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Melatonin Ameliorates Liver Fibrosis Induced by Carbon Tetrachloride in Rats via Inhibiting TGF-β1/Smad Signaling Pathway*

Yu-rong WANG, Ru-tao HONG#, Yuan-yuan XIE, Jian-ming XU
Department of Gastroenterology, First Affiliated Hospital of Anhui Medical University, Key Laboratory of Digestive Disease of Anhui Province, Hefei 230022, China

Summary: Melatonin has been reported to inhibit hepatic fibrosis and the mechanism may be correlated to its anti-oxidant effect. Nevertheless, the mechanism is not completely identified. This study was conducted to investigate the effects of melatonin on TGF-β1/Smad signaling pathway in liver fibrosis in rats. The liver fibrosis model was made by the subcutaneous injection of CCl_4. The liver pathology changes were detected using hematoxylin and eosin (H&E) staining and Van Gieson (VG) staining. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were measured with an autoanalyzer. Glutathione peroxidase (GPx) activities and levels of malondialdehyde (MDA) and hydroxyproline (Hyp) in liver were evaluated by spectrophotometry. Expression levels of TGF-β1, Smad2/3, phosphorylated Smad2/3 (p-Smad2/3) and Smad7 in liver were detected by immunohistochemistry and Western blot analysis. Results showed that melatonin suppressed CCl_4-induced liver fibrosis, along with an improvement in histological changes, significant decreases in pathologic grading sores and obvious decreases in Hyp levels in liver. Melatonin improved liver function indicated by decreased serum ALT and AST activities. In addition, melatonin exerted its anti-oxidant effects, as supported by decreased MDA levels and increased GPx activities in liver. Furthermore, melatonin inhibited TGF-β1/Smad pathway, as evidenced by decreased TGF-β1, Smad2/3 and p-Smad2/3 expression and increased Smad7 expression in liver. In conclusion, melatonin may suppress CCl_4-induced hepatic fibrosis in rats via inhibiting TGF-β1/Smad pathway. It is possible for melatonin to be a potential reagent to treat and cure liver fibrosis.
Key words: melatonin; liver fibrosis; TGF-β1; Smad

As a universal feature of various chronic liver diseases, hepatic fibrosis is characterized by the excessive synthesis and accumulation of extracellular matrix (ECM) and collagen. Furthermore, the progression of liver fibrosis can lead to cirrhosis and even hepatocellular carcinomas, with organ failure and the risk of mortality. However, mounting evidence suggests that liver fibrosis is dynamic and can be reversed[1]. But no effective treatment is available for liver fibrosis in human at present. Increasing evidence has demonstrated that varieties of events and numerous cytokines are involved in hepatic fibrosis[2]. TGF-β1 has been recognized as the most important one during numerous cytokines in the progression of liver fibrosis[3]. TGF-β1 is excreted by Kuffer cells, sinus endothelial cells and platelets in response to acute and chronic liver injuries[4]. TGF-β1 promotes the synthesis of new matrix components by activating quiescent hepatic stellate cells (HSCs). On the other hand, it inhibits the degradation of ECM, through increasing the synthesis of enzymes such as tissue inhibitor of metalloproteinases (TIMPs) and decreasing the synthesis of matrix-degrading proteases such as matrix-metalloproteinases (MMPs)[5]. Overexpression of the cytokine can lead to the excessive and uncontrolled deposition of ECM in liver. TGF-β1 functions mainly through TGF-β1/Smad signaling pathway. Smad

Yu-rong WANG, E-mail: 1029182985@qq.com
#Corresponding author, E-mail: hongrutao@163.com
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proteins are functionally classified into: receptor-regulated protein, common mediator and inhibitor[6, 7].

Melatonin, the chief product secreted by the pineal gland, takes part in many important physiological functions, including modulating the circadian rhythm, immune-enhancing, anti-inflammation and antioxidation[8]. Melatonin declines numerous free radicals and various reactive oxygen intermediates remarkably, such as singlet oxygen, hydroxyl radical, nitricoxid and peroxynitrite anion[9]. Furthermore, melatonin is 10 times more powerful than vitamin E and vitamin C in disrupting the chain reaction of lipid peroxidation. On the other hand, melatonin raises the levels of potential antioxidants, such as glutathione peroxidase (GPx), glutathione (GSH) and superoxide dismutase (SOD)[10]. Recently, the anti-fibrotic effects of melatonin have been found. For instance, our previous studies showed that melatonin inhibited hepatic fibrosis and that the mechanism may be relevant to its anti-oxidant effects[11, 12]. Nevertheless, the mechanisms underlying the anti-fibrotic effect of melatonin have remained elusive. Until now, there is no report about the relationship between melatonin and the TGF-β1/Smad pathway in liver fibrosis. Therefore, we investigated further the anti-fibrotic effect of melatonin on CCl_4-induced hepatic fibrosis in rats and the relationship between melatonin and the TGF-β1/Smad pathway protein expression in liver in this study.

1 MATERIALS AND METHODS

1.1 Reagents

Melatonin was supplied by Sigma (USA). It was dissolved with a mixture of ethyl alcohol (0.01%, v/v) and saline, and was stored at –20°C. Commercial kits used for measuring malondialdehyde (MDA), GPx and hydroxyproline (Hyp) were provided by Nanjing Jiancheng Biological Engineering (China). The primary antibodies: rabbit polyclonal anti-TGF-β1 (CAT: sc-146), anti-Smad2/3 (CAT: sc-8332), anti-p-Smad2/3 (CAT: sc-11769) and mouse monoclonal anti-Smad7 (CAT: sc-365846) were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Rabbit polyclonal anti-β-actin antibody and horseradish peroxidase (HRP)-conjugated secondary antibodies, including normal goat anti-rabbit and goat anti-mouse IgG, were provided by Beijing Zhongshan Biological Technology Co, Ltd. (China).

1.2 Animals and Drug Treatment

The protocols for the study were approved by Ethic Committee and Animal Experimental Committee in Anhui Medical University. Totally, 75 male Sprague-Dawley (SD) rats (180–220 g), were bought from the Laboratory Animal Center in Anhui Medical University in Hefei of China. All rats were allowed to acclimate to the temperature (22±1°C) and humidity of (50±10)% of controlled rooms, under a 12-h:12-h light-dark cycle for 1 week before experiments. The animals were freely accessible to enough food and water. Furthermore, the rats were treated in a humane way, in accordance to National Guidelines for Caring Laboratory Animals in China. All the animals were divided randomly into 5 groups: normal group (n=15), model group (n=15), MEL-1 group (n=15), MEL-2 group (n=15) and MEL-3 group (n=15). The normal group was treated subcutaneously with peanut oil (3 mL/kg) and the other groups were administered subcutaneously with the same amount of 40% CCl_4 dissolved in peanut oil semiweekly for 6 weeks. At the start of the treatment of CCl_4, different doses of melatonin were intraperitoneally administered to the groups: the MEL-1 group (2.5 mg/kg), the MEL-2 group (5.0 mg/kg) and the MEL-3 group (10.0 mg/kg) daily for 6 weeks. After 6 weeks, all animals were fasted overnight and anesthetized with 10% chloral hydrate. After obtaining blood from the abdominal aorta, all the rats were sacrificed and the livers were removed. Serum samples were obtained by a centrifugation (4°C, 1500 g, 10 min) and then saved at –80°C. After obtaining samples for histological evaluation, the other liver tissues were stored in liquid nitrogen.

1.3 Liver Function Analysis

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities in serum samples were detected using Hitachi Automatic Analyzer (Japan).

1.4 Measurement of Levels of MDA and Activities of GPx in Liver

Firstly, liver tissues were homogenized (1:9, w:v) with 0.9% ice-cold saline. Then, the homogenates were subjected to the centrifugation (3000 r/min, 10 min, 4°C) and MDA levels and GPx activities in the supernatant were assayed in accordance with instructions of the manufacturer. The reaction between MDA and thiobarbituric acid (TBA) was used to determine the levels of MDA in liver. The absorbance (A) value was detected at 532 nm by a spectrophotometer. Levels of MDA were shown as nmol/mg protein (nmol/mg prot), using 1,1,3,3-tetramethoxypropane as a standard. The activities of GPx were measured as the amount of GSH oxidized by hydroperoxide. The A value was detected at 412 nm. Then the activities of GPx were shown as units per mg protein (U/mg prot) tissue.

1.5 Measurement of Hyp

The concentration of liver collagen was measured by determining Hyp content in fresh liver samples using a commercial kit in accordance with its manufacturer's instructions. 5 μg/mL Hyp was used as the standard and the A value was read at 550 nm by a spectrophotometer. The levels of Hyp in liver homogenate were expressed as μg/mg weight.

1.6 Histopathological Evaluation

Hematoxylin and eosin (H&E) staining was

used to detect the severity of liver damage, while VG staining was performed for investigating the deposition of liver collagen and ECM. After fixation with formalin (10%), liver specimens were subjected to embedding in paraffin. After cutting into 5 μm-thick slices, liver sections were stained by H&E according to the standard procedure. VG staining was performed according to the instruction of the manufacturer (Baso diagnostics, Inc. Zhuhai, China). Finally, the METAVIR scoring system was applied to evaluate the extent of hepatic fibrosis[13]: 0, no fibrosis; 1, portal fibrosis without septa; 2, few septa; 3, numerous septa without cirrhosis; 4, cirrhosis. Two expert pathologists, knowing nothing about the sources of each tissue sample, examined the stained slides independently. The severity of fibrosis was shown as the average grade of 10 different regions randomly selected from each section.

1.7 Immunohistochemistry

After the deparaffinage with xylene and rehydration with graded ethanol, 5-μm-thick liver tissue sections were soaked in 0.5% periodic acid for 30 s–1 min to block the activity of endogenous peroxidase and then were subjected to the boiled citrate buffer in a microwave oven for 5×2 min to retrieve antigen. Following incubating with normal goat serum for 15 min, sections were subjected to incubation with primary antibodies: anti-TGF-β1 antibody (1:450), anti-Smad2/3 antibody (1:200), anti-p-Smad2/3 antibody (1:550) overnight at 4°C. Negative controls were incubated only with PBS. After washing 3×5 min with phosphate-buffered saline (PBS, pH 7.2–7.4), sections were subjected to incubation with secondary antibodies. Then the sections were stained for 5–10 min with diaminobenzidine (DAB) (Beijing Zhongshan Biological Technology Co, Ltd. China) in accordance with the manufacture's instruction. Yellow, brown or tan staining was seen as the positive signal. After staining with DAB, the slides were stained with hematoxylin. Then an inversed fluorescent microscope was applied to capture the images. We selected randomly 5 high-power fields (400× magnification) from each slide. The assessment of the *A* value was used to evaluate the sections. Finally, the results were shown as the average density of unit area measured by Image-Pro Plus 6.0 software.

1.8 Western Blotting

Liver samples were homogenized and were then subjected to centrifugation at 15 000× g, 4°C for 15 min. Bicinchoninic Acid assay (Beyotime Biotechnology, China) was applied to measure the protein concentrations in the supernatants and bovine serum albumin was identified as the standard protein. The obtained supernatants were boiled for 10 min in sample buffer (SDS). After separating with SDS-PAGE (12.5% acrylamide), protein samples were then transferred to PVDF membranes (Millipore, USA). After incubating with blocking buffer (5% nonfat milk) for 2 h at room temperature, membranes were subjected to the incubation with antibodies: anti-TGF-β1 antibody (1:450), anti-Smad2/3 antibody (1:200), anti-p-Smad2/3 antibody (1:450), anti-Smad7 antibody (1:500) and anti-β-actin antibody (1:1000) overnight at 4°C. Membranes were washed with PBST (0.01 mol/L PBS, pH 7.2–7.4, 0.05% Tween 20) for 3×10 min and PBS for 1×10 min and then incubated with the HRP-conjugated secondary antibodies (1:5000) for 2 h at room temperature. After the extensive wash with PBST and PBS, the membranes were detected by the ECL Western blotting detection system (Tanon Science & Technology Co., Ltd., China). The ratio between integrated *A* (I*A*) of target protein and I*A* of β-actin was quantified by Quantity One software.

1.9 Statistical Analysis

SPSS 17.0 was applied to analyze all data. Quantitive data were presented as $\bar{x}\pm s$. The statistical comparisons of quantitive data were made by one-way ANOVA and the LSD test. The comparisons of the qualitative data were made using the Ridit analysis. A P value less than 0.05 was considered statistically significant.

2 RESULTS

2.1 Liver Function

An observable increase was found in the activities of enzymes (AST and ALT) in serum samples from the group treated with CCl_4 only, in comparison with the normal group, whereas the increase was partially eliminated by melatonin administration (2.5, 5.0, 10 mg/kg) (P<0.01) (table 1) .

Table 1 Effect of melatonin on the activities of serum ALT and AST in CCl_4-induced hepatic fibrotic rats ($\bar{x}\pm s$)

Groups	Melatonin (mg/kg)	*n*	ALT (U/L)	AST (U/L)
Normal	-	15	48.00±13.37	157.27±16.18
Model	-	13	336.92±26.48**	438.15±26.79**
MEL-1	2.5	13	250.54±21.59▲▲	351.31±20.97▲▲
MEL-2	5.0	15	222.27±28.43▲▲	313.80±21.23▲▲
MEL-3	10.0	15	202.20±27.09▲▲	281.53±25.57▲▲

**P<0.01 *vs*. the normal group; ▲▲P<0.01 *vs*. the model group

2.2 Effect of Melatonin on the Markers of Oxidative Stress in Liver Tissues

An evident increase in MDA levels and a significant decrease in GPx activities were observed in liver homogenates from the group administrated with CCl_4 only as compared with the normal group (P<0.01), whereas treatment of melatonin (2.5, 5.0, 10 mg/kg) suppressed partially the effects induced by CCl_4 (P<0.05) (table 2).

Table 2 Effect of melatonin on the levels of MDA and the activities of GPx in CCl_4-induced hepatic fibrotic rats ($\bar{x}\pm s$)

Groups	Melatonin (mg/kg)	MDA (nmol/mg prot)	GPx (U/mg prot)
Normal	-	2.51±0.62	79.51±12.49
Model	-	4.60±0.66**	37.61±14.28**
MEL-1	2.5	3.84±0.60▲▲	47.92±13.05 ▲
MEL-2	5.0	3.41±0.60▲▲	56.62±11.53▲▲
MEL-3	10.0	2.95±0.56▲▲	69.51±12.66▲▲

**P<0.01 *vs*. the normal group; ▲P<0.05, ▲▲P<0.01 *vs*. the model group

2.3 Hyp Levels in Liver Homogenates

Liver homogenates from the model group showed an observable increase in Hyp levels as compared with the normal group, whereas treatment of melatonin (2.5, 5.0, 10 mg/kg) inhibited partially the effect induced by CCl_4 (P<0.01) (table 3).

Table 3 Effect of melatonin on the levels of Hyp in liver tissues in CCl_4-induced hepatic fibrotic rats ($\bar{x}\pm s$)

Group	Melatonin (mg/kg)	MDA Hyp (μg/mg)
Normal	-	0.25±0.06
Model	-	0.54±0.06**
MEL-1	2.5	0.42±0.07▲▲
MEL-2	5.0	0.35±0.07▲▲
MEL-3	10.0	0.30±0.07▲▲

**P<0.01 *vs*. the normal group; ▲▲P<0.01 *vs*. the model group

2.4 Effect of Melatonin on Liver Histopathology

The normal group presented regular parenchymal architecture with no deposition of liver collagen and ECM (fig. 1A and fig. 2A). Liver tissues from the model group showed significant necrosis, infiltration of inflammatory cells and significant fibrosis (fig. 1B and fig. 2B). Treatment of melatonin (2.5, 5.0, 10.0 mg/kg) reduced the degree of morphological changes and the degree of fibrosis significantly (fig. 1C-1E and fig. 2C-2E).

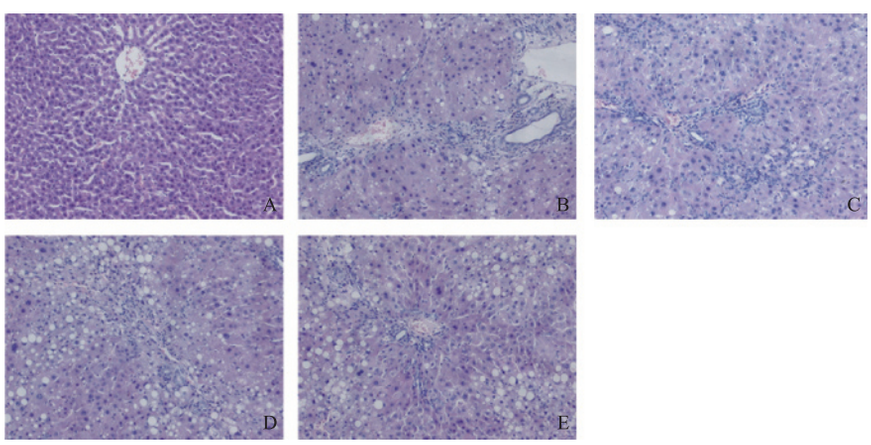

Fig. 1 Effect of melatonin on liver histopathology determined by H&E staining (magnification, ×200)
A: the normal group, normal liver; B: In the model group, significant necrosis and inflammatory cells were found in the space of liver tissues. C: the MEL-1 group (melatonin, 2.5 mg/kg); D: the MEL-2 group (melatonin, 5.0 mg/kg); E: the MEL-3 group (melatonin, 10.0 mg/kg). Administration of melatonin (especially 5.0 mg/kg and 10.0 mg/kg) reduced the liver injury and inflammation significantly.

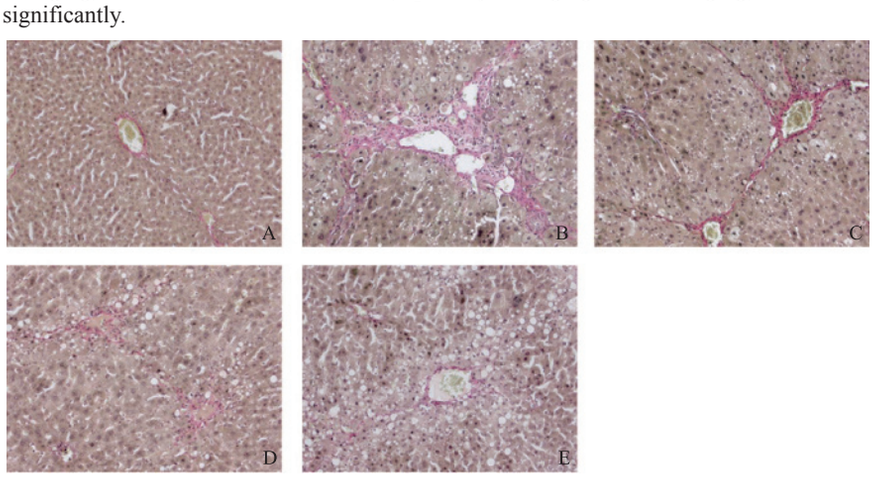

Fig. 2 Effect of melatonin on liver histopathology determined by VG staining (magnification, ×200)
A: the normal group, no fibrosis; B: the model group, significant fibrosis; C: the MEL-1 group (melatonin, 2.5 mg/kg); D: The MEL-2 group (melatonin, 5.0 mg/kg); E: the MEL-3 group (melatonin, 10.0 mg/kg). Treatment of melatonin significantly reduced hepatic fibrosis induced by CCl_4.

Consistently with the results shown by H&E staining and VG staining, an evident increase was shown in pathologic grading in the group administrated with CCl_4 only, as compared with the normal group (P<0.01). When compared with the group administrated with CCl_4 only, treatment of melatonin (10.0 mg/kg) reduced significantly the grading of hepatic fibrosis (P<0.05). However, the differences were not significant in pathologic grading scores between the melatonin-treated groups (2.5 mg/kg, 5.0 mg/kg) and the model group (both P>0.05) (table 4).

Table 4 Effect of melatonin on pathological grading of hepatic fibrosis induced by CCl_4 in rats

Group	Melatonin (mg/kg)	n	Pathologic grading of hepatic fibrosis					u value
			0	1	2	3	4	
Normal	-	15	15	0	0	0	0	4.5709▲▲
Model	-	13	0	0	2	8	3	-
MEL-1	2.5	13	0	0	7	4	2	1.5939
MEL-2	5.0	15	0	1	6	5	3	1.2658
MEL-3	10.0	15	0	2	8	3	2	2.2737▲

u>1.96 indicate P<0.05, u>2.58 indicate P<0.01;
▲P<0.05, ▲▲P<0.01 *vs*. the model group

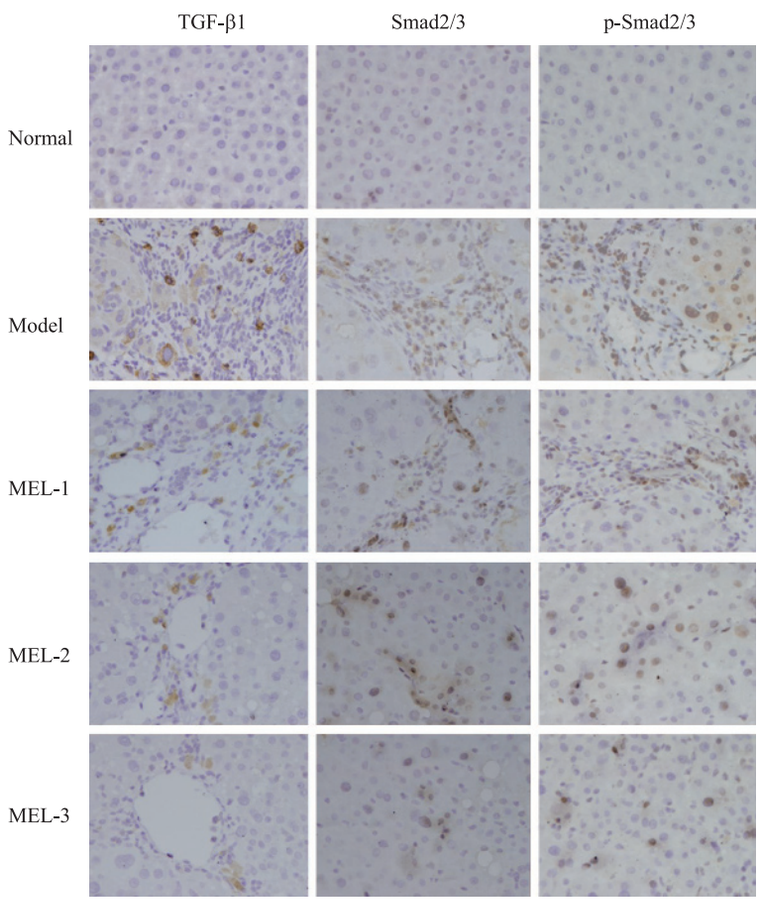

Fig. 3 Effect of melatonin on the expression of TGF-β1/Smad pathway in liver tissue determined by immunohistochemical staining (magnification, ×400)
TGF-β1 and Smad2/3 were located mostly in the cytoplasm, while p-Smad2/3 was located in the nuclei. The normal group showed weak positive target proteins. Positive areas of TGF-β1, Smad2/3 and p-Smad2/3 were prominent in the model group. Treatment of melatonin reduced the positive areas of target proteins *vs*. the model group.

2.5 Effect of Melatonin on TGF-β1/Smad Expression in Liver Tissues (Immunochemistry)

TGF-β1 and Smad2/3 were mostly expressed in the cytoplasm of inflammatory cells and fibrocytes, while p-Smad2/3 expression was mostly in the nuclei not only in inflammatory cells and fibrocytes but also in some normal hepatocytes. As we can see from fig. 3 and table 5, the expression of TGF-β1 was increased significantly in the group administrated with CCl4 only, especially in areas around the central veins, as compared with the normal group, while administration of melatonin (2.5, 5.0 and 10.0 mg/kg) suppressed the effect induced by CCl_4 (P<0.01). Furthermore, the staining results of Smad2/3 and p-Smad2/3 was similar to that of TGF-β1 (P<0.01) (fig. 3, table 5).

Table 5 Effect of melatonin on the expression of TGF-β1, Smad2/3 and p-Smad2/3 in liver tissue ($\bar{x}\pm s$), n=6)

Groups	Melatonin (mg/kg)	TGF-β1	Smad2/3	p-Smad2/3
Normal	-	0.2135±0.0142	0.2315±0.0208	0.2411±0.0175
Model	-	0.8016±0.0159**	0.6475±0.0337**	0.6614±0.0297**
MEL-1	2.5	0.5576±0.0198▲▲	0.5221±0.0200▲▲	0.5111±0.0214▲▲
MEL-2	5.0	0.4429±0.0290▲▲	0.4019±0.0163▲▲	0.4201±0.0197▲▲
MEL-3	10.0	0.3102±0.0208▲▲	0.3318±0.0184▲▲	0.3093±0.0205▲▲

**P<0.01 *vs*. the normal group, ▲▲P<0.01 *vs*. the model group

2.6 Effect of Melatonin on TGF-β1/Smad Pathway in Liver Tissues (Western Blotting)

As shown in fig. 4A and fig. 4C, TGF-β1 expression was prominent in the group administrated with CCl_4 only as compared to the normal group, whereas administration of melatonin (2.5, 5.0 and 10.0 mg/kg) suppressed the expression of TGF-β1, as compared to the model group (P<0.01). Furthermore, the results about Smad2/3 and p-Smad2/3 expression were similar to those of TGF-β1 (fig. 4B, fig. 4D-4E and fig. 4G) (P<0.01). Nevertheless, fig. 4F and fig. 4H displayed that the expression of Smad7 was reduced in the model group and increased significantly in melatonin-treated groups (P<0.01) (fig. 4).

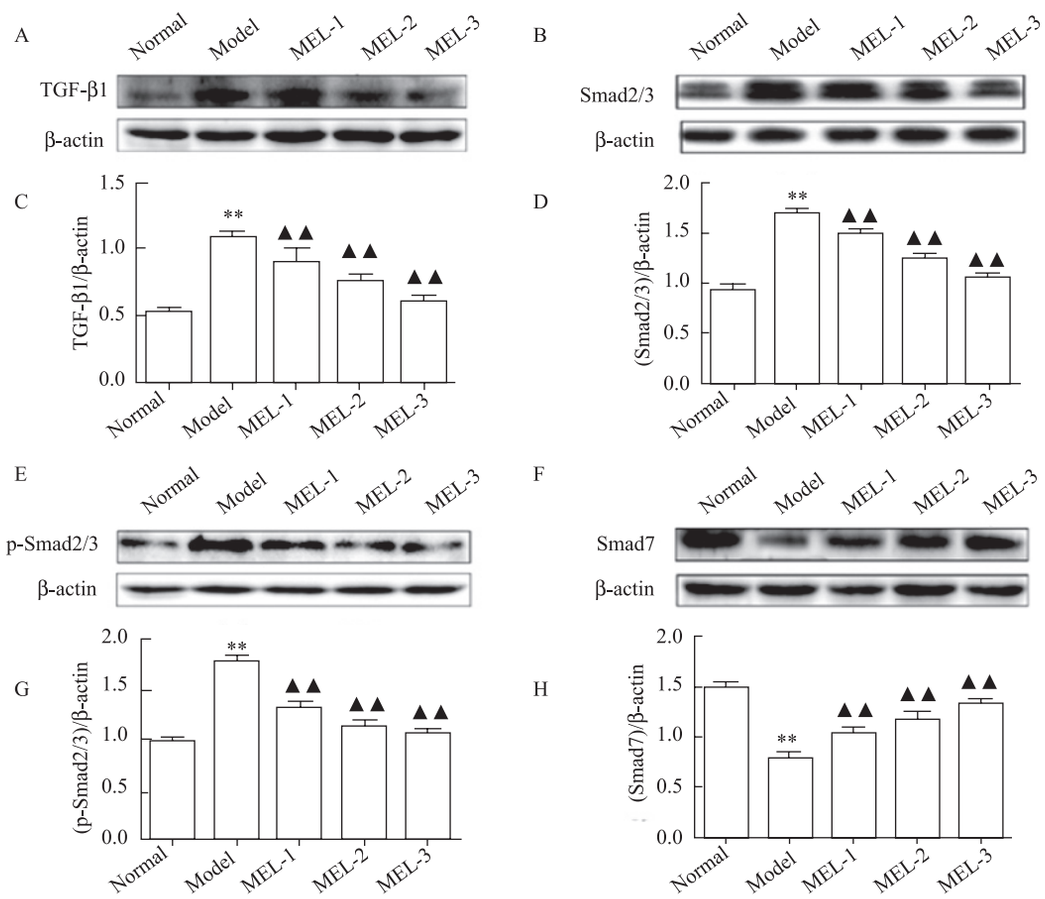

Fig. 4 Effect of melatonin on the expression of TGF-β1/Smad pathway in liver tissue (Western blotting)
A: representative Western blots for TGF-β1 protein; B: representative Western blots for Smad2/3 protein; C: the ratio of TGF-β1 normalized to β-actin protein levels; D: the ratio of Smad2/3 normalized to β-actin protein levels; E: representative Western blots for p-Smad2/3 protein; F: representative Western blots for Smad7 protein; G: the ratio of p-Smad2/3 normalized to β-actin protein levels; H: the ratio of Smad7 normalized to β-actin protein levels. **P<0.01 *vs*. the normal group, ▲▲P<0.01 *vs*. the model group ($\bar{x}\pm s$, n=3).

3 DISCUSSION

Hepatic fibrosis is considered as a universal pathological process in a lot of chronic liver diseases, which results in the loss of liver function and the extensive deposition of ECM. CCl_4 is widely applied to induce the experimental model of liver fibrosis, because CCl_4-induced liver fibrosis is similar to

human liver fibrosis in morphology[14]. As we known, oxidative stress is a major mechanism involved in liver fibrosis model induced by CCl_4. CCl_4 is metabolized by cytochrome P-450 to the trichloromethyl radical (CCl3•) and the trichloromethylperoxyl radical (OOCCl3•), producing free radicals that lead to the degeneration of hepatocytes, the apoptosis of hepatocytes and the infiltration of inflammatory cells, finally resulting in liver fibrosis[15, 16]. In this study, the model of liver fibrosis induced by CCl_4 for 6 weeks was established successfully, which was indicated by increased enzyme activities of ALT and AST in serum samples, elevated concentrations of Hyp in liver tissues, increased pathological scores and the significant deposition of ECM in liver. Furthermore, the level of MDA, an index of lipid peroxidation, was significantly increased in the model group, as compared to the normal group. The activities of GPx in liver from the model group were reduced, in comparison to the normal group.

As we know, oxidative stress plays a pivotal role in the etiopathogenesis of hepatic fibrosis. Reports have demonstrated that oxidative stress aggravates liver fibrosis through activating HSCs and that lipid peroxidation stimulates the transcription of the collagen genes[17, 18]. More evidence showed that antioxidants could ameliorate liver fibrosis[19]. Melatonin, the main production of the pineal gland, has multiple physiological functions, for instance, regulation of circadian rhythms, sleep, stress response, process of aging, and immunity[20, 21]. Furthermore, melatonin is a well-known powerful antioxidant. On the one hand, melatonin detoxifies reactive oxygen intermediates and a variety of free radicals directly. On the other hand, melatonin increases indirectly the activities and/or the expression of the main antioxidant enzymes, for instance, superoxide dismutase, catalase, GPx and glutathione reductase, and thus improves the antioxidative capacity of the organism[22, 23]. Hu *et al* reported that melatonin attenuated liver injury induced by alcohol and it was correlated to the anti-oxidative effect, indicated by decreased MDA levels and increased GPx and SOD activities in liver[24]. Hatzis *et al* found that melatonin ameliorated fatty liver damage in rats induced by high fat diet through countering lipid peroxidation and oxidative stress[21]. Our previous studies suggested that melatonin could ameliorate acute alcoholic liver injury in rats via its antioxidant capability[25]. Recently, some reports showed that protective effects of melatonin on liver injuries may involve other mechanisms[26, 27]. Increasing reports suggested that melatonin could exert anti-fibrotic effects via inhibition of oxidative stress[28]. Our previous works showed the antifibrotic effect of melatonin on CCl_4-induced hepatic fibrosis in experimental rats and deduced that the mechanism may be related with its anti-oxidant activities[11, 12]. In this study, administration of melatonin significantly attenuated liver fibrosis induced by CCl_4, as demonstrated by improved changes in liver histopathology, reduced pathological scores, decreased Hyp levels in liver homogenates and reduced serum activities of ALT and AST. Melatonin exerted its antioxidant effect, as evidenced by decreased MDA levels and significantly increased GPx activities in liver homogenates. So, we concluded that melatonin ameliorated liver fibrosisly induced by CCl_4 and its mechanism may be partially related to the suppression of oxidative stress.

The pathological process of liver fibrosis involves multiple cellular events and numerous cytokines. TGF-β1 has been demonstrated as the most potent pro-fibrotic cytokine in the pathogenesis of liver fibrosis, mostly in the development and progression of fibrogenesis and ECM metabolism[29]. TGF-β1 regulates the proliferation of hepatocyte negatively and induces the apoptosis of hepatocytes during hepatic fibrosis. The main mechanism of TGF-β1 is to activate and promote the transformation of HSCs[30–32]. TGF-β1 performs its functions mainly via the Smad signaling transduction pathway[33]. According to different structures and functions, Smads have been classified into common Smad (Co-Smad), inhibitory Smad (I-Smad) and activated Smads (R-Smads). After the activation of serine-threonine kinase in TβRI, Smad2/3 is phosphorylated and then combines with Co-Smad. Then the newly formed complex migrates into cell nucleus for the transcription and expression of the target genes, including MMPs, several fibrillar ECM proteins (fibronectin, collagen) and plasminogen activator inhibitor type 1[5, 14, 33]. In this pathway, Smad 2 and 3 are stimulatory, and p-Smad2/3 proteins play a pivotal role in transmitting signals from TGF-β1 receptors to the nucleus, while Smad7, as a negative feedback mediator, seems to be antagonistic to TGF-β1 by forming a stable complex with activated TβRI[6, 34]. In this regard, TGF-β1/Smad signaling pathway may be an appropriate therapeutic target for treating liver fibrosis. Increasing evidence indicated that downregulating TGF-β1/Smad signaling way had been an effective therapeutic strategy in liver fibrosis models[35]. Yao *et al* reported that curcumin significantly ameliorated liver inflammation and fibrosis induced by CCl_4 via inhibiting TGF-β1/Smad signaling pathway and downregulating CTGF expression[6]. Li *et al* reported that luteolin attenuated hepatic fibrosis through inhibition of AKT/mTOR/p70S6K and TGFbeta/Smad signaling pathways[36]. Our previous studies indicated that melatonin could prevent liver fibrosis[11], so we hypothesized that the mechanism by which melatonin suppresses liver fibrosis is related to the inhibition of TGF-β1/Smad pathway. In this study, immunohistochemistry and Western blotting were applied to evaluate TGF-β1/

Smad pathway expression. We found that compared to the normal group, TGF-β1, Smad2/3, p-Smad2/3 expression was increased and Smad7 expression was decreased in the group treated with CCl_4 only, whereas administration of melatonin (2.5, 5.0, 10.0 mg/kg) reversed these effects induced by CCl_4. These results showed that melatonin administration significantly downregulated the TGF-β1/Smad pathway. So we drew a conclusion that the preventive effect of melatonin on liver fibrosis induced by CCl_4 perhaps was correlated to its inhibition of TGF-β1/Smad pathway.

In conclusion, this study demonstrated that melatonin significantly ameliorated CCl_4-induced liver fibrosis, the mechanisms of which may be correlated to its downregulation of TGF-β1/Smad pathway and its anti-oxidant effects. However, further studies are indispensable for the confirmation of the mechanism. Related work is in progress in our laboratory.

Conflict of Interest Statement

The authors declare that there is no conflict of interest with any financial organization or corporation or individual that can inappropriately influence this work.

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