* 2000; 110: 90-103.
16. Dimitroulopoulos D, Dourakis SP, Xinopoulos D, Tsamakidis K, Paraskevas E. Immune thrombocytopenic purpura in a patient treated with interferon alfacon-1. *J Viral Hepatitis* 2004; 11: 477-8.
17. Sevastianos VA, Deutsch M, Dourakis SP, Manesis EK. Pegylated interferon-2b-associated autoimmune thrombocytopenia in a patient with chronic hepatitis C. *Am J Gastroenterol* 2003; 98: 706-7.
18. Mylvaganam R, Ahn Y, Harrington W, Kim CI. Immune modulation by danazol in autoimmune thrombocytopenia. *Clin Immunol Immunopathol* 1987; 42: 281-6.

19. Mylvaganam R, Ahn Y, Garcia R, Kim C, Harrington W. Very low-dose danazol in idiopathic thrombocytopenic purpura and its role as an immune modulator. *Am J Med Sci* 1989; 298: 215-20.
20. Blanco R, Martínez-Taboada VM, Rodríguez-Valverde V, Sánchez-Andrade A, González-Gay MA. Successful therapy with danazol in refractory autoimmune thrombocytopenia associated with rheumatic diseases. *Br J Rheumatol* 1997; 36: 1095-9.
21. Nakhoul IN, Kozuch P, Varma M. Management of adult idiopathic thrombocytopenic purpura. *Clin Adv Hematol Oncol* 2006; 4: 136-153.
22. Stasi R, Pravan D. Management of thrombocytopenic purpura in adults. *Mayo Clin Proc* 2004; 79: 504-22.
23. Cines DB, McKenzie SE, Siegel DL. Mechanisms of action of therapeutics in idiopathic thrombocytopenic purpura. *J Pediatric Hematol Oncol* 2003; 25: S52-S56.
24. Committee on Care and Use of Laboratory Animals of the Institute for Laboratory Animal Resources, Commission on Life Sciences, National Research Council: Guide for the Care and Use of Laboratory Animals. Publication 86-23, revised. *National Academy of Sciences*, Washington DC, EEUU.
25. Rojkind M, Gonzalez E. An improved method for determining specific radioactivities of proline-¹⁴C and hydroxyproline-¹⁴C in collagen and noncollagenous protein. *Anal Biochem* 1974; 57: 1-7 [PMID: 4817495].
26. Chomozynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987; 162: 157-63.
27. Derynck R, Jarrett J, Chen E, Goeddel D. The murine transforming growth factor-beta precursor. *J Biol Chem* 1986; 261: 4377-9.
28. Sun J, Wang XD, Liu H, Xu JG. Ketamine suppresses intestinal NF-kappa B activation and proinflammatory cytokine in endotoxemic rat. *World J Gastroenterol* 2004; 10: 1028-31.
29. Pietsch K, Ehlers S, Jacobs E. Cytokine gene expression in the lungs of BALB/c mice during primary and secondary intranasal infection with *Mycoplasma pneumoniae*. *Microbiology* 1994; 140: 2043-8.
30. Méndez-Herrera MC, Támez L, Candido A, Reyes-Esparza J, Pedernera E. Follicle stimulating hormone increases somatic and germ cell number in the ovary during chick embryo development. *Gen Comp Endocrinol* 1998; 111: 207-15.
31. Poynard T, Imbert-Bismut F, Ratziu V, Chevreton S, Jardel C, Moussalli J, Messous D, et al. GERMED cyt04 group. Biochemical markers of liver fibrosis in patients infected by hepatitis C virus: Longitudinal validation in a randomized trial. *J Viral Hepat* 2002; 9: 128-33.
32. Poynard T, Ratziu V, Charlotte F, Goodman Z, McHutchison J, Albrecht J. Rates and risk factors of liver fibrosis progression in patients with chronic hepatitis C. *J Hepatol* 2001; 34: 730-9.
33. Shimotohno K. Hepatitis C virus and its pathogenesis. *Semin Cancer Biol* 2000; 10: 233-40.
34. Paleka NA, Harrison SA. Extrahepatic manifestations of hepatitis C. *South Med J* 2005; 98: 1019-23.
35. Giannini EG. Review article: thrombocytopenia in chronic liver disease and pharmacologic treatment options. *Aliment Pharmacol Ther* 2006; 23: 1055-1065.
36. Cines DB, Lanchette VS. Immune thrombocytopenia purpura. *N Engl J Med* 2002; 346: 995-1008.
37. Demirturk N, Cevik F, Demirdal T, Aykin N, Aslan V. Autoimmune thrombocytopenia induced by PEG-IFN-alpha plus ribavirin in hepatitis C. *Platelets* 2006; 17: 340-3.
38. Maloisel F, Andres E, Zimmer J, Noel E, Zamfir A, Koumariou A, Dufour P. Danazol therapy in patients with chronic idiopathic thrombocytopenic purpura: long-term results. *Am J Med* 2004; 116: 590-4.
39. Lancia A, Colella F, Nicoletta U, Grandioso P, Di Virgilio D. Hepatic damage after danazol treatment. *Ital J Gastroenterol* 1991; 23: 15-16.
40. Yaginuma T, Okamura T, Takeuchi T, Nishii O, Fujimori R. Preventive effect of traditional herbal medicine, shosaiko-to, on danazol-induced hepatic damage. *Int J Gynaecol Obstet* 1989; 29: 337-4.
41. Silva MO, Reddy KR, McDonald T, Jeffers LJ, Schiff ER. Danazol-induced cholestasis. *Am J Gastroenterol* 1989; 84: 426-8.
42. Alvaro D, Piat C, Francia C, Franchitto A, Furfaro S, Valente C, Capocaccia L, et al. Ultrastructural features of danazol-induced cholestasis: a case study. *Ultrastruct Pathol* 1996; 20: 491-5.
43. Estrada Rodríguez JL, Jorquera Plaza, Gozalo Reques F, Álvarez Puebla MJ. Hepatitis por danazol en paciente con déficit funcional de C1 inhibidor. *Anales de Medicina Interna* 2001; 18: 605-6.
44. Confavreux C, Se'vé P, Broussole C. Danazol-induced hepatocellular carcinoma. *Q J Med* 2003; 96: 315-8.
45. Alvaro D, Piat C, Francia C, Franchitto A, Furfaro S, Valente C, Capocaccia L, et al. Ultrastructural features of danazol-induced cholestasis: a case study. *Ultrastruct Pathol* 1996; 20: 491-5.
46. Bartley J, Loddenkemper C, Lange J, Mechsner S, Radke C, Neuhaus P, Ebert AD. Hepatocellular adenoma and focal nodular hyperplasia after long-term use of danazol for endometriosis: a case report. *Arch Gynecol Obstet* 2004; 269: 290-3.
47. Hayashi T, Takahashi T, Minami T, Akaike J, Kasahara K, Adachi M, Hinoda Y, et al. Fatal acute hepatic failure induced by danazol in a patient with endometriosis and aplastic anemia. *J Gastroenterol* 2001; 36: 783-6.
48. Cicardi M, Bergamaschini L, Tucci A, Agostoni A, Tornaghi G, Coggi G, Colombi R, et al. Morphologic evaluation of the liver in hereditary angioedema patients on long-term treatment with androgen derivatives. *J Allergy Clin Immunol* 1983; 72: 294-8.
49. Monnier N, Ponard D, Duponchel C, Csopaki F, Bouillet L, Tosi M, Lunardi J, et al. Characterisation of a new C1 inhibitor mutant in a patient with hepatocellular carcinoma. *Mol Immunol* 2006; 43: 2161-8.
50. Friedman SL. Mechanisms of disease: Mechanisms of hepatic fibrosis and therapeutic implications. *Nat Clin Pract Gastroenterol Hepatol* 2004; 1: 98-105.
51. Pinzani M, Rombouts K, Colagrande S. Fibrosis in chronic liver diseases: diagnosis and management. *J Hepatol* 2005; 42: S22-S36.
52. Castet V, Fournier C, Soulier A, Brillet R, Coste J, Larrey D, Dhumeaux D. Alpha interferon inhibits hepatitis C virus replication in primary human hepatocytes infected *in vitro*. *J Virol* 2002; 76: 8189-99.
53. Poynard T, McHutchison J, Manns M, Trepo C, Lindsay K, Goodman Z, Ling MH, et al. Impact of pegylated interferon alfa-2b and ribavirin on liver fibrosis in patients with chronic hepatitis C. *Gastroenterology* 2002; 122: 1303-13.
54. Horie S, Harada T, Mitsunari M, Taniguchi F, Iwabe T, Terakawa N. Progesterone and progestational compounds attenuate tumor necrosis factor alpha-induced interleukin-8 production via nuclear factor kappaB inactivation in endometriotic stromal cells. *Fertil Steril* 2005; 83: 1530-5.
55. Neuman MG, Benhamou J, Malkiewicz IM, Akremi R, Shear NH, Asselah T, et al. Cytokines as predictors for sustained response and as markers for immunomodulation in patients with chronic hepatitis C. *Clin Biochem* 2001; 34: 173-82.
56. Kowala-Piaskowska A, Mozer-Lisewska I, Figlerowicz M, Machowska L, Szuzewski W. Interleukin 6 and 12, alanine aminotransferase activity, and HCV viral load in children with chronic hepatitis C treated with interferon and ribavirin. *Inflammation* 2004; 28: 320-4.
57. Marín-Serrano E, Rodríguez-Ramos C, Diaz F, Martín-Herrera L, Giron-Gonzalez JA. Modulation of the anti-inflammatory interleukin 10 and of proapoptotic IL-18 in patients with chronic hepatitis C treated with interferon alpha and ribavirin. *J Viral Hepat* 2006; 13: 230-34.
58. Vigano P, Di Blasio AM, Busacca M, Sabbadini MG, Vignali M. Danazol suppresses both spontaneous and activated human lymphocyte-mediated cytotoxicity. *Am J Reprod Immunol* 1992; 28: 38-42.
59. Matalliotakis I, Neonaki M, Zolindaki A, Hassan E, Georgoulis V, Koumantakis E. Changes in immunologic variables (TNF-a, sCD8 and sCD4) during danazol treatment in patients with endometriosis. *Int J Fertil Womens Med* 1997; 42: 211-4.
60. Liu Y, Luo L, Zhao H. *In vitro* effect of danazol on cytokine production of macrophages in peritoneal fluid of infertile patients with endometriosis and its relationship with cytosolic free calcium concentration. *Zhonghua Fu Chan Ke Za Zhi* 2000; 35: 479-81.