

Pegylated interferon-alpha plus taurine in treatment of rat liver fibrosis

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CONCLUSION: Peginterferon-alpha 2b exerts anti-fibrotic effects on rat liver fibrosis. It seems ineffective against oxidative stress *in vivo*. Peginterferon-alpha 2b in combination with taurine seems to be an antifibrotic strategy.

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Key words: Liver fibrosis; Liver cirrhosis; Pegylated interferons; Taurine; Oxidative stress; Hepatic stellate cells; Apoptosis; Malondialdehyde; Glutathione peroxidase; Superoxide dismutase

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Abstract

AIM: To investigate the antifibrotic effects of peginterferon-alpha 2b and taurine on oxidative stress markers and hepatocellular apoptosis.

METHODS: Sixty rats with CCl₄-induced liver fibrosis were divided into 4 groups ($n = 15$). Group 1 was left for spontaneous recovery (SR). Groups 2-4 received peginterferon-alpha 2b, taurine, and their combination, respectively, for four weeks. Histological fibrosis scores, histomorphometric analysis, tissue hydroxyproline, tissue MDA, GPx and SOD activities were determined. Activated stellate cells and hepatocellular apoptosis were also evaluated.

RESULTS: The degree of fibrosis decreased in all treatment groups compared to spontaneous recovery group. Taurine alone and in combination with peginterferon-alpha 2b reduced oxidative stress markers, but peginterferon-alpha 2b alone did not. Apoptotic hepatocytes and activated stellate cells were higher in groups 2-4 than in group 1. Combined taurine and peginterferon-alpha 2b further reduced fibrosis and increased activated stellate cell apoptosis, but could not improve oxidative stress more than taurine alone.

INTRODUCTION

Liver fibrosis, an early stage of cirrhosis, occurs as a response to chronic injury from many causes, and is characterized by overproduction and diminished degradation of extracellular matrix proteins in the sinusoids^[1,2]. Excessive matrix proteins are synthesized primarily by hepatic stellate cells (HSCs), which activate and acquire a myofibroblast like phenotype in damaged areas of the liver.

Oxidative stress (OS) is associated with liver fibrosis as evidenced both in experimental^[3-6] and in clinical studies^[7]. Therefore, antioxidant treatment approaches have been studied in a series of experiments. *In vitro* investigations and studies on animals have revealed beneficial effects of antioxidants in terms of reduction in collagen production and matrix accumulation in the liver^[8-11], though, significant improvements in the degree of fibrosis were not found in the clinical setting^[12].

Interferons (IFNs) are a family of cytokines with pleiotropic effects on target cells, including induction of an anti-viral state inhibition of cell growth and modulation of the immune response^[13]. IFN-alpha (2a and 2b) is the hallmark of treatment in viral hepatitis, which is a

major cause of liver cirrhosis and hepatocellular cancer worldwide. Standard IFNs are inferior to the pegylated interferons (peginterferon) in terms of tolerability and attaining sustained response rates in patients with viral hepatitis^[14-16]. On the other hand, IFNs display *in vitro* and *in vivo* antifibrotic actions with IFN-alpha being the least potent^[17-21]. It was reported that the antifibrotic effects of IFN-alpha in rats and patients with hepatitis C virus (HCV) infection are independent from their antiviral activity, i.e., it reduces the degree of fibrosis even when there is no sufficient antiviral response^[10,22]. It was also reported that IFN-alpha decreases the production of reactive oxygen species (ROS) in stimulated hepatocytes and HSCs^[23], and inhibits OS in patients with HCV infection^[24]. However, the activity of recombinant IFN-alpha against oxidative damage has not been confirmed in a rat model of short-term liver injury^[10]. It was reported that peginterferons increase the rates of sustained response in patients with viral hepatitis^[15,25,26], but their antifibrotic behavior has not been examined widely.

Taurine is a potent antioxidant which prevents DNA damage at concentrations normally present in cells^[27]. It was reported that taurine prevents experimental diabetic neuropathy^[28], lead-induced oxidative damage^[29], CCl₄-induced OS^[30] as well as experimental liver fibrosis^[6,11] through antioxidant mechanisms.

Reversibility of liver fibrosis has been defined in animals and proposed to be associated with loss of activated HSCs mainly through apoptosis in the liver^[31,32]. It is also known that the degree of fibrosis decreases after successful treatment of viral hepatitis or haemochromatosis, cessation of alcohol, and biliary decompression^[33]. Therefore, enhancement of spontaneous regression capacity in the liver may contribute to etiological treatment providing faster and better reconstitution of liver functions. In this work, we studied the effects of peginterferon-alpha 2b, taurine and their combination on hepatic histology, activation and apoptosis of HSCs, and OS parameters in rat liver fibrosis. Given the evidence of high regeneration capacity in the liver, efficacy of both agents was compared with spontaneous recovery (SR).

MATERIALS AND METHODS

Animals and study protocol

One hundred male Sprague-Dawley rats (250-400 g) were kept at 20-25°C in a 12 h light/12 h dark cycle with free access to food and water. The animals were injected s.c with 0.2 mL/100 g of CCl₄ (dissolved 1:1 in sterile olive oil) for two consecutive days per week for 12 wk. Development of fibrosis was confirmed by killing three animals every 6 wk. A total of 60 animals survived the induction period and were divided into four groups (*n* = 15). Treatment protocols were as follows: group 1 was left for SR for 4 wk without any treatment, group 2 received 1.5 µg/kg peginterferon-alpha 2b per week^[32], group 3 was treated with 1200 mg/kg taurine^[11] intraperitoneally each day, and group 4 was treated with a combination of these two agents at the same doses. At the end of 4 wk of treatment the animals were killed

under i.p. pentobarbital (Nembutal, Abbott) anesthesia, and their livers were rapidly removed. Liver samples were processed for histological and biochemical studies. The experiments, approved by the Institutional Animal Use and Care Committee of the Gulhane Medical Academy, Ankara, Turkey, were performed according to the criteria of the National Institutes of Health guidelines for the care and handling of animals.

Assessment of fibrosis

Histological scoring: Liver samples were fixed in 4% buffered formaldehyde, embedded in paraffin, and stained with hematoxylin-eosin. Masson's trichrome staining was performed for extracellular matrix evaluation. Histologic evaluation was performed twice by two pathologists blinded to the protocol on five low-power fields per slide. Modified Ishak scoring system was used with minor changes in order to define the degree of fibrosis^[34] (Table 1).

Histomorphometric analysis: Percentage of fibrosis was determined by a computer system composed of the following components: an IBM compatible computer running Microsoft Windows NT 4.0 service pack 6 operating system, a light microscope with motorized stage (Zeiss axioscope, Gottingen, Germany), frame grabber card (Matrox Meteor, Matrox imaging Dorval, Quebec, Canada), a digital camera attached to the microscope (Sony AVT Horn 3 CCD, Tokyo), a 21 inch color video monitor (Philips, Chungli, Taiwan), and a special image analysis software (Karl Zeiss KS 400 version 3.0 for Windows, Zeiss, Gottingen, Germany). With the aid of KS 400 for Windows, a software using a semiautomatic, interactive "macro" (a batch file containing all of the instructions to accomplish a predetermined set of measurements) was prepared.

Measurement was started with selection of the area of interest under a plan 2.5X objective. Then, this area was scanned automatically as a consecutive rectangular area under a plan 20X objective. The fibrotic areas and parenchyma were counted separately and summed up by the software at the end of each measurement. The results were expressed as the percentage of fibrosis.

Measurement of hydroxyproline: Liver tissue hydroxyproline, which represents a reliable indicator of tissue collagen content in the liver, was quantified as previously described^[35]. Small modifications in the duration of incubation of liver tissues in Ehrlich's solution (35 min at 60°C instead of 25 min at 60°C) and dissolving hydroxyproline in the homogenates by concentrated HCl at 110°C were applied.

Measurement of oxidative stress

Liver tissue malondialdehyde (MDA), glutathione peroxidase (GPx) and superoxide dismutase (SOD) measurements were performed as described previously^[36-38] to determine the oxidative status in the liver specimens. MDA levels were expressed as nmol/g tissue. GPx and SOD activities were expressed as U/g tissue dye.

Immunohistochemical tests

Detection of activated stellate cells: Alpha-smooth

Table 1 Histological scoring model

0	No fibrosis or minimal fibrous expansion of occasional portal areas
1	Moderate fibrous expansion of some portal areas with short fibrous septae
2	Moderate fibrous expansion of most portal areas, occasional portal-to-portal bridging
3	None or minimal expansion of portal areas, porto-portal fibrosis, incomplete bridging nodules
4	Marked portal fibrosis, portal to portal fibrosis, occasional micronodules
5	Mild to moderate fibrous expansion in portal areas, micronodules, incomplete cirrhosis
6	Marked fibrosis in portal areas, macro- and micronodules with thick fibrous septae, complete cirrhosis

Table 2 Results of fibrosis, oxidative stress parameters, activated HSC counts and apoptotic cells in groups 1-4

	Spontaneous recovery (n = 15)	Peginterferon-alpha (n = 15)	Taurine (n = 15)	Peginterferon-alpha plus Taurine (n = 15)
Histological score	5 (5-6)	3 (2-4)	2 (1-3)	3 (2-4)
Percentage fibrosis (%)	14.3 ± 0.8	7.9 ± 0.5	7.9 ± 0.6	6.1 ± 0.3
Hydroxyproline (µg/g wet weight)	1.03 ± 0.07	0.58 ± 0.04	0.64 ± 0.03	0.32 ± 0.04
MDA (nmol/g tissue)	47.8 ± 1.0	43.3 ± 1.8	20.2 ± 0.8	18.4 ± 0.6
GPx (U/g tissue)	128 ± 3	168 ± 6	271 ± 13	270 ± 11
SOD (U/g tissue)	343 ± 5	385 ± 8	644 ± 14	619 ± 19
alpha-SMA (+) cell count	120.0 ± 9.4	59.6 ± 2.7	46.7 ± 3.5	32.6 ± 3.0
Apoptotic hepatocyte count	19.1 ± 1.0	31.3 ± 2.6	34.8 ± 3.9	43.9 ± 4.0
Apoptotic activated HSC count	4.6 ± 0.5	14.1 ± 1.5	11.0 ± 0.8	21.5 ± 2.0

MDA: Malondialdehyde; GPx: Glutathione peroxidase; SOD: Superoxide dismutase; alpha-SMA: alpha-smooth muscle actin.

muscle actin (alpha-SMA) immunostaining was used to detect and count the activated HSCs^[39]. For this purpose, the tissue samples were stained for monoclonal rat anti-alpha-SMA (Sigma) with lbeled streptavidin-biotin (LSAB, Dako) complex method as described elsewhere^[31], and analyzed by two blinded observers for the number of apoptotic HSCs in 30 random high-power fields in each specimen.

Detection of apoptotic hepatocytes and hepatic stellate cells: Hepatocytes and activated HSCs undergoing apoptosis were detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining^[11,31] using a commercial ready-to-use kit (In situ cell death detection kit, AP, Roche Diagnostics, Germany) as previously described^[32]. Slides were analyzed by two blinded observers who counted the number of TUNEL positive hepatocytes and HSCs in 30 random high-power fields from each specimen.

Statistical analysis

Parametric results were expressed as mean ± SE, and the significance of differences was assessed by one-way ANOVA and Tukey's HSD as post hoc. Nonparametric results (histological scores) were expressed as median and percentiles (25%-75%) and assessed by Mann-Whitney U test. Correlation analysis was assessed with Pearson Correlation procedure. The differences were accepted as statistically significant when *P* value was less than 0.05.

RESULTS

Effects on fibrosis

Histological scores: Peginterferon-alpha 2b (group 2), taurine (group 3) and their combination (group 4) reduced

fibrosis scores when compared to SR group (group 1) (*P* < 0.01) (Table 2). Fibrosis scores were similar in groups 2-4. Combination treatment did not reduce fibrosis scores compared to individual treatments. The histological sections of fibrotic rat livers from four groups are shown in Figure 1.

Histomorphometry: The percentage of fibrotic areas was lower in groups 2-4, in line with histological scores and hydroxyproline measurement than in group 1 (*P* < 0.01), whereas it was similar in individual treatment groups. Combined treatment reduced percent fibrosis significantly when compared to peginterferon-alpha 2b or taurine alone (*P* < 0.05) (Table 2).

Hydroxyproline: Liver tissue hydroxyproline decreased in groups 2-4 when compared to group 1 (*P* < 0.01) (Table 2). Hydroxyproline contents were similar in groups 2 and 3. Combination treatment reduced tissue hydroxyproline more significantly than individual treatments (*P* < 0.05 and *P* < 0.01 for combination *vs* peginterferon-alpha 2b and taurine, respectively).

Effects on oxidative stress

Compared to group 1, liver tissue MDA levels decreased, and GPx and SOD activities increased in groups 3 and 4 (*P* < 0.01), but not in group 2 (Table 2). Similar results were also obtained in groups 3 and 4 when compared to group 2 (*P* < 0.01). Decrease in MDA and increase in GPx and SOD activities in groups 3 and 4 were comparable.

Correlation analysis

We evaluated the predictive value of histological fibrosis scores or OS markers regarding hydroxyproline content and found that the amount of liver tissue hydroxyproline content correlated with the percentage of fibrosis and

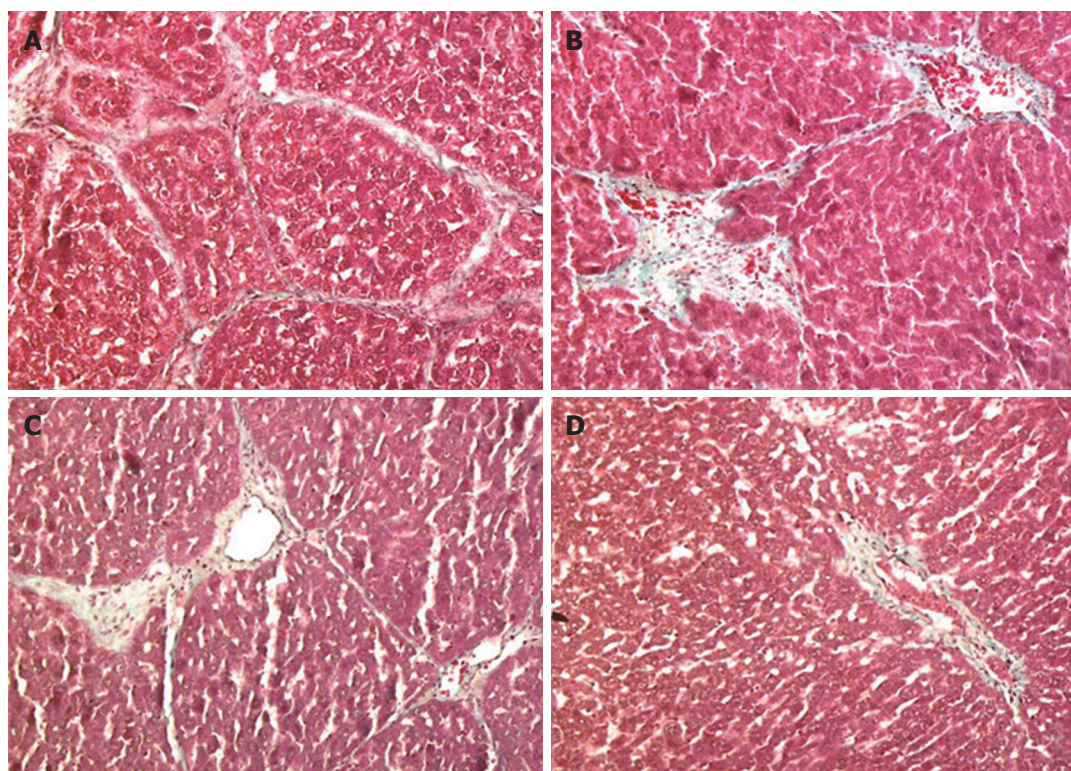


Figure 1 Sections of fibrotic rat livers from four groups. **A:** Mild-moderate fibrous-expansion in portal areas and several micronodules in group 1 (Masson's trichrome stain, $\times 100$); **B:** Moderate expansion of portal areas and porto-portal fibrosis in group 2 (Masson's trichrome stain, $\times 200$); **C:** Moderate fibrous expansion of most portal areas and porto-portal bridging in group 3 (Masson's trichrome stain, $\times 200$); **D:** Mild fibrous expansion of some portal areas in group 4 (Masson's trichrome stain, $\times 200$).

histological score as well as tissue MDA, GPx and SOD ($P < 0.05$).

Effects on the number of alpha-smooth muscle actin positive HSCs

The number of alpha-SMA (+) HSCs was lower in groups 2-4 than in group 1 ($P < 0.01$). Groups 2 and 3 had similar alpha-SMA (+) HSC counts ($P > 0.05$) (Table 2). The number of alpha-SMA (+) HSCs was lower in group 4 than in group 2 ($P < 0.05$), whereas no difference was found between groups 4 and 3 ($P > 0.05$).

Effects on the number of apoptotic hepatocytes and activated hepatic stellate cells

TUNEL positive hepatocytes were comparable in groups 2 and 3 (Table 2). The number of hepatocytes undergoing apoptosis was higher in groups 2-4 than in group 1 ($P < 0.05$, respectively). The apoptotic hepatocyte number was also higher in group 4 than in group 2 ($P < 0.05$). There was no difference between groups 3 and 4.

The number of alpha-SMA (+) apoptotic HSCs (dually stained with anti-alpha-SMA and TUNEL technique) was increased in groups 2-4 in comparison with group 1 ($P < 0.05$). The sinusoidal lining as well as activated and apoptotic stellate cells are shown in Figure 2. Combined treatment increased activated HSC apoptosis more significantly than either peginterferon-alpha 2b or taurine alone ($P < 0.01$). Apoptotic HSC count was similar in groups 2 and 3.

The cause(s) and therapeutic alternatives for liver fibrosis are shown in Figure 3.

DISCUSSION

In the present investigation, peginterferon-alpha 2b and

taurine improved experimental liver fibrosis in an equal pattern, and their combination yielded further benefits. Improvement of fibrosis in the liver was accompanied with decrease in OS as well as increase in the number of activated HSCs undergoing apoptosis. This study was designed to investigate the therapeutic efficacy, but not the preventive effects of peginterferon-alpha 2b and taurine in chronically injured liver.

Involvement of OS in activation of HSCs and initiation of liver fibrosis has been well described^[40,41]. Moreover, an increased state of oxidative activity^[24,42-44] and decreased antioxidant levels^[45] have also been shown in patients with chronic HCV infection and cirrhosis. Upon injury, major reactive oxygen intermediates (ROIs) and aldehydic end products of lipid peroxidation, which are released by injured hepatocytes and activated inflammatory cells such as neutrophils, macrophages and Kupffer cells, deteriorate cellular signal transduction as well as proliferative and functional responses^[46]. Macrophages, known to activate with a variety of stimuli including exposure to abnormally deposited collagenous matrix^[47-49], have recently been shown to play an important role in both fibrogenesis and its resolution in the liver. It was reported that ROIs released by these cells can limit activation of cytotoxic lymphocytes by IFN-alpha^[50], suggestive of usefulness of antioxidants, such as taurine, in enhancing immunostimulatory efficiency of this agent.

In chronic liver diseases, an imbalance occurs between the antioxidant defenses and OS, which may result in cytotoxicity, apoptotic cell death and fibrosis^[51]. It was reported that several antioxidants can prevent activation of HSCs, decrease collagen synthesis and improve fibrosis in the liver of animals^[9-11,52]. However, the results provided by clinical trials with antioxidants are less impressive^[53], in particular, when histological changes are concerned^[7,12,54].

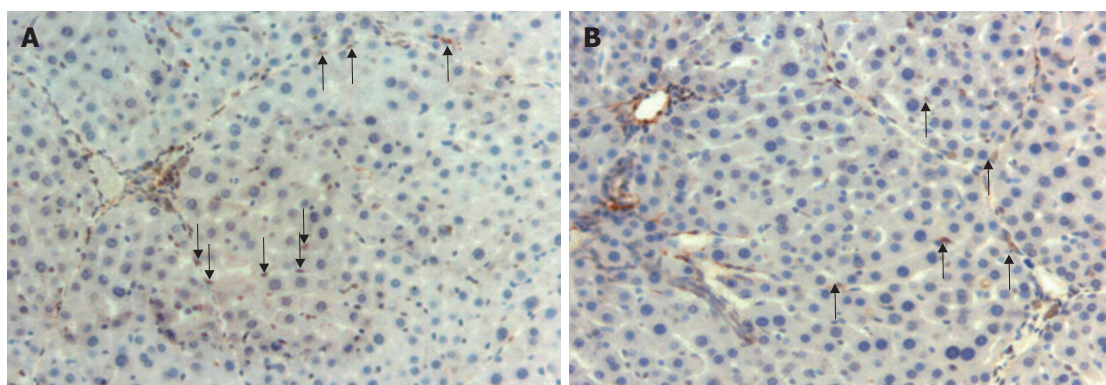


Figure 2 Activated and apoptotic hepatic stellate cells. **A:** α -SMA (+) stellate cells in the sinusoids in a section from group 3 (arrows) ($\times 200$); **B:** Apoptotic activated stellate cells positively stained with TUNEL technique in the sample from group 2 (arrows) ($\times 200$).

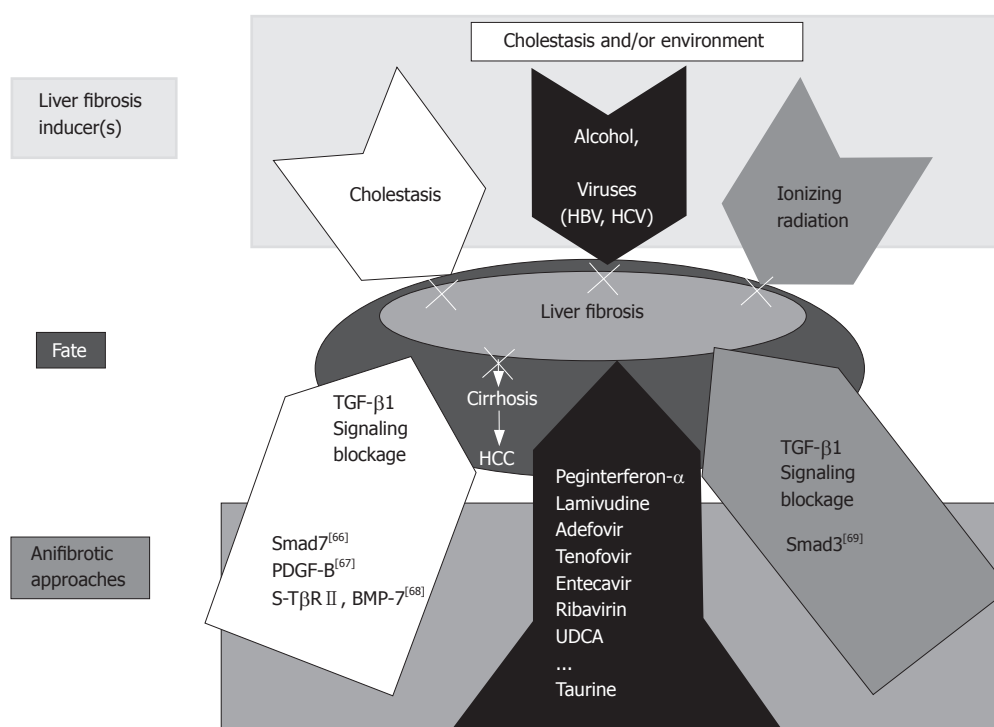


Figure 3 Working hypothesis: cause(s) and therapeutic alternatives for liver fibrosis. In the present study, a combined peginterferon- α plus taurine treatment for fibrotic livers was applied successfully. Though different etiologies are responsible for chronic liver diseases, viruses and alcohol prevail worldwide. Our results can represent a therapeutic option to prevent progression of the underlying disease to fibrosis in the liver. Other experimental models are also presented including TGF- β 1 signaling blockage by Smad7 overexpression^[66], PDGF-B^[67], and a soluble receptor TGF- β 2 (T β R II) and BMP-7^[68]. Liver fibrosis affected by ionizing radiation can be inhibited by Smad knock out [Smad3 (-)]^[69].

Nevertheless, marked improvements in OS parameters reported in experimental studies indicate that these agents work in the liver. But, to get clearer results on fibrosis, optimal starting time, dose and duration of the treatment should be fully established. Of note, more potent antioxidants and combined treatment approaches with agents with proven antifibrotic activity might also be more useful.

Taurine is a potent antioxidant which has been shown to improve liver histology in experimental conditions mainly through decreasing the production of ROS^[6,11]. The present study confirms the previous findings that showed taurine's ability to exert an antioxidant activity at the tissue level in fibrotic rat livers. Therefore, histological recovery in the taurine-treated group in the present study could be attributed to the decrease in OS. Indeed, increased levels of OS markers exist not only in the early stages of liver diseases but also in advanced states such as cirrhosis^[43]. Moreover, although experimental studies have revealed controversial results^[55], it is possible that natural defense mechanisms against oxidant injury may fail to prevent progression of the disease further. Thus, attempts to

decrease OS even in the advanced stages of liver diseases could be beneficial. However, Look *et al*^[12] reported that decreases in markers of OS is not accompanied with histological improvement, though the duration of treatment in their study was relatively short for adaptation of antioxidant systems.

Since there is evidence that supports the existence of an increased oxidative status in patients with chronic viral hepatitis^[42,43], several authors have studied the effects of IFN treatment on the parameters of OS. In dimethylnitrosamine-induced liver fibrosis, recombinant IFN- α was found to exert no significant antioxidant activity^[10]. In contrast, Lu *et al*^[23] have reported *in vitro* antioxidant effects of IFN- α on isolated rat hepatocytes and HSCs. Though the exact mode of antifibrotic action of IFN- α has not been fully elucidated to date, a so called direct antioxidant effect may also be involved in the mechanism of its antifibrotic features. However, the present *in vivo* investigation also confirms that peginterferon- α 2b does not display any effect on markers of OS in the liver.

Our results indicate that combination of peginterferon-

alpha 2b with taurine could result in significant improvement in the degree of fibrosis, but could not enhance the resultant antioxidant efficacy. Several experimental and clinical studies partly confirming our results have previously addressed the biochemical and histological changes after treatment with IFN-alpha combined with antioxidants. Addition of N-acetylcysteine could not result in further decrease in liver collagen content in dimethyl nitrosamine-induced rat fibrosis^[10]. In a pilot study, similar results were also found in patients with HCV infection treated with standard IFN-alpha plus antioxidants. But, no further decrease was observed in the serum markers of OS when compared to IFN-alpha alone^[12]. However, a decrease in OS correlated with fibrosis grade has been reported in IFN-alpha plus antioxidant-treated hepatitis C patients^[24]. These contradictory reports suggest that more reliable data are needed regarding the clinical expression of IFN effects over oxidative damage. Moreover, as yet there is no clinical study on combination of peginterferon-alpha 2b with an antioxidant.

It was reported that liver fibrosis can regress spontaneously mainly through increased rate of apoptosis of activated HSCs^[31]. Therefore, inducing apoptosis of activated HSCs in the liver might be an effective treatment approach. It has been shown that taurine acts as an antiapoptotic agent in some types of cells under different conditions^[56,57]. However, no effect on isolated HSCs^[58] and even *in vivo* proapoptotic effects^[11] were reported. Also in the present study, taurine induced apoptosis of hepatocytes and activated HSCs in the resolution period of liver fibrosis, which possibly resulted from the decrease in the degree of OS. ROIs released from resident liver cells and activated inflammatory cells like macrophages are potential inducers of apoptosis in the healing liver. While severe OS results in rapid cell necrosis, less severe OS has been suggested to cause liver cell apoptosis^[51], which is a desired and a proposed condition in recovery of liver fibrosis^[31]. Therefore, reducing OS may prevent liver cell loss by necrosis, but induce programmed death of phenotypically changed cells in the liver.

There is also a delicate interaction of ROIs with nitric oxide (NO), which is produced by liver cells, including HSCs, as well as other cell types such as macrophages in conditions with chronic inflammation^[59]. NO can reduce liver injury by decreasing generation of OH^[60] and lipid peroxidation products^[61] with a so-called antioxidant activity. However, in the states of increased OS where high levels of ROIs are present, NO can interact with ROIs to produce cytotoxic compounds^[61]. Similarly, increased levels of NO can lead to apoptosis of several cell types in the presence of superoxide anions that react with NO to produce peroxynitrite, which seems to be an inducer of apoptosis^[59,62]. It was reported that NO synthesis is up-regulated *in vitro* in IFN-alpha treated monocytes through induction of nitric oxide synthase^[63]. It was also reported that IFN-treated HCV patients have significantly higher nitric oxide synthase activity in their mononuclear cells, and serum nitrite and nitrate levels are increased in IFN-alpha responder patients^[64]. Moreover, though activated HSCs play an *in vitro* direct antiapoptotic role in liver diseases^[65], IFN-alpha-associated increase in NO

concentration might induce apoptosis of these cells. The increased number of hepatocytes and HSCs undergoing apoptosis in peginterferon-alpha 2b and taurine-treated animals as well as in the combined treatment group in our study seems to be associated with multiple, but as yet unidentified mechanisms.

In conclusion, the results of this study indicate that peginterferons, like the conventional IFNs, effectively reduce fibrosis in a rat model of liver fibrosis. But, peginterferon-alpha 2b has no antioxidant action. The reduction in OS is accompanied with an increase in activated HSC apoptosis. The additive antifibrotic effects of peginterferons with taurine may have clinical implications.

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