# Melatonin limits the expression of profibrogenic genes and ameliorates the progression of hepatic fibrosis in mice

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**We investigated whether melatonin ameliorates fibrosis and limits the expression of fibrogenic genes in mice treated with carbon tetrachloride ($CCl_4$). Mice in treatment groups received $CCl_4$ 5 $\mu$L/g body weight intraperitoneally twice a week for 4 or 6 weeks. Melatonin was given at 5 or 10 mg/kg/d intraperitoneally, beginning 2 weeks after the start of $CCl_4$ administration. Treatment with $CCl_4$ resulted in fibrosis evidenced by the staining of Van Gieson and $\alpha$-smooth muscle actin ($\alpha$-SMA) positive cells in the liver. At both 4 and 6 weeks, $CCl_4$ induced an increase in the messenger RNA levels of collagens I and III, transforming growth factor (TGF)-$\beta$, platelet-derived growth factor (PDGF), connective tissue growth factor (CTGF), amphiregulin, matrix metalloproteinase (MMP)-9, and tissue inhibitor of metalloproteinase (TIMP)-1. Protein concentrations of CTGF, amphiregulin, MMP-9, TIMP-1, and phospho-Smad3 were also significantly augmented in fibrotic mice. Melatonin successfully attenuated liver injury, as shown by histopathology and decreased levels of serum transaminases. Immunohistochemical staining of $\alpha$-SMA indicated an abrogation of hepatic stellate cell activation by the indol. Furthermore, melatonin treatment resulted in significant inhibition of the expression of collagens I and III, TGF-$\beta$, PDGF, CTGF, amphiregulin, and phospho-Smad3. The MMP-9 activity decreased and the expression of nuclear factor erythroid–2–related factor 2 (Nrf2) increased in mice receiving melatonin. Data obtained suggest that attenuation of multiple profibrogenic gene pathways contributes to the beneficial effects of melatonin in mice with $CCl_4$-induced liver fibrosis. (Translational Research 2015;165:346–357)**

**Abbreviations:** $\alpha$-SMA = $\alpha$-smooth muscle actin; $CCl_4$ = carbon tetrachloride; CTGF = connective tissue growth factor; HSC = hepatic stellate cell; MMP-9 = matrix metalloproteinase 9; Nrf2 = nuclear factor erythroid 2–related factor 2; PDGF = platelet-derived growth factor; TGF-$\beta$ = transforming growth factor $\beta$; TIMP-1 = tissue inhibitor of metalloproteinase 1

Hepatic fibrosis is a reversible wound-healing response to either acute or chronic cellular injury from a wide variety of etiologies, characterized by an excessive deposition of extracellular matrix (ECM) resulting in liver dysfunction and irreversible cirrhosis. During liver fibrogenesis, hepatic stellate cells (HSCs) undergo activation to a $\alpha$-smooth muscle actin (SMA)-positive myofibroblastic phenotype and synthesize excess ECM components, particularly collagen.[1] Among the numerous

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## AT A GLANCE COMMENTARY

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#### Background

Melatonin reduces liver damage in animal models of experimentally induced liver fibrosis such as carbon tetrachloride administration. However, changes in the expression of fibrogenic factors have not been tested, and only a preventive effect before the onset of liver toxicity has been demonstrated.

#### Translational Significance

Melatonin given 2 weeks after the start of chronic carbon tetrachloride treatment delays the development of fibrosis in mice through effects involving the inhibition of hepatic stellate cell activation, the suppression of various profibrogenic mediators, and the promotion of extracellular matrix degradation. Results suggest that melatonin might be an effective antifibrotic drug in the prevention of liver disease progression.

profibrogenic factors, transforming growth factor (TGF)-$\beta$ is a key mediator that activates Smad2/3 to induce fibrosis. Other cytokines, such as platelet-derived growth factor (PDGF) or connective tissue growth factor (CTGF), and the epidermal growth factor receptor amphiregulin, play an important fibrogenic role.[2] Moreover, fibrogenesis is a dynamic process involving not only net accumulation of ECM but also its ongoing remodeling by proteases, including the balance between matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinase (TIMPs).[3]

Recent clinical and experimental evidence indicates that hepatic fibrosis may be reversed on removal of the underlying etiologic agent.[4] The prospect that fibrosis is reversible has generated great interest for researchers to develop antifibrotic therapies, although an effective therapeutic approach is still required and there is a need for searching antifibrotic strategies that can prevent, halt, or reverse hepatic fibrosis. Oxidative stress aggravates liver fibrosis.[5] Thus, inhibiting oxidative stress has been considered a potential useful strategy to prevent the development of hepatic fibrogenesis, and it has been reported that antioxidants such as epigallocatechin-3-gallate,[6] polyprenols,[7] quercetin,[8] or proanthocyanidin,[9] among others, may prevent liver injury in different animal models of fibrosis.

Melatonin is a versatile molecule endowed with an abrogated activation of HSCs induced by reactive oxygen species in vitro,[10] and different studies have shown that the pineal hormone prevents liver damage in rats with fibrosis induced by bile duct ligation,[11] dimethylnitrosamine,[12] or thioacetamide.[13] The most commonly used approach to cause experimental liver fibrosis is the periodic administration of carbon tetrachloride ($CCl_4$) in mice or rats.[14] $CCl_4$-induced liver fibrosis in rodents can be completely resolved within several weeks after withdrawal of the toxic treatment, and it resembles all important properties of human liver fibrosis, including inflammation, regeneration, fiber formation, and potentially fibrosis regression.[15] Using this toxin-mediated model, it has been found that melatonin administration, at doses ranging from 2.5 to 20 mg/kg body weight, prevents liver histopathologic changes, reduces hepatic hydroxyproline content, inhibits oxidative stress and apoptosis, increases antioxidant enzyme levels, or reduces proinflammatory cytokine production, when administered intraperitoneally to rats or mice.[16-22]

However, in these in vivo studies, effects of melatonin on the activation of HSCs and changes in the expression of fibrogenic factors or molecules involved in ECM degradation have not been tested. Moreover, because melatonin was always given before or in parallel to $CCl_4$ administration, only a preventive effect before the onset of liver toxicity was demonstrated. Thus, in the present research, it was decided to assess if melatonin treatment, beginning 2 weeks after the start of the toxic injection to allow initial activation of HSCs, could attenuate the development of liver fibrosis in the progression of chronic $CCl_4$-induced liver injury in mice. HSCs' turnover, ECM components, profibrogenic cytokines, and molecules involved in ECM degradation were evaluated. We showed that melatonin treatment impaired HSC activation, reduced the MMP-9 activity, and resulted in a significant inhibition of the expression of profibrogenic factors in a dose dependent-manner, leading to the improvement in liver function and amelioration of fibrosis.

## MATERIAL AND METHODS

**Animal experiments and drug treatment.** Male C57BL/6J mice (Harlan Laboratories, Barcelona, Spain) weighing 20–25 g were used in this study. The animals were acclimated to the temperature (22 $\pm$ 2°C) and humidity (55 $\pm$ 5%) of controlled rooms with a 12–12 hour light-dark cycle for at least a week before experiments. They were allowed access to mice chow and water ad libitum. Mice in treatment groups received $CCl_4$ at a dose of 5 $\mu$L/g body weight (10% $CCl_4$ in corn oil) via intraperitoneal injection twice a week for 4 or

6 weeks. Melatonin (Sigma, St. Louis, Missouri) was administered via intraperitoneal injection (5 or 10 mg/kg/d), beginning 2 weeks after the start of $CCl_4$ administration. Melatonin was dissolved into absolute ethanol and further dilutions were made in saline; the final concentration of ethanol was 5%. Mice that received corn oil injection or melatonin injection only served as sham controls. Each group consisted of 8 mice. The study protocol was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and was specifically approved by the Ethics Committee of the University of León. At the end of the experiment, mice were anesthetized with ketamine-xylazine cocktail and sacrificed. Serum samples were collected from each mouse and stored at −80°C to determine the serum biochemical parameters. Livers were harvested 24 hours after the last injection of $CCl_4$ for 3 uses: (1) fixed with 10% buffered formalin for histologic examinations; (2) preserved at −80°C for Western blot; and (3) homogenized in Trizol for RNA isolation.

**Biochemical determinations.** The levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in serum of mice were determined in the Instrumental Techniques Laboratory of the University of León using standard techniques.

**Histologic analysis.** Tissue samples were recovered, fixed in 10% buffered formalin, and embedded in paraffin. For the microscopic analysis, the liver fragment slides were stained with Van Gieson. Fibrosis was quantified with WinRoof version 6.3 software (Visual System Division, Mitani Corporation) by analyzing 10 nonconsecutive and randomly histologic fields. Results were expressed as the percentage of fibrotic area in each field.

**Immunohistochemical staining.** Immunohistochemistry using polyclonal antibody $\alpha$-SMA was performed as a marker of activated HSCs. Tissue samples were recovered, fixed in 10% buffered formalin, and embedded in paraffin. Sections (4 $\mu$m) were dewaxed and hydrated through graded ethanol, cooked in 25 mM citrate buffer, pH 6.0, in a pressure cooker for 10 minutes, transferred into boiling deionized water, and let to cool for 20 minutes. Tissue sections were then treated with 3% hydrogen peroxide to inactivate endogenous peroxidase activity. The slides were incubated with antibody $\alpha$-SMA (Abcam, Cambridge, UK) at its working dilution of 1:200 overnight at 4°C.[23] The specificity of the technique was evaluated by negative controls (omitting the incubation with the primary antibody and incubating it with nonimmune sera). Areas staining positive for $\alpha$-SMA were analyzed by WinRoof version 6.3 software with 10 nonconsecutive randomly chosen histologic fields. Results were expressed as the percentage of stained area in each field.

**Real-time Reverse Transcription-Polymerase Chain Reaction (RT-PCR).** Total RNA was obtained from frozen mouse liver using a Trizol reagent (Life Technologies, Madrid, Spain) and quantified using a Nano Drop1000 spectrophotometer (Thermo Scientific, Wilmington, Delaware). Residual genomic DNA was removed by incubating RNA with RNA Quantified (RQ1) RNase-free DNase (Promega, Madison, Wisconsin). RNA integrity was confirmed by formaldehyde gel electrophoresis. Total RNA (1 $\mu$g) was reverse transcribed as described[24] and messenger RNA (mRNA) was determined by real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis using Taqman Universal PCR MasterMix (Roche Diagnostics GmbH, Mannheim, Germany). Taqman primers and probes for collagen I (GenBank accession no. NM_007742.3 and Mm00801666_g1), collagen III (GenBank accession no. NM_009930.2 and Mm01254476_m1), TGF-$\beta$ (GenBank accession no. NM_009367.3 and Mm00436955_m1), PDGF (GenBank accession no. NM_011057.3 and Mm00440677_m1), CTGF (GenBank accession no. NM_010217.2 and Mm01192933_g1), MMP-9 (GenBank accession no. NM_013599.2 and Mm00442991_m1), TIMP-1 (GenBank accession no. NM_0001044384.1 and Mm00441818_m1), and glyceraldehide-3-phosphate dehydrogenase (GenBank accession no. NM_008084.2 and Mm99999915_g1) genes were derived from the commercially available TaqMan Gene Expression Assay (Applied Bio-systems). Relative changes in gene expression levels were determined using the $2^{-\Delta\Delta Ct}$ method.[25,26] The cycle number at which the transcripts were detec-table (Ct) was normalized to the cycle number of glyceraldehide-3-phosphate dehydrogenase gene detection, referred to as $\Delta$Ct.

**Western blot analysis.** For Western blot analysis, liver tissue (25 mg) was homogenized in 1 mL radioimmunoprecipitation assay buffer (RIPA) buffer containing protease and phosphatase inhibitor cocktails (Roche Diagnostics GmbH), maintaining temperature at 4°C throughout all procedures. Then the homogenate was incubated on ice for 30 minutes and finally the samples were centrifuged at 13,000 × $g$ for 30 minutes at 4°C. The supernatant fraction was stored at −80°C in aliquots until use. Protein concentration was measured by Bradford assay. Equal amounts of protein extracts (30 $\mu$g) were separated by 7%–12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred electrically to polyvinylidene difluoride membranes (Millipore, Bedford, Massachusetts). The membranes were then blocked with 5% nonfat dry

**Table I.** Effect of $CCl_4$ and treatment with Mel on serum ALT and AST levels

| Marker | Control | Control + Mel | $CCl_4$ 4 wk | $CCl_4$+Mel5 4 wk | $CCl_4$+Mel10 4 wk | $CCl_4$ 6 wk | $CCl_4$+Mel5 6 wk | $CCl_4$+Mel10 6 wk |
|---|---|---|---|---|---|---|---|---|
| ALT (IU/L) | 25.6 ± 0.5 | 22.3 ± 0.7 | 1474 ± 152* | 753 ± 198*,† | 499 ± 70*,†,‡ | 3915 ± 587* | 1917 ± 529*,†,‡ | 1473 ± 242*,† |
| AST (IU/L) | 57.8 ± 5.9 | 52.5 ± 9.6 | 940 ± 134* | 664 ± 75*,† | 416 ± 100*,†,‡ | 2721 ± 163* | 1137 ± 264*,† | 860 ± 86*,†,‡ |

*Abbreviations: ALT,* alanine aminotransferase; *AST,* aspartate aminotransferase; *Mel,* melatonin.
Mel was given for 4 or 6 wk to mice receiving $CCl_4$ and Mel. Values are expressed as means ± standard error of the mean.
*$P < 0.05$, compared with control.
†$P < 0.05$, compared with $CCl_4$ same period.
‡$P < 0.05$, compared with Mel5 same period.

milk in Tris-buffered saline containing 0.05% Tween 20 for 30 minutes at 37°C and probed overnight at 4°C with polyclonal anti-amphiregulin, Smad3, p-Smad3, nuclear factor erythroid 2-related factor (Nrf2) (Santa Cruz Biotechnology, Santa Cruz, California), CTGF, MMP-9, and TIMP-1 (Abcam) antibodies at 1:200–1:1000 dilution with phosphate buffered saline with 0.05% Tween 20 (PBST) containing 2.5% nonfat dry milk. Equal loading of protein was demonstrated by probing the membranes with a rabbit anti-$\beta$-actin polyclonal antibody (1:2000; Sigma). After washing with Tris-buffered saline containing 0.05% Tween 20, the membranes were incubated for 1 hour at room temperature with secondary *horseradish peroxidase*–conjugated antibody (1:5000; Dako, Glostrup, Denmark), and visualized using enhanced chemiluminescence (ECL) detection kit (Amersham Pharmacia, Uppsala, Sweden).[27] The density of the specific bands was quantified with an imaging densitometer (Scion Image J Software 1.46a, Bethesda, Maryland).

**Zymography assays.** MMP-9 activities were measured by gelatin zymography. Thirty milligrams of liver tissue extracts were loaded onto SDS-polyacrylamide gel electrophoresis gels containing 0.01% wt/vol gelatin from bovine skin (Sigma) as a substrate in a 10% polyacrylamide under nonreducing conditions and were run at 100 V for 45 minutes. After electrophoresis, gels were equilibrated in 2.5% Triton X-100 to remove SDS and incubated in 50 mM of Tris-HCl (pH 7.5), 10 mM of $CaCl_2$, 150 mM of NaCl, 1 mM of $ZnCl_2$, and 0.02% $NaN_3$ for 18 hours at 37°C. Then, gels were stained with Coomassie R250.

**Statistical analysis.** Results are expressed as mean values ± standard error of the mean. Data were compared by analysis of variance; when the analysis indicated the presence of a significant difference, the means were compared with the Newman-Keul's test. Pathologic grading of hepatic fibrosis was analyzed using the Kruskal-Wallis test on ranks, and group comparisons were performed using the nonparametric rank-based Mann-Whitney *U* test. Significance was accepted when *P* value was less than 0.05. Values were analyzed using the statistical package SPSS 19.0 (IBM Corporation, Armonk, New York).

## RESULTS

Mice treated with $CCl_4$ showed a marked increase in hepatic enzyme AST and ALT levels in serum. As observed in Table I, melatonin treatment (5 or 10 mg/kg body weight) resulted in an attenuation of liver injury, with a significant reduction in AST and ALT levels at both 4 and 6 weeks.

Liver tissue samples from mice in control groups presented normal lobular architecture with central veins and radiating hepatic cords. Animals receiving $CCl_4$ for 4 or 6 weeks developed significant fibrosis, with deposition of connective tissue and formation of fibrotic septa. However, $CCl_4$-treated mice administered melatonin displayed thinner septa and a more preserved parenchyma, being significantly reduced the fibrotic area (Fig 1).

We used immunohistochemical staining of $\alpha$-SMA to evaluate the degree of HSC activation. Image analysis demonstrated that chronic $CCl_4$ treatment significantly increased the accumulation of activated HSCs. Compared with the $CCl_4$ groups, melatonin treatment induced a significant decrease in HSC activation in the liver at both 4 and 6 weeks (Fig 2).

We next analyzed the expression of genes related to fibrogenesis using quantitative real-time PCRs. Collagen deposition is the result of HSC activation, and collagen fibers are major components of the ECM in the fibrotic liver. We found increased mRNA levels of collagens I and III in $CCl_4$-treated mice, and this increase was significantly prevented by melatonin administration at both time points (Table II). The effect of melatonin on the expression of the important profibrogenic molecules TGF-$\beta$, PDGF, and CTGF was also investigated. The 3 cytokines were significantly overexpressed at 4 and 6 weeks. The fibrogenic process also involves inhibition of ECM degradation through an imbalance between MMPs and their inhibitors. We observed a significant induction of MMP-9 expression in liver of animals treated with $CCl_4$ that was

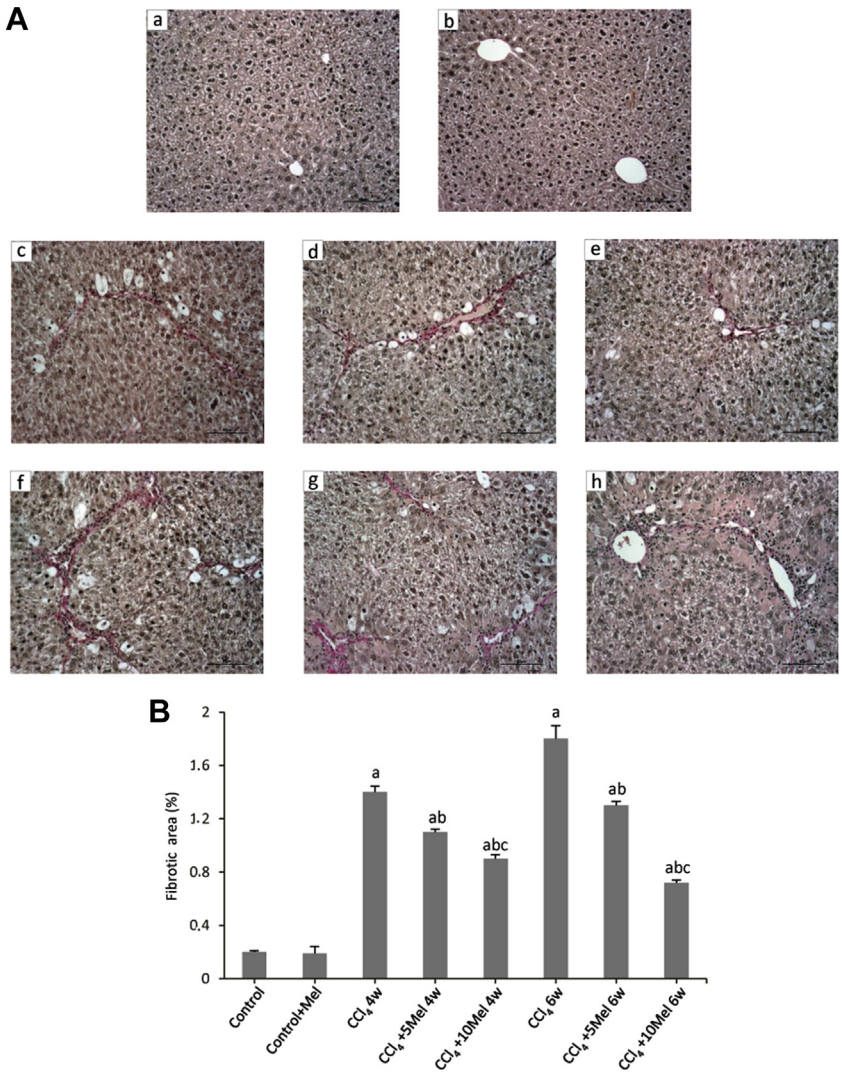


**Fig 1.** Histologic assessment of liver fibrosis in mice with $CCl_4$ and melatonin treatment. (**A**) Photomicrographs of sections of liver samples taken at 2 and 4 weeks from control (a), control + Mel (b), $CCl_4$ 4 weeks (c), $CCl_4$+Mel 5 mg/kg 4 weeks (d), $CCl_4$+Mel 10 mg/kg 4 weeks (e), $CCl_4$ 6 weeks (f), $CCl_4$+Mel 5 mg/kg 6 weeks (g), and $CCl_4$+Mel 10 mg/kg 6 weeks (h) mice. Paraffin-embedded sections were stained with Van Gieson. The livers of control and control + Mel mice had normal architecture. Deposition of connective tissue and the development of fibrotic septa were observed in $CCl_4$-treated mice. Treatment with melatonin resulted in a significant decrease in the fibrotic area associated with the $CCl_4$ intoxication. (**B**) Image analysis of the area of fibrosis. Values are expressed as means ± standard error of the mean. $^{a}P < 0.05$, compared with control. $^{b}P < 0.05$, compared with $CCl_4$ same period. $^{c}P < 0.05$, compared with Mel5 same period. $CCl_4$, carbon tetrachloride; Mel, melatonin; w, week.

accompanied by an upregulation of the inhibitor TIMP-1. Administration of melatonin significantly attenuated the expression of both MMP-9 and TIMP-1 (Table II).

The transcriptional effect on the expression of profibrogenic genes was also present at a posttranslational level, as confirmed by Western blot. Analyses demonstrated that the expression of CTGF, amphiregulin, MMP-9, TIMP-1, and phospho-Smad3 increased in mice treated with $CCl_4$. Administration of melatonin to $CCl_4$-injected mice, however, significantly decreased the levels of the different profibrogenic mediators. Changes in MMP-9 activity, measured by zymography, are shown in Fig 4; the strongest gelatinase activity, as detected by the lytic zones in the zymography, was observed in $CCl_4$-treated mice, whereas activity values decreased significantly in animals treated with melatonin (Fig 4). Moreover, melatonin administration resulted in a significantly increased protein level of Nrf2, an important regulator of the cellular antioxidant response (Fig 3).

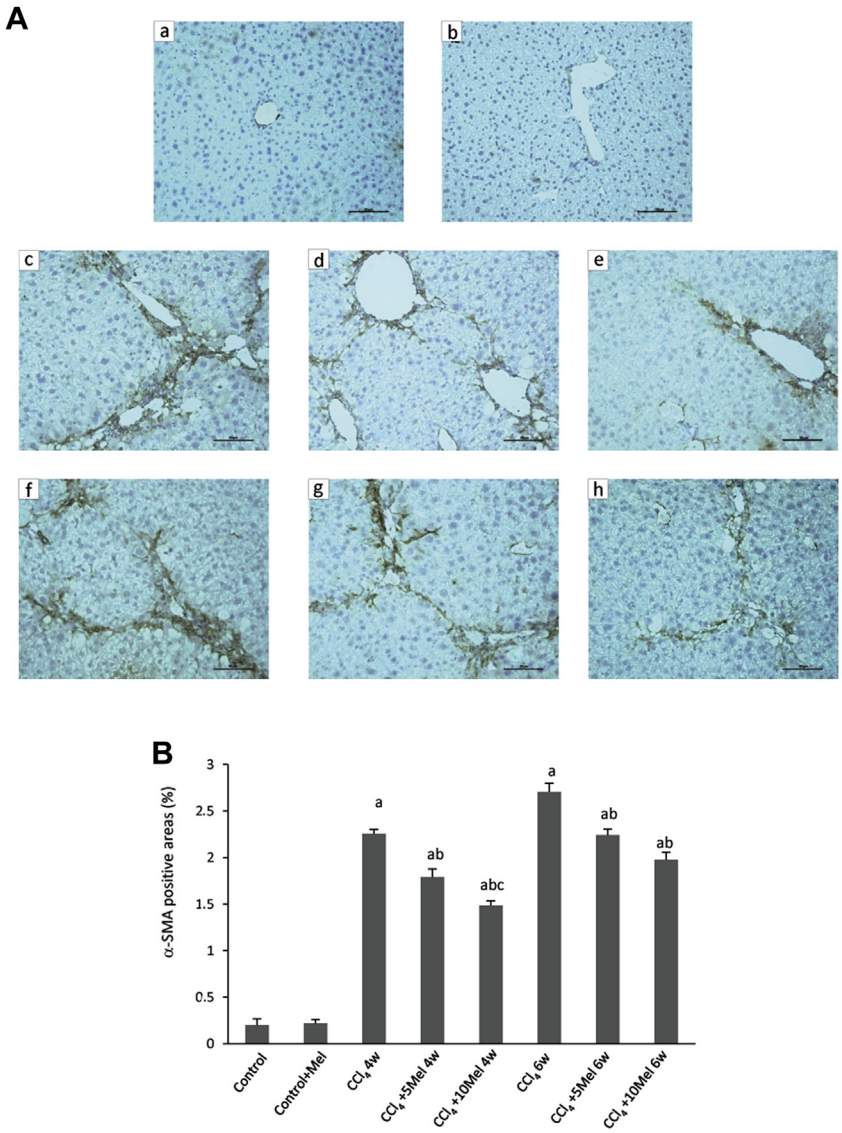


**Fig 2.** Effect of $CCl_4$ and treatment with melatonin on liver $\alpha$-SMA immunohistochemistry. (**A**) Photomicrographs of sections of liver samples taken at 2 and 4 weeks from control (a), control + Mel (b), $CCl_4$ 4 weeks (c), $CCl_4$+Mel 5 mg/kg 4 weeks (d), $CCl_4$+Mel 10 mg/kg 4 weeks (e), $CCl_4$ 6 weeks (f), $CCl_4$+Mel 5 mg/kg 6 weeks (g), and $CCl_4$+Mel 10 mg/kg 6 weeks (h) mice. Paraffin-embedded sections were stained with a $\alpha$-SMA antibody. (**B**) Image analysis of the area of $\alpha$-SMA staining. Treatment with melatonin resulted in a significant decrease in the amount of $\alpha$-SMA immunostaining associated with the $CCl_4$ intoxication. Values are expressed as means ± standard error of the mean. $^{a}P < 0.05$, compared with control. $^{b}P < 0.05$, compared with $CCl_4$ same period. $^{c}P < 0.05$, compared with Mel5 same period. $CCl_4$, carbon tetrachloride; Mel, melatonin, SMA, smooth muscle actin; w, week.

Effects induced by melatonin on the activation of HSCs and the expression of factors involved in the fibrogenic process were dose dependent, reaching values that were in most cases significantly lower in mice treated with 5 mg/kg body weight melatonin when compared with those receiving the pineal hormone at 10 mg/kg body weight (Table II, Figs 2 and 3).

## DISCUSSION

Although beneficial effects of melatonin on hepatic fibrogenesis induced by $CCl_4$ and other toxins have been reported by different authors, this is the first investigation demonstrating that in mice with liver fibrosis induced by $CCl_4$ injection, melatonin significantly suppresses the activation of HSCs and reduces the expression of collagen proteins, effects that are accompanied

**Table II.** Effect of $CCl_4$ and treatment with melatonin (Mel) on messenger RNA levels of genes related to fibrosis

| Marker | Control | Control + Mel | $CCl_4$ 4 wk | $CCl_4$+Mel5 4 wk | $CCl_4$+Mel10 4 wk | $CCl_4$ 6 wk | $CCl_4$+Mel5 6 wk | $CCl_4$+Mel10 6 wk |
|---|---|---|---|---|---|---|---|---|
| Col I | 100 ± 11 | 81 ± 7 | 1121 ± 148* | 940 ± 46*,† | 655 ± 19*,†,‡ | 1360 ± 87* | 1198 ± 75* | 1063 ± 208*,† |
| Col III | 100 ± 18 | 88 ± 9 | 331 ± 34* | 318 ± 34* | 239 ± 14*,†,‡ | 349 ± 20* | 185 ± 18*,† | 104 ± 18†,‡ |
| TGF-$\beta$ | 100 ± 9 | 92 ± 7 | 280 ± 21* | 218 ± 15*,† | 165 ± 75†,‡ | 317 ± 16* | 216 ± 12*,† | 158 ± 35*,†,‡ |
| PDGF | 100 ± 19 | 101 ± 12 | 408 ± 15* | 294 ± 15*,† | 213 ± 28*,†,‡ | 558 ± 18* | 437 ± 15*,† | 394 ± 19*,†,‡ |
| CTGF | 100 ± 9 | 97 ± 8 | 257 ± 26* | 213 ± 18* | 175 ± 7*,† | 308 ± 15* | 234 ± 22*,† | 167 ± 22*,†,‡ |
| MMP-9 | 100 ± 8 | 102 ± 9 | 322 ± 455* | 298 ± 16* | 162 ± 9*,†,‡ | 363 ± 32* | 168 ± 22*,† | 122 ± 18*,†,‡ |
| TIMP-1 | 100 ± 6 | 53 ± 11 | 1081 ± 101* | 744 ± 25*,† | 664 ± 30*,† | 1215 ± 102* | 532 ± 45*,† | 295 ± 75*,†,‡ |

*Abbreviations: Col I*, collagen I; *Col III*, collagen III; *CTGF*, connective tissue growth factor; *MMP-9*, matrix metalloproteinase 9; *PDGF*, platelet-derived growth factor; *TGF-β*, transforming growth factor β; *TIMP-1*, tissue inhibitor of metalloproteinase 1.
Mel was given for 4 or 6 wk to mice receiving $CCl_4$ and Mel. Values are expressed as means ± standard error of the mean.
*$P < 0.05$, compared with control.
†$P < 0.05$, compared with $CCl_4$ same period.
‡$P < 0.05$, compared with Mel5 same period.

by the downregulation of TGF-$\beta$/Smad signaling and by changes in the expression of other profibrogenic mediators.

In our study, after $CCl_4$ administration, mice consistently developed histologic evidence of fibrosis together with increased levels of ALT and AST in serum. Formation of fibrotic septa and increase in enzyme levels were impaired in animals treated with melatonin (5 and 10 mg/kg body weight), which is in line with previous reports on the effect of the pineal hormone in mice or rats with $CCl_4$-induced fibrosis.[16-22] The critical step in the generation of liver fibrosis is the activation of HSCs resulting in $\alpha$-SMA and collagen deposition.[28] The increased $\alpha$-SMA expression in the livers of $CCl_4$-treated mice was significantly diminished by melatonin administration, as indicated by immunohistochemical staining. Consistent with the histology staining results, RT-PCR analysis revealed decreased expression of collagens I and III. These data indicate that melatonin induces antifibrogenic actions related in part to a lower activation of HSCs during the course of the fibrogenic process. A similar antifibrogenic action, with decreases in the formation of collagen in the liver and in the expression of $\alpha$-SMA, has been previously reported in rats with $CCl_4$ liver injury receiving epigallocatechin-3-gallate[6] or after the administration of proanthocyanidins to rats with dimethylnitrosamine-induced liver damage.[9]

The role of profibrogenic cytokines is central for the development of fibrosis, with progression greatly dependent on the production of TGF-$\beta$, which is the master cytokine in liver fibrogenesis and one of the primary targets in the development of antifibrotic agents.[29] TGF-$\beta$ is downregulated in animal models of fibrosis by treatment with curcumin,[30] epigallocatechin-3-gallate,[6] or polyprenols.[7] Present data confirm that melatonin also decreases TGF-$\beta$ expression in $CCl_4$-treated mice, a fact that *undoubtedly* contributes to the interference of the indol with the activation of HSCs. TGF-$\beta$ signals stimulate through transmembrane receptors cytoplasmic Smad proteins, which in turn modulate the transcription of target genes, including those encoding ECM components.[2] In the context of liver fibrosis, Smad3 is pathogenic because mice null for Smad3 are protected against dimethylnitrosamine-induced hepatic fibrosis.[31] Our data demonstrate that the upregulation of TGF-$\beta$ was associated with increased phosphorylation of Smad3, effects which were partially abrogated by melatonin. These results similar to those previously reported in rats treated with $CCl_4$ for asiatic acid, a natural product from *Centella asiatica*,[32] and curcumin[30] suggest that restoring the balance of TGF-$\beta$/Smad signaling may be a central mechanism by which melatonin inhibits liver fibrosis.

PDGF is the predominant mitogen for activated HSCs, and its expression may act synergistically with TGF-$\beta$ to enhance collagen synthesis via Smad pathways. In rats with $CCl_4$-induced fibrosis, PDGF is significantly downregulated by polyprenols from *Taxus chinensis*[7] or extracts from *Artemisia capillaris* and *Artemisia iwayomogi*.[33] Results from the present research suggest that the downregulation of PDGF could also be a contributor to the antifibrotic properties of melatonin. TGF-$\beta$ and PDGF have traditionally been considered as the key fibrogenic and proliferative stimuli to HSCs, respectively; however, newer players and pathways are gaining attention. For example, CTGF is a cytokine induced by TGF-$\beta$ that acts synergically to promote matrix protein deposition and fibrogenesis, both in vitro and in vivo.[34] Smad3 signaling has been identified as the responsible pathway inducing CTGF expression in hepatocytes of $CCl_4$-treated mice,[35] and knockdown of CTGF by injection of small interfering RNA into the portal veins of rat livers prevents $CCl_4$-induced fibrosis by inhibiting TGF-$\beta$ induction and HSC activation.[36] It has been reported that in rats treated

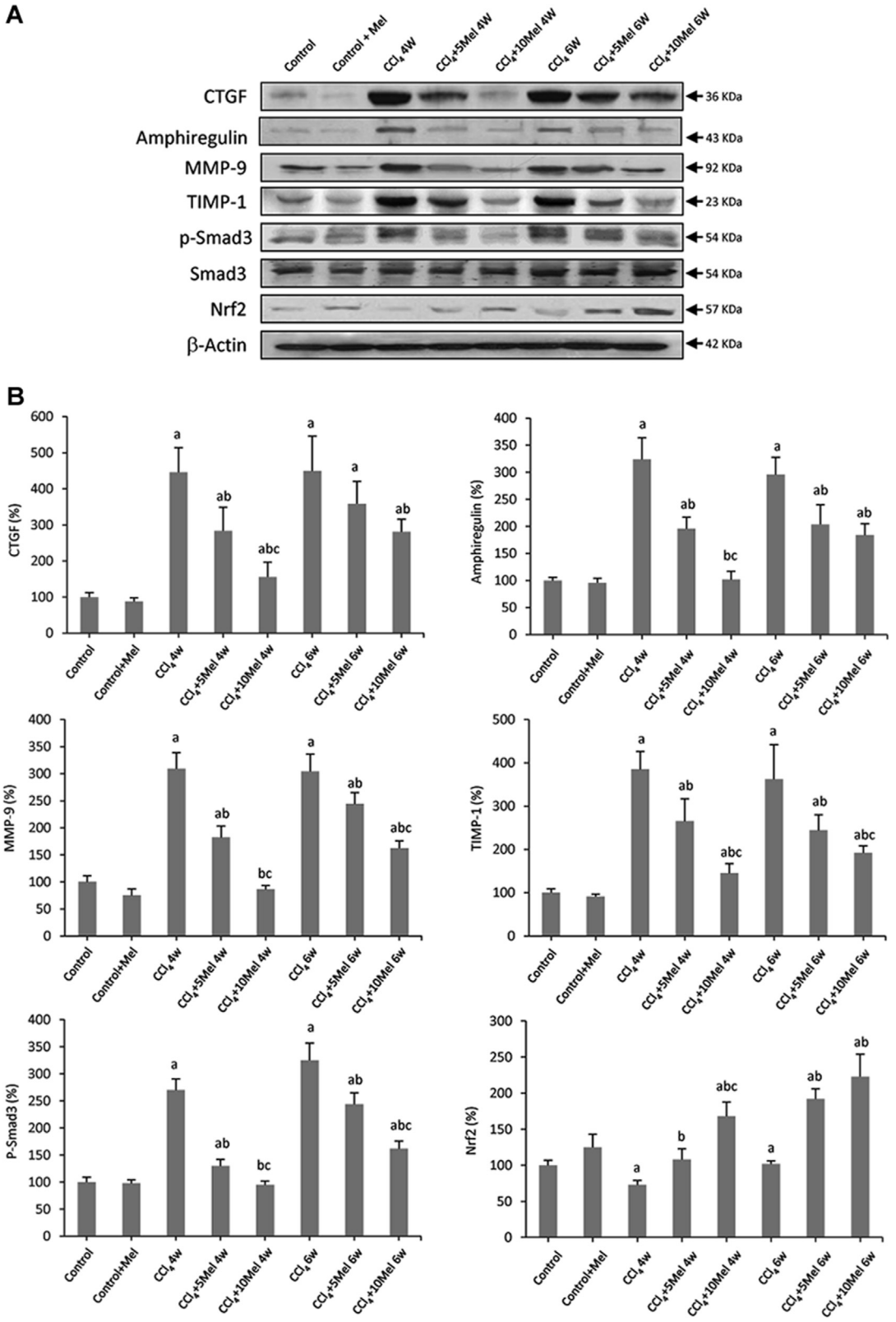


**Fig 3.** Effect of $CCl_4$ and treatment with melatonin on protein concentration of CTGF, amphiregulin, MMP-9, TIMP-1, phospho-Smad3, and Nrf2. Protein from liver extracts taken at 4 and 6 weeks was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, followed by immunoblotting. Equal loading of proteins is

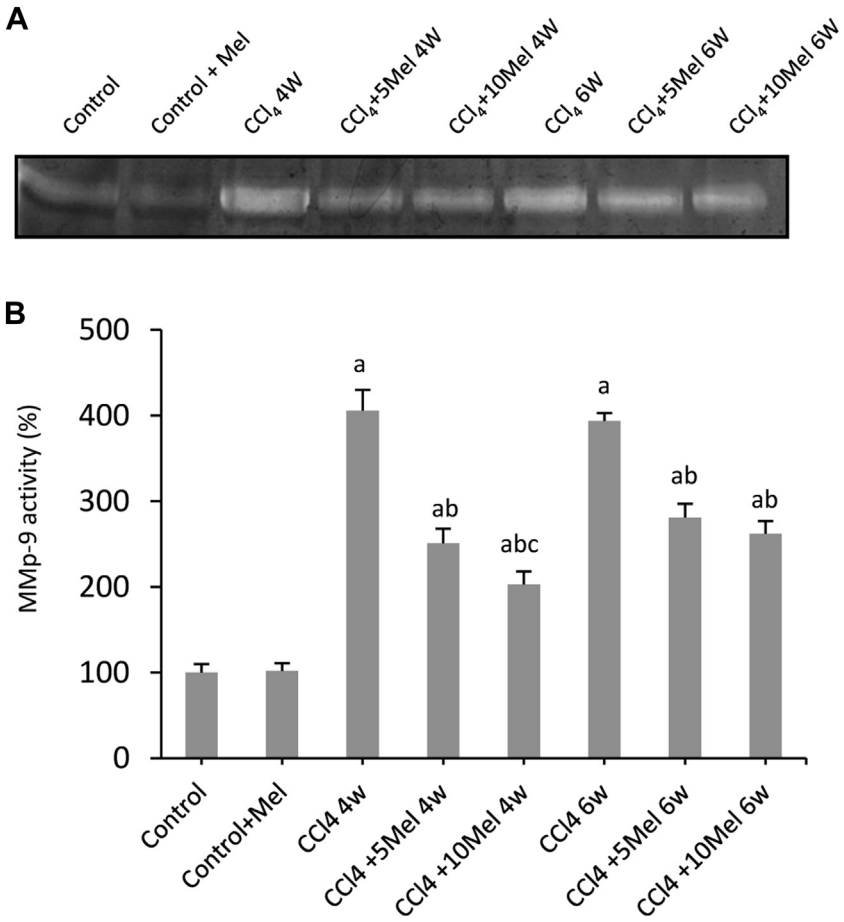


**Fig 4.** Effect of $CCl_4$ and treatment with melatonin on MMP-9 activity. The MMP-9 gelatinase activities in liver tissue extracts taken at 4 and 6 weeks were measured by gelatin zymography assays as described. Melatonin (Mel) was given for 4 or 6 weeks. (**A**) Representative zymography photographs. (**B**) Densitometric quantification. Values are expressed as means ± standard error of the mean. $^{a}P < 0.05$, compared with control. $^{b}P < 0.05$, compared with $CCl_4$ same period. $^{c}P < 0.05$, compared with Mel5 same period. $CCl_4$, carbon tetrachloride; MMP-9, matrix metalloproteinase 9; w, week.

with $CCl_4$ silymarin decreases CTGF to improve liver fibrosis[37] and curcumin significantly attenuates the severity of liver damage through inhibition of CTGF expression.[30] Our findings confirmed that chronic $CCl_4$ administration markedly enhanced the intrahepatic expression of CTGF mRNA and protein, and that melatonin significantly reduced these $CCl_4$-induced increases.

The ligand of the epidermal growth factor amphiregulin is another molecule, which plays a specific role in liver fibrosis, contributing to the expression of fibrogenic mediators and to the proliferation of fibrogenic cells.[38] The expression of amphiregulin increases markedly in liver injury induced by $CCl_4$,[39] and amphiregulin deficient-mice develop significantly less collagen accumulation.[40] We have previously reported that suppression of amphiregulin signals contributes to the protective effects of quercetin in cirrhotic rats with common bile duct ligation,[7] and in mice fed a methionine-choline–deficient diet.[2] It is also known that amphiregulin amplifies the HSC response through increased production of CTGF and TIMP-1.[39] Thus, the inhibition of amphiregulin expression by melatonin in $CCl_4$-treated mice supports the suggestion that

illustrated by $\beta$-actin bands. Melatonin (Mel) was given for 4 or 6 weeks. (**A**) Representative Western blot photographs. (**B**) Densitometric quantification. Values are expressed as means ± standard error of the mean. $^{a}P < 0.05$, compared with control. $^{b}P < 0.05$, compared with $CCl_4$ same period. $^{c}P < 0.05$, compared with Mel5 same period. $CCl_4$, carbon tetrachloride; CTGF, connective tissue growth factor; MMP-9, matrix metalloproteinase 9; Nrf2, nuclear factor erythroid 2; TIMP-1, tissue inhibitor of metalloproteinase 1; w, week.

suppression of the amphiregulin signaling system could be a new target in the prevention of liver fibrosis.

An additional finding from our study was the reversal of changes in the expression of MMP-9 and TIMP-1, and the decrease in MMP-9 activity induced by melatonin after $CCl_4$ administration. Increases in MMPs and TIMPs are common in fibrotic diseases, whereas an increase in the MMP/TIMP expression ratio is known to be critical in discouraging matrix degradation and remodeling and favors ECM deposition.[28] Previous studies have shown that melatonin inhibits MMP-9 activity via binding to its active site and via MT1 receptor signaling pathway.[41,42] Consequently, data obtained indicate that reduction in ECM deposition by melatonin could contribute to the alleviation of liver fibrosis, confirming results from rats treated with $CCl_4$ that were given gallic acid[43] or extracts from *Acanthopanax koreanum*.[44]

Oxidative stress is one the most important stimuli to activate HSCs and plays a key role in the development of fibrosis.[5] Melatonin has been reported in different studies to significantly reduce the production of lipid peroxides and increase the activity of different antioxidant enzymes, which alleviates the oxidative damage caused by $CCl_4$.[16,17,20,21] Effects of melatonin could therefore be dependent, at least in part, on the modulation of the profibrogenic signals related to its free radical scavenging ability. Nrf2 is a member of the cap'n'collar family of basic leucine-zipper transcription factors, which protects against oxidative stress through antioxidant response element-mediated induction of diverse antioxidant enzymes.[45] It has been reported that Nrf2-deficient mice exhibit an increased susceptibility to fibrosis induced by acetaminophen,[46] and activation of Nrf2 by curcumin attenuates dimethylnitrosamine-induced hepatic fibrosis in rats.[47] The antioxidant sulforaphane suppresses TGF-$\beta$–enhanced expression and TGF-$\beta$–stimulated phosphorylation of Smad2 and Smad3 in human immortalized HSCs through an effect that is significantly abolished by Nrf2 knockdown.[48] Because Nrf2 expression in significantly stimulated by melatonin, data obtained in the present research suggest the possibility that suppression of fibrogenic gene expression by melatonin could be mediated, at least in part, through Nrf2-dependent inhibition of the canonical TGF-$\beta$/Smad signaling. A direct antifibrogenic effect of melatonin on HSC cannot be ruled out, given the fact that the expression of $\alpha$-SMA is significantly reduced by the indol. In this respect, it has been recently reported that melatonin induced augmentation of collagen deposition in fibroblasts and myofibroblasts through an effect that is prevented by luzindole, the blocker of $MT_1$ and $MT_2$ melatonin membrane receptors.[49]

## CONCLUSIONS

Our study demonstrates that melatonin administered 2 weeks after $CCl_4$ treatment to allow the initial activation of HSCs protects mouse liver from fibrogenesis. Beneficial effects, delaying the progression of fibrosis, involve inhibition of HSC activation, the suppression of TGF-$\beta$/Smad signaling and various profibrogenic mediators, and changes in ECM remodeling. Although the precise mechanisms of melatonin in liver fibrosis remain to be elucidated and further studies are required, our results suggest that melatonin might be an effective antifibrotic drug in the prevention of liver disease progression.

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Conflicts of Interest: All authors have read the journal's policy on disclosure of potential conflicts of interest and have none to declare.

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