Melatonin ameliorates experimental hepatic fibrosis induced by carbon tetrachloride in rats

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Abstract

AIM: To investigate the protective effects of melatonin on carbon tetrachloride (CCl₄)-induced hepatic fibrosis in experimental rats.

METHODS: All rats were randomly divided into normal control group, model control group treated with CCl₄ for 12 wk, CCl₄ + NAC group treated with CCl₄ + NAC (100 mg/kg, i.p.) for 12 wk, CCl₄ + MEL-1 group treated with CCl₄ + melatonin (2.5 mg/kg) for 12 wk, CCl₄ + MEL-2 group treated with CCl₄ + melatonin (5.0 mg/kg) for 12 wk, and CCl₄ + MEL-3 group treated with CCl₄ + melatonin (10 mg/kg). Rats in the treatment groups were injected subcutaneously with sterile CCl₄ (3 mL/kg, body weight) in a ratio of 2:3 with olive oil twice a week. Rats in normal control group received hypodermic injection of olive oil at the same dose and frequency as those in treatment groups. At the end of experiment, rats in each group were anesthetized and sacrificed. Hematoxylin and eosin (HE) staining and Van Gieson staining were used to examine changes in liver pathology. Serum activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and protein concentration were measured with routine laboratory methods using an autoanalyzer. Hydroxyproline (HYP) content in liver and malondialdehyde (MDA) and glutathione peroxidase (GPx) levels in liver homogenates were assayed by spectrophotometry. Serum hyaluronic acid (HA), laminin (LN), and procollagen III N-terminal peptide (PⅢNP) were determined by radioimmunoassay.

RESULTS: Pathologic grading showed that the fibrogenesis was much less severe in CCl₄ + MEL3 group than in model control group (\( \mu = 2.172, P < 0.05 \)), indicating that melatonin (10 mg/kg) can significantly ameliorate CCl₄-induced hepatic fibrotic changes. The serum levels of ALT and AST were markedly lower in CCl₄ + MEL treatment groups (5, 10 mg/kg) than in model control group (ALT: 286.23 ± 121.91 U/L vs 201.15 ± 101.16 U/L and 178.67 ± 103.14 U/L, \( P = 0.028, P = 0.007 \); AST: 431.00 ± 166.35 U/L vs 321.23 ± 162.48 U/L and 292.42 ± 126.23 U/L, \( P = 0.043, P = 0.013 \)). Similarly, the serum laminin (LN) and hyaluronic acid (HA) levels and hydroxyproline (HYP) contents in liver were significantly lower in CCl₄ + MEL-3 group (10 mg/kg) than in model control group (LN: 45.89 ± 11.71 μg/L vs 55.26 ± 12.30 μg/L, \( P = 0.012 \); HA: 135.71 ± 76.03 μg/L vs 201.10 ± 68.46 μg/L, \( P = 0.020 \); HYP: 0.42 ± 0.08 mg/g tissue vs 0.51 ± 0.07 mg/g tissue, \( P = 0.012 \)). Moreover, treatment with melatonin (5, 10 mg/kg) significantly reduced the MDA content and increased the GPx activity in liver homogenates compared with model control group (MDA: 7.89 ± 1.49 nmol/mg prot vs 6.29 ± 1.42 nmol/mg prot and 6.25 ± 2.27 nmol/mg prot, respectively, \( P = 0.015, P = 0.015 \); GPx: 49.13 ± 8.72 U/mg prot vs 57.38 ± 7.65 U/mg prot and 61.39 ± 13.15 U/mg prot, respectively, \( P = 0.035, P = 0.003 \)).

CONCLUSION: Melatonin can ameliorate CCl₄-induced hepatic fibrosis in rats. The protective effect of melatonin on hepatic fibrosis may be related to its antioxidant activities.

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Key words: Melatonin; Hepatic fibrosis; Oxidative stress; Hyaluronic acid; Laminin; Malondialdehyde; Glutathione peroxidase

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INTRODUCTION

Hepatic fibrosis, a common pathological process of chronic hepatic disease, can lead to irreversible cirrhosis, and involves multiple cellular and molecular events that ultimately result in accumulation of collagen and extra cellular matrix protein in space of Disse. If treated properly at fibrosis stage, cirrhosis can be prevented\(^4\). However, no effective antifibrosis drugs are available at present. Several lines of evidence suggest that oxidative stress plays an important role in the etiopathogenesis of hepatic fibrosis\(^[2,3]\).

Melatonin (N-acetyl-5-methoxytryptamine), a secretory product of the pineal gland, is a powerful endogenous antioxidant, regulates circadian rhythms, sleep and immune system activity, behaves as a free radical scavenger\(^6\), eliminates oxygen free radicals and reactive intermediates\(^{[8,9]}\). Both in vitro and in vivo experiments have shown that melatonin can protect cells, tissues, and organs against oxidative damage induced by a variety of free-radical-generating agents and processes, such as safrole, lipopolysaccharide (LPS), carbon tetrachloride (CCL\(_4\)), ischemia-reperfusion, amyloid-protein, and ionizing radiation\(^{[10-12]}\). In addition, melatonin also has an indirect antioxidant effect by enhancing the levels of potential antioxidants such as glutathione peroxidase (GPx), superoxide dismutase (SOD), and glutathione (GSH)\(^{[10-12]}\). Recent studies showed that melatonin exerts its cytoprotective effects in various experimental models of acute liver injury and reduces fibroblast proliferation and collagen synthesis\(^{[12,13]}\), indicating that melatonin may have therapeutic effects on acute and chronic liver injury, through its antioxidant action.

The aim of our present study was to evaluate the possible antifibrotic effect of melatonin on a hepatic fibrosis model of rats. In addition, the antioxidant and anti-inflammatory properties of melatonin were investigated in rats with liver fibrosis.

MATERIALS AND METHODS

Drugs and materials

Crystalline melatonin was purchased from Sigma Chemical Company (St. Louis, MO, USA). The solvent used for melatonin was a mixture of ethanol (1%, v/v) and NaCl (0.9%). N-acetyl-L-cysteine (NAC) was purchased from Shanghai Sinopharm Chemical Reagent Co. Ltd (Shanghai, China). Commercial kits used for determining malondialdehyde (MDA), glutathione peroxidase (GPx) and hydroxyproline (HYP) were obtained from Jiancheng Institute of Biotechnology (Nanjing, China). Commercial kits for radioimmunoassay of procollagen \(\text{N}\)-terminal peptide (P\(\text{\text{III}}\)NP), laminin (LN), and hyaluronic acid (HA) were obtained from Beijing North Institute of Biological Technology (Beijing, China). Other commercial chemicals used in experiments were of analytical grade.

Animal experiments and drug treatment

Male Sprague-Dawley rats, weighing 170-240 g at beginning of the study, purchased from Anli Experimental Animal Limited Company (Anhui, China), were kept at a constant temperature (22°C) in a 12-h light and dark cycle, with free access to food and water. All animals were treated humanely according to the National Guidelines for the Care of Animals in China. Rats were randomly divided into normal control group \((n = 11)\), model control group \((n = 20)\) treated with CCL\(_4\) for 12 wk, CCL\(_4\) + NAC group \((n = 20)\) treated with CCL\(_4\) + NAC (100 mg/kg, i.p.) for 12 wk, CCL\(_4\) + MEL-1 group \((n = 20)\) treated with CCL\(_4\) + melatonin (2.5 mg/kg) for 12 wk, CCL\(_4\) + MEL-2 group \((n = 20)\) treated with CCL\(_4\) + melatonin (5.0 mg/kg) for 12 wk, and CCL\(_4\) + MEL-3 group \((n = 20)\) treated with CCL\(_4\) + melatonin (10 mg/kg) for 12 wk. Rats in treatment groups were injected subcutaneously with sterile CCL\(_4\) (3 mL/kg of body weight) in a ratio of 2:3 with olive oil twice a week. Rats in normal control group received hypodermic injection of olive oil at the same dose and frequency as those in the treatment groups. At the beginning of CCL\(_4\) injection, rats received intraperitoneal melatonin daily whereas rats that did not receive melatonin were given the same volume of vehicle (1% ethanol) at the same time point. After 12 wk, a laparotomy was performed and blood was drawn from the abdominal aorta under 3% pentobarbital sodium (1 mL/kg) anesthesia. The animals were then killed with their livers removed. Blood was collected into tubes and centrifuged. Serum was aspirated and stored at -80°C. Liver tissue was fixed in formalin for routine histological examination, or stored at -80°C until required.

Histopathological examination

Liver tissue samples, fixed in 40 g/L paraformaldehyde and embedded in paraffin, were cut into 5-µm thick sections, which were stained with hematoxylin and eosin (HE) and Van Gieson (VG) according to the standard procedure. Van Gieson’s method was used to detect collagen fibers. Hepatic fibrosis was divided into the following stages as previously described\(^{[14]}\): stage 0: no fibrosis; stage 1: expansion of portal tracts without linkage; stage 2: portal expansion with portal to portal linkage; stage 3: expansive portal to portal and focal portal to central linkage; and stage 4: cirrhosis. Two pathologists with no knowledge of liver sources examined the stained sections independently.
Analysis of liver function
Serum activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and protein concentration were measured with routine laboratory methods using an autoanalyzer (Hitachi Automatic Analyzer, Japan).

Measurement of MDA and GPx levels in liver homogenates
Liver samples were thawed, weighed and homogenized (1:9 w:v) in 0.9% saline. The homogenates were centrifuged at 1000 × g for 10 min at 4°C and supernatant was taken for assay of MDA and GPx with a commercial kit (Jiancheng Institute of Biotechnology, Nanjing, China) following its manufacturer’s instructions. MDA was assayed by measuring the levels of thiobarbituric acid reactive substances (TBARS) at 532 nm and expressed as nmol/mg protein. GPx assay was based on its ability to inhibit oxidation of oxyamine by the xanthine-xanthine oxidase system. Total protein concentration in liver homogenates was determined using the Coomassie blue method with bovine serum albumin as a standard.

Detection of hydroxyproline content in liver
Total collagen content in fresh liver samples was determined by hydroxyproline assay. Hydroxyproline content was detected with a commercial hydroxyproline detection kit (Jiancheng Institute of Biotechnology, Nanjing, China) following its manufacturer’s instructions.

Detection of liver function
Serum ALT and AST levels were significantly higher in experimental and model groups than in normal control group (P < 0.01). The ALT and AST levels were significantly higher in model group than in CCl4 + NAC and CCl4 + MEL groups (5, 10 mg/kg) (P < 0.05, P < 0.01). Melatonin (5, 10 mg/kg) significantly decreased the elevated serum transaminase levels (Table 1, Figure 1C-F).

MDA content and GPx activity in liver homogenates
The MDA level was significantly higher while GPx activity was significantly lower in liver homogenates of CCl4 + NAC and CCl4 + MEL groups than in those of normal control group (P < 0.01). The MDA level was significantly higher in model control group than in CCl4 + NAC and CCl4 + MEL groups (5, 10 mg/kg) (P < 0.05). Melatonin (5, 10 mg/kg) significantly decreased the elevated MDA level. GPx activity was significantly lower in the model control group than in CCl4 + NAC and CCl4 + MEL groups (5, 10 mg/kg) (P < 0.05, P < 0.01, Table 3).

HYP contents in liver tissue
Hepatic fibrosis was quantified by measuring hepatic

### Table 1  Pathologic grading of hepatic fibrosis in different groups

| Group         | Dose (mg/kg) | n | Pathologic grading of hepatic fibrosis | A/G value |
|---------------|--------------|---|---------------------------------------|-----------|
|               |              |   | 0          | I          | II         | III        | IV        |
| Normal        | -            | 11| 11         | 0          | 0          | 0          | 0         | 5.5681†   |
| Model         | -            | 13| 0          | 0          | 1          | 6          | 6         | -         |
| NAC           | 100          | 12| 2          | 5          | 3          | 2          | 1.8838    |
| MEL           | 2.5          | 11| 0          | 2          | 4          | 2          | 3         | 1.5568    |
| MEL           | 5            | 13| 0          | 2          | 4          | 3          | 4         | 1.3662    |
| MEL           | 10           | 12| 0          | 4          | 4          | 1          | 3         | 2.1720‡   |

* represents the Ridit value of the two groups, † P < 0.05 indicates a > 1.96; P < 0.01 indicates a > 2.58; ‡ P < 0.05, ‡‡ P < 0.01 vs model group.

### Table 2  Effect of melatonin on serum ALT, AST levels and A/G value in different groups (mean ± SD)

| Group         | Dose (mg/kg) | n | ALT (U/L) | AST (U/L) | A/G |
|---------------|--------------|---|-----------|-----------|-----|
| Normal        | -            | 11| 70.00 ± 35.27| 139.82 ± 72.83| 0.94 ± 0.40 |
| Model         | -            | 13| 286.23 ± 121.91| 431.00 ± 166.35| 0.74 ± 0.09 |
| NAC           | 100          | 12| 194.42 ± 90.83c| 293.33 ± 94.60c| 0.78 ± 0.11 |
| MEL           | 2.5          | 11| 211.09 ± 97.03c| 357.09 ± 153.32c| 0.68 ± 0.15 |
| MEL           | 5            | 13| 201.15 ± 101.16c| 321.23 ± 162.48c| 0.77 ± 0.15 |
| MEL           | 10           | 12| 178.67 ± 103.14c| 292.42 ± 162.23c| 0.73 ± 0.07 |

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; A: Albumin; G: Globulin. a < 0.01 vs normal control group; b P < 0.05 vs model control group; c P < 0.01 vs model control group.
hydroxyproline. The hydroxyproline content was significantly higher in model control, CCl₄ + NAC and CCl₄ + MEL groups than in normal control group (P < 0.01), and significantly higher in model group than in CCl₄ + NAC and CCl₄ + MEL groups (10 mg/kg) (P < 0.05). Treatment with melatonin (10 mg/kg) or NAC reduced the hydroxyproline content in liver homogenates, and therefore prevented hepatic fibrosis induced by CCl₄ (Figure 2).

Measurement of serum HA, LN, and PⅢNP levels
The serum LN and HA levels were significantly higher in model control, CCl₄ + NAC, and CCl₄ + MEL groups than in normal control group (P < 0.05, P < 0.01), and significantly decreased after treatment with melatonin (10 mg/kg) (P < 0.05). Treatment with NAC significantly reduced the serum HA level (P < 0.05). The serum PⅢNP level was significantly higher in model control, CCl₄ + NAC and CCl₄ + MEL groups than in normal control group (P < 0.05). However, no significant difference was observed among the five groups (Table 4).

Figure 1  Pathological changes. Light microscopy of liver tissue sections showing normal liver lobular architecture with central veins in the normal control group (HE staining, × 200) (A), no collagen deposition in liver of normal control group (VG staining, × 200) (B), degenerated and necrotic liver cells associated with inflammatory cells in model group (HE staining, × 200) (C), formation of fibrotic septa in model group (VG staining, × 200) (D), and pathological change in liver of CCl₄ + melatonin (10 mg/kg) group was rather milder compared with the model group (HE staining, × 200; VG staining, × 200) (E, F).

Table 3  MDA content and GPx activity in liver homogenates of different groups (mean ± SD)

| Group    | Dose (mg/kg) | n  | MDA (nmol/mg prot) | GPx (U/mg prot) |
|----------|--------------|----|--------------------|-----------------|
| Normal   | -            | 11 | 4.3 ± 1.87         | 80.68 ± 10.76   |
| Model    | -            | 13 | 7.89 ± 1.49         | 49.13 ± 8.72    |
| NAC      | 100          | 12 | 6.29 ± 1.36         | 64.68 ± 8.22    |
| MEL      | 2.5          | 11 | 6.84 ± 1.10         | 53.44 ± 9.35    |
| MEL      | 5            | 13 | 6.29 ± 1.42         | 57.38 ± 7.65    |
| MEL      | 10           | 12 | 6.25 ± 2.27         | 61.39 ± 13.13   |

MDA: Malondialdehyde; GPx: Glutathione peroxidase. *P < 0.01 vs normal control group; **P < 0.05 vs model control group; ***P < 0.01 vs model control group.
efficiency than vitamin E and GSH, which are known as powerful antioxidants\cite{10}. The antioxidant properties of melatonin prevent acute liver injury induced by ischemia-reperfusion\cite{23}, irradiation\cite{24}, bile duct ligation\cite{25-27}, and toxins\cite{18,28,29}. Several lines of evidence suggest that melatonin plays an important role in regulation of collagen levels and inhibition of collagen accumulation. Ostrowska et al\cite{30} showed that melatonin is negatively related with urine hydroxyproline levels in fasting rats. Cunnane et al\cite{31} demonstrated that primary biliary cirrhosis is related with melatonin deficiency in pinealectomized rats. Tahan et al\cite{32} reported that daily melatonin injection at pharmacological doses is effective against liver damage in a rat liver fibrosis model induced by a 14-d dimethylsulfoxide regimen. In the present study, liver injury was assessed with histological and biochemical parameters. The results suggest that melatonin could decrease the scores of hepatic fibrosis and serum ALT and AST levels in rats with hepatic injury caused by CCl\textsubscript{4}. Melatonin at a dose of 10 mg/kg was as effective as 100 mg/kg NAC in reducing serum ALT and AST levels, indicating that melatonin can protect liver and alleviate the progression of hepatic fibrosis. However, further study is needed on the liver function protective effect of melatonin in cirrhotic patients.

HA and LN are known to be good serum markers of hepatic fibrogenesis\cite{12,14}. HYP in liver is an important index reflecting the degree of hepatic fibrosis and hepatic fibrosis can be quantified by measuring hepatic hydroxyproline\cite{33,35}. In the present study, treatment with melatonin (10 mg/kg) could significantly reduce HA and LN in serum and HYP in liver. The decreased of hepatic hydroxyproline and serum LN and HA levels indicate that melatonin can inhibit collagen deposition in liver.

Oxidative stress plays an important role in the formation of hepatic fibrosis \textit{via} increasing stellate cell activation and collagen synthesis. MDA is the main product of lipid peroxidation and its concentration is generally presented as the total level of lipid peroxidation products\cite{36}. As an end product of lipid peroxidation, MDA can produce ozone, which reacts rapidly with cellular structures, generates hydrogen peroxide and other reactive oxygen species, leading to peroxidation and denaturation of membranes\cite{37}. It has been shown that MDA can activate stellate cells that produce collagen. The results of this study suggest that treatment with melatonin (5, 10 mg/kg) could significantly block increased MDA, suggesting that melatonin decreases lipid peroxidation and plays an anti-oxidative role in hepatic fibrosis induced by CCl\textsubscript{4} in rats.

Melatonin is not only a direct antioxidant but also an indirect antioxidant through enhancement of antioxidant enzyme activities in liver\cite{39}. It was reported that melatonin can reduce free radical damage by elevating GPs activation\cite{11,30}. Montilla et al\cite{32} reported that acute ligation of the bile duct is accompanied with decreased GSH levels both in plasma and in liver, as well as significantly reduced antioxidant enzyme activities. Treatment with melatonin is associated with a significant recovery of anti-oxidative enzymes such as

### Table 4 Serum HA, LN and PIII NP levels in different groups (mean ± SD)

| Group   | Dose (mg/kg) | n  | HA (µg/L) | LN (µg/L) | PIII NP (µg/L) |
|---------|--------------|----|-----------|-----------|----------------|
| Normal  | -            | 11 | 71.65 ± 27.64 | 37.65 ± 6.09 | 26.41 ± 7.28 |
| Model   | -            | 13 | 210.10 ± 68.40 | 55.26 ± 12.30 | 35.88 ± 5.92 |
| NAC     | 100          | 12 | 131.31 ± 58.58 | 34.89 ± 6.40 | 32.89 ± 3.90 |
| MEL     | 2.5          | 11 | 174.41 ± 72.99 | 34.89 ± 5.92 | 32.89 ± 3.90 |
| MEL     | 10           | 12 | 135.71 ± 66.03 | 45.89 ± 11.71 | 31.99 ± 6.09 |

HA: Hyaluronic acid; LN: Laminin; PIII NP: Procollagen III N-terminal peptide.
1. *P < 0.05 vs model control group; 2. *P < 0.01 vs normal control group;
   3. *P < 0.05 vs model control group.

**DISCUSSION**

CCl\textsubscript{4} is widely used to induce hepatic fibrosis and cirrhosis in animal models. In this study, hepatic fibrosis was successfully induced by subcutaneous injection of sterile CCl\textsubscript{4} twice weekly for 12 wk. Through this hepatic fibrosis model, the effects of melatonin on hepatic fibrosis induced by CCl\textsubscript{4} in rats were examined.

N-acetylcycteine (NAC), a free radical scavenger, is a glutathione precursor which increases glutathione levels in hepatocytes\cite{35,36}. Increased glutathione levels limit the production of reactive oxygen species (ROS) which can cause hepatocellular injury. NAC can also inhibit the proliferation of hepatic stellate cells\cite{37,38}. Therefore, it was used as a positive control in this study.

It is well known that oxidative damage can induce hepatic fibrogenesis. ROS, such as H\textsubscript{2}O\textsubscript{2}, O\textsubscript{2}−, and OH\textsuperscript{−}, are implicated in the development and pathological progress of hepatic fibrosis\cite{18,19}. Free radicals and biomolecular reaction products promote phagocytic and myofibroblastic activities. Lipid peroxidation accelerates collagen synthesis by stimulating stellate cells\cite{39}. It has been shown that melatonin is an effective antioxidant and a free radical scavenger. Due to its small size and high lipophilicity, melatonin can cross biological membranes easily and reach all compartments within the cell\cite{31}, thus protecting DNA, proteins, and biological membrane lipids from the deleterious effects of free radicals\cite{20}. It has been found that melatonin has a higher antioxidant

**HA: Hyaluronic acid; LN: Laminin; PIII NP: Procollagen III N-terminal peptide.**
GPx\textsuperscript{[20,21]} found that melatonin can restore GPx activity in a rat liver fibrosis model induced by a 14-d dimethylnitosamine regimen. In this study, the GPx activity was significantly lower in model control group than in CCl\textsubscript{4} + NAC and CCl\textsubscript{4} + MEL groups (5, 10 mg/kg), indicating that melatonin can protect liver against CCl\textsubscript{4}-induced hepatic fibrosis in rats, possibly through its direct and indirect antioxidant effects.

In conclusion, melatonin may have beneficial effects on hepatic fibrosis induced by CCl\textsubscript{4} in rats. The protective effect of melatonin on hepatic fibrosis may be related to its antioxidant activities.

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**COMMENTS**

**Background**

In China, the incidence of liver cirrhosis is still high. Liver cirrhosis results from fibrosis. If treated properly at fibrosis stage, cirrhosis can be prevented. However, no effective antifibrosis drugs are available at present. Several lines of evidence suggest that oxidative stress plays an important role in the etiopathogenesis of hepatic fibrosis. Melatonin can protect cells, tissues, and organs against oxidative damage induced by a variety of free-radical-generating agents and processes. The possible fibrosuppressant effect of melatonin on hepatic fibrosis was evaluated in this study. In addition, the antioxidant and anti-inflammatory properties of melatonin were investigated in rats with fibrosis.

**Research frontiers**

Although the exact pathogenesis of hepatic fibrosis is still obscure, the role of free radicals and lipid peroxides in the development of hepatic fibrosis has attracted considerable attention. If treated properly at this stage, cirrhosis can be successfully prevented. However, it remains a problem to prevent hepatic fibrosis or to control its progression. Great efforts have been made to find safe and effective drugs. Recent experiments demonstrate that melatonin may have therapeutic effects on acute and chronic liver injury, possibly through its antioxidant activities.

**Innovations and breakthroughs**

Melatonin may have beneficial effects on hepatic fibrosis induced by carbon tetrachloride in rats. The protective effect of melatonin may be related to its antioxidant activities.

**Applications**

Melatonin can be used as an antioxidant drug, protect liver cells against fibrosis and inhibits collagen fiber deposition in liver, thus providing a basis for further studies on its therapeutic effect on hepatic fibrosis.

**Terminology**

Melatonin (N-acetyl-5-methoxytryptamine), a secretory product of the pineal gland, is a powerful endogenous antioxidant. It regulates circadian rhythms, sleep and immune system activity, behaves as a free radical scavenger, and eliminates oxygen free radicals and reactive intermediates.

**Peer review**

This is a well-designed study describing the protective effect of melatonin on fibrosis induced by carbon tetrachloride. Methods are appropriate and results are consistent with the conclusion. The study is very interesting with a great amount of data, which corroborate the major conclusion.
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