

## Original Article

# The protective effects of melatonin on peritoneal fibrosis induced by high glucose peritoneal dialysis in rats

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**Abstract:** The prevention and reduction of peritoneal fibrosis are the keys to long-term successful dialysis in peritoneal dialysis. In this study, we investigated the protective effects of melatonin (MT) on peritoneal fibrosis in rats. The rats were injected intraperitoneally with 4.25% peritoneal dialysis fluid at 100 ml/kg daily to build a peritoneal fibrosis model. MT (5, 10, 20 mg·kg<sup>-1</sup>·d<sup>-1</sup>) was given through an intraperitoneal injection for 4 weeks. In a 4 h peritoneal equilibration test, MT could increase ultrafiltration (UF) as well as the ratio of glucose concentration in dialysate at 4 h and at the initiation of the dwell (D/D0) and could decrease the dialysate-to-plasma ratio of creatinine (D/PCr). The peritoneum pathological changes were markedly reduced by MT intervention. The expressions of TGF-β1, Col-I and α-SMA were significantly more increased in the model group than they were in the control group. They were significantly decreased by treatment with MT. A Western blot analysis showed that the expressions of peritoneal TGF-β1 and p-Smad 2/3 were significantly increased and that Smad 7 was significantly decreased in the model group compared to the control group, MT intervention might reduce TGF-β1 and p-Smad 2/3 and elevate Smad 7 expressions. These data show MT can inhibit peritoneal fibrosis in rats by improving the structure and function of the peritoneum and by inhibiting the expressions of the TGF-β1, α-SMA, and Col-I. And it may exert its anti-fibrosis effects through the TGF-β1/Smad pathway.

**Keywords:** Melatonin, peritoneal fibrosis, peritoneal dialysis, TGF-β1/Smad pathway

## Introduction

Peritoneal dialysis (PD) is an established form of renal replacement therapy for patients suffering from end stage renal disease (ESRD) [1]. It has been demonstrated that the mortality risk of patients on PD may be lower than patients on hemodialysis, especially during the first two years for those on renal replacement therapy [2, 3]. In patients undergoing peritoneal dialysis, the peritoneum is exposed to hyperglycemic, hypertonic, and non-biocompatible peritoneal dialysis fluid at a low pH, which can lead to peritoneal fibrosis over a long period of time, resulting in the loss of peritoneal structure and function [4-6]. Long-term PD induces morphologic and functional changes to the peritoneum, leading to peritoneal ultrafiltration failure (UF) [7]. Therefore, the prevention and reduction of peritoneal fibrosis is the key to long-term successful dialysis in peritoneal dialysis patients.

Recent studies showed that the TGF-β/Smad signaling pathway plays an important role in the pathogenesis of peritoneal changes induced by PD [8, 9]. TGF-β can promote the production of an extracellular matrix component deposition. Some studies showed that blocking TGF-β can prevent the progression of peritoneal fibrosis. The induction of profibrotic genes by TGF-β signal transduction is mediated by the phosphorylation of Smad 2 and Smad 3. Meanwhile, Smad 6 and Smad 7 are transcriptionally induced by TGF-β and negatively regulate these pathways [9].

Melatonin (N-acetyl-5-methoxytryptamine) is predominantly synthesized by the pineal gland of the human brain in response to darkness [10, 11]. It is an indolamine derived from tryptophan. This indole and its metabolites have been shown to have many physiological functions, especially those working against inflammation and oxidative stress [12-14]. Meanwhile, some

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studies found that melatonin could play an anti-fibrosis role in some experimental models [15]. Also, it was demonstrated that melatonin could inhibit collagen deposition and regulate the maintenance of collagen levels [16].

In this study, melatonin intervention was given in order to observe its impact on peritoneal dialysis-related peritoneal fibrosis. We hope to acquire some experimental evidence for the prevention and treatment of peritoneal dialysis-related fibrosis.

### Materials and methods

#### *Drugs and reagents*

Peritoneal dialysis solution (lactate-G 4.25%) was purchased from Huaren Pharmaceutical Co., Ltd. (Qingdao, China). Melatonin was purchased from Sigma Chemical Co. (St. Louis, Mo, USA). Hematoxylin-eosin (HE) and Masson dye were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). TGF- $\beta$ 1, Col-I,  $\alpha$ -SMA, p-Smad 2/3 and Smad 7 polyclonal antibody were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti- $\beta$ -actin antibody, anti-mouse IgG, and anti-rabbit IgG horseradish-peroxidase (HRP) were obtained from Wuhan Sanying Biotechnology (Wuhan, China). The immunohistochemistry kit (PV-9000) and DAB chromogenic kit were bought from Beijing Zhongshan Biotechnology Inc (Zhongshan, China). Nitrocellulose membrane and the chemiluminescence kit were obtained from Amersham Life Science (Little Chalfont, UK).

#### *Animals*

Forty male Sprague-Dawley rats were purchased from the Anhui Medical University Animal Center, each weighing from 180 g~230 g. The animals were housed in rodent cages in a 24°C room with a 12 h light-dark cycle. They had free access to food and drinking water. The animals were housed for 1 week to adapt to the environment. The study was approved by the Animal Protection Committee of Anhui Medical University.

#### *Experimental design*

Forty male SD rats were randomly divided into a control group (C, n=8), a peritoneal fibrosis model group (M, n=8), a peritoneal fibrosis+MT 5 mg·kg<sup>-1</sup>·d<sup>-1</sup> administration group (MT 5

mg·kg<sup>-1</sup>·d<sup>-1</sup>, n=8), a peritoneal fibrosis+MT 10 mg·kg<sup>-1</sup>·d<sup>-1</sup> administration group (MT 10 mg·kg<sup>-1</sup>·d<sup>-1</sup>, n=8), and a peritoneal fibrosis+MT 20 mg·kg<sup>-1</sup>·d<sup>-1</sup> administration group (MT 20 mg·kg<sup>-1</sup>·d<sup>-1</sup>, n=8). Rats in the peritoneal fibrosis model group were intraperitoneally injected with 4.25% peritoneal dialysate daily at 100 ml/kg for 4 weeks. Rats in the melatonin intervention groups were given an intraperitoneal injection daily. The melatonin was dissolved in physiological saline containing 2% ethanol and given as an intraperitoneal injection daily for 4 weeks.

#### *Peritoneal equilibration test (PET)*

Forty-eight hours after stopping dialysis, a peritoneal equilibration test (PET) was performed in all the animals to evaluate the peritoneal membrane transport function. Each rat was intraperitoneally injected with 4.25% peritoneal dialysis solution 20 mL, and intraperitoneal retention was carried out for 4 hours after the intraperitoneal injection of 10% chloral hydrate (3 ml/kg) for anesthesia. The amount of ultrafiltration was measured. The remaining dialysate was then completely drained and collected. At the same time, blood samples were taken for the analysis of the rats' serum creatinine and glucose levels. The peritoneal transport characteristic was determined from the dialysate-to-plasma ratio of creatinine (D/PCr) and the ratio of glucose concentration in dialysate at 4 h and the initiation of the dwell (D/DO).

#### *Pathological and morphological observation of peritoneal tissue*

We removed the parietal peritoneums, then we fixed them with 10% neutral buffered formalin for 16-18 hours and followed this with the conventional dehydration of gradient alcohol, xylene transparent, immersion wax and paraffin embedding. 4  $\mu$ m sections were stained with hematoxylin and eosin (HE) and Masson staining, and the morphological changes were observed under a lighted microscope.

#### *Immunohistochemistry*

The 4  $\mu$ m paraffin sections were baked at 65°C for 3 hours, then this was followed by conventional dewaxing hydration, immersion in a 3% hydrogen peroxide solution for 10 minutes at room temperature and then phosphate buffer saline (PBS) washed 3 times. We added goat serum for 10 minutes at room temperature.

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**Table 1.** Changes in the body weights of the rats in each group (g) ( $\bar{x} \pm s$ )

Group	n	Dose	Day 1	Day 7	Day 14	Day 21	Day 28
C	8	-	206.65±4.19	246.8±5.75	287.17±4.02	336.49±3.8	378.19±3.95
M	7	-	204.54±4.57	247.57±3.91	287.3±4.76	337.42±3.64	378.37±5.42
MT	7	5 mg·kg <sup>-1</sup> ·d <sup>-1</sup>	208.08±5.04	249.02±4.48	283.86±4.15	335.99±10.71	377.07±3.63
MT	8	10 mg·kg <sup>-1</sup> ·d <sup>-1</sup>	208.12±3.95	246.42±4.48	286.3±4.11	337.02±7.1	380.45±4.02
MT	8	20 mg·kg <sup>-1</sup> ·d <sup>-1</sup>	207.18±4.18	247.78±4.34	287.65±1.61	339.17±6.5	377.52±3.86

**Table 2.** Changes in peritoneal function in each group ( $\bar{x} \pm s$ )

Group	n	Dose	UF (ml)	D4/D0	D/PCr
C	8	-	9.86±0.39	0.59±0.03	0.27±0.02
M	7	-	2.21±0.33* (*P=0.000)	0.40±0.01* (*P=0.000)	0.36±0.28* (*P=0.000)
MT	7	5 mg·kg <sup>-1</sup> ·d <sup>-1</sup>	2.9±0.28# (#P=0.013)	0.43±0.02# (#P=0.011)	0.16±0.03# (#P=0.000)
MT	8	10 mg·kg <sup>-1</sup> ·d <sup>-1</sup>	4.33±0.37# (#P=0.000)	0.47±0.02# (#P=0.000)	0.20±0.01# (#P=0.000)
MT	8	20 mg·kg <sup>-1</sup> ·d <sup>-1</sup>	5.28±0.23# (#P=0.000)	0.52±0.36# (#P=0.000)	0.21±0.03# (#P=0.000)

Compared with the control group, \*P<0.05; compared with the model group, #P<0.05. Data are represented as Mean ± SD.

The serum was then removed, and we added the TGF- $\beta$ 1 polyclonal antibody, the Col-I polyclonal antibody, and the  $\alpha$ -SMA polyclonal antibody. PBS was used instead of the corresponding antibody as a negative control. Then we rewarmed the serum with PV9000 Reagent 1 (Polymer Helper) and PV9000 Reagent 2 (poly peroxidase-anti-mouse/rabbit IgG) in a 37°C incubator. Then we stained it with DAB. A high-resolution color pathological image analysis system developed by Beijing University of Aeronautics and Astronautics was used to capture images, and the results were analyzed by image-Pro Plus 6.0 image analysis software.

### Western blots analysis

The parietal peritoneum proteins were lysed and combined with a loading buffer, boiled, then separated by 10-12% SDS-PAGE. The proteins were electroblotted onto a nitrocellulose membrane, incubated with primary antibody TGF- $\beta$ 1, p-Smad 2/3 and Smad 7 overnight at 4°C after being blocked with skim milk for 1 hour. The combination of secondary antibody (the goat anti-rabbit IgG) was handled once the membrane was washed. Finally, there came the observation of the image which needed the help of the enhanced chemiluminescence, and image J software was used for the gray-scale semi-quantitative analysis.

### Statistical analysis

The data were analyzed with the help of SPSS 16.0, and the continuous variables were expressed as the mean ± SD. All the compared

data were subjected to an ANOVA analysis. The differences between groups were tested by LSD, and the Levene method was used for the homogeneity test of variance, in which a P value under 0.05 was considered significant.

## Results

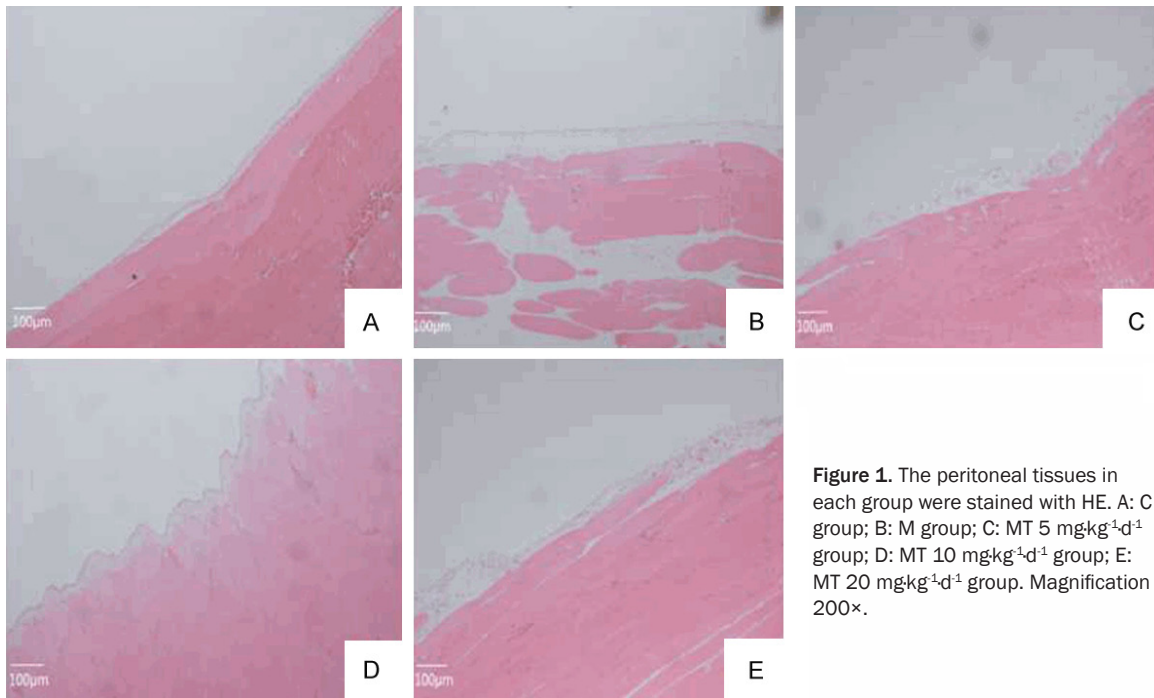
### Rat general situation

Forty rats were injected intraperitoneally once daily for 4 weeks. Two rats were withdrawn from the experiment, including one rat in the M group and one in the MT 5 mg·kg<sup>-1</sup>·d<sup>-1</sup> group. At the beginning of the experiment, there were no significant differences between the body weights of the rats in each group. After the MT intervention, the rats in each group were weighed every weekend. There were no significant differences between each group in the same period, and there were no significant differences between the same group in the different periods (**Table 1**).

### Peritoneal function

**Table 2** showed changes of peritoneal function. Compared with the C group, the UF and D4/D0 in the M group were decreased, D/PCr was increased, and the difference was statistically significant (P<0.05). UF and D4/D0 in the MT 5, 10, and 20 mg·kg<sup>-1</sup>·d<sup>-1</sup> intervention groups were increased compared with the M group and the differences were statistically significant (P<0.05), but D/PCr was decreased and the

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**Figure 1.** The peritoneal tissues in each group were stained with HE. A: C group; B: M group; C: MT 5 mg·kg<sup>-1</sup>·d<sup>-1</sup> group; D: MT 10 mg·kg<sup>-1</sup>·d<sup>-1</sup> group; E: MT 20 mg·kg<sup>-1</sup>·d<sup>-1</sup> group. Magnification 200×.

difference was also statistically significant ( $P < 0.05$ ).

### *Pathological and morphological changes in the peritoneum*

**Figure 1** showed that the peritoneal tissues were stained with HE staining. In the C group, HE staining showed that the peritoneal surface was covered with a layer of continuously flat mesothelial cells. In the M group, the shape of the mesothelial cells on the peritoneal surface changed from flat to round or columnar, and some mesothelial cells were missing. Inflammatory cell infiltration and neovascularization could be seen simultaneously. But the mesothelial cell detachment situation in the MT intervention groups was significantly improved compared with the M group and the peritoneal thickness was thinner compared with the M group. **Figure 2** showed that the peritoneal tissues were stained with Masson staining. Masson staining showed that the layer of peritoneal mesothelial cells in the C group was thin and continuous. Compared to the C group, visible subcutaneous collagen deposition and peritoneal thickening could be observed in the M group. The peritoneal thickness of the MT intervention groups were thinner than the M group. And the mesothelial cell collagen deposition decreased compared with the M group.

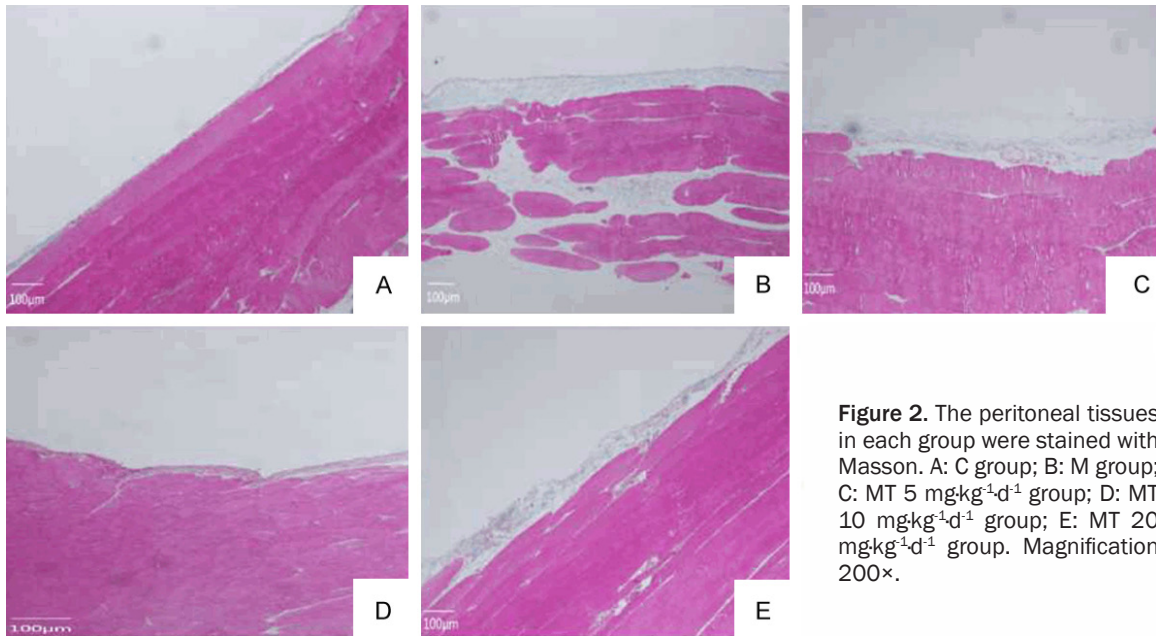
### *Immunohistochemistry*

**Figures 3-6** showed TGF- $\beta$ 1, Col-I and  $\alpha$ -SMA protein immunostaining as well as quantitative analyses in the parietal peritoneum. The immunohistochemistry showed that only a small amount of TGF- $\beta$ 1, Col-I and  $\alpha$ -SMA were expressed in the mesothelial cells of the C group. A large number of brown-yellow particles were found in the mesothelial cells in the M group. Compared with the C group, the intensity of TGF- $\beta$ 1, Col-I and  $\alpha$ -SMA immunostaining in the M group was significantly increased ( $P = 0.000$ ,  $P = 0.000$ ,  $P = 0.000$ ,  $P < 0.05$ ). Compared with the M group, the intensity of TGF- $\beta$ 1, Col-I and  $\alpha$ -SMA immunostaining in the 5 mg·kg<sup>-1</sup>·d<sup>-1</sup> group ( $P = 0.000$ ,  $P = 0.000$ ,  $P = 0.000$ ,  $P < 0.05$ ), 10 mg·kg<sup>-1</sup>·d<sup>-1</sup> group ( $P = 0.000$ ,  $P = 0.000$ ,  $P = 0.000$ ,  $P < 0.05$ ) and the 20 mg·kg<sup>-1</sup>·d<sup>-1</sup> group ( $P = 0.000$ ,  $P = 0.000$ ,  $P = 0.000$ ,  $P < 0.05$ ) was significantly decreased parallel to the concentration of MT given to the rats, proving that the treatment with MT 5, 10, and 20 mg·kg<sup>-1</sup>·d<sup>-1</sup> can lower the expression of TGF- $\beta$ 1, Col-I, and  $\alpha$ -SMA in peritoneal fibrosis models.

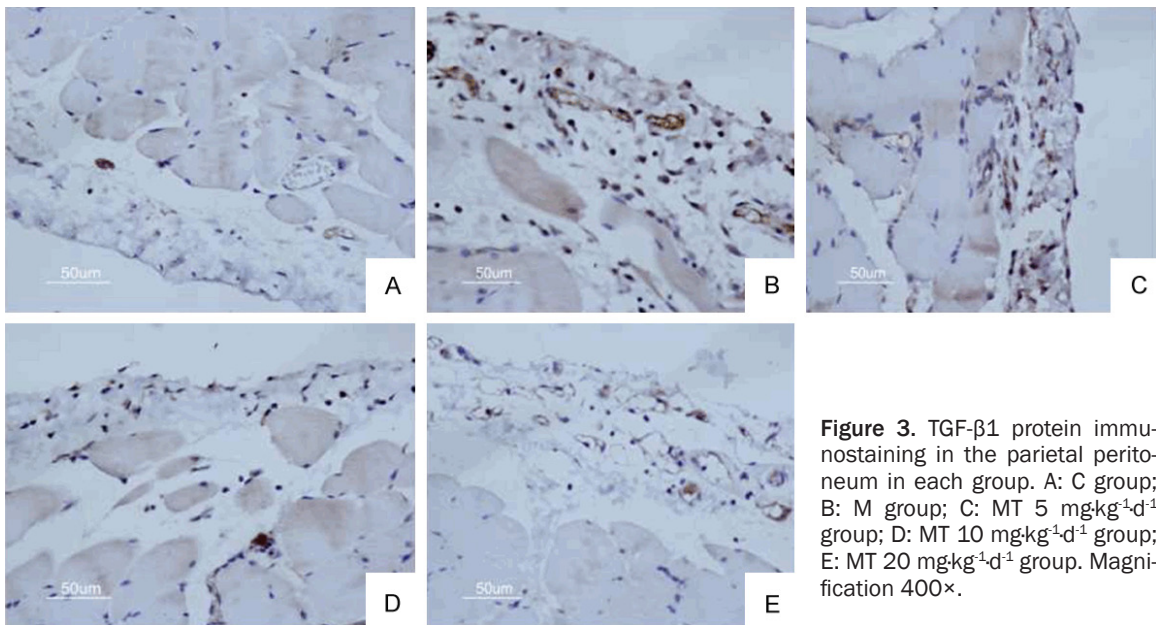
### *Western blot analysis*

The protein levels of TGF- $\beta$ 1, p-Smad 2/3 and Smad 7 were assessed by Western blot analysis (**Figure 7**). The Western blot analysis show-

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**Figure 2.** The peritoneal tissues in each group were stained with Masson. A: C group; B: M group; C: MT 5 mg·kg<sup>-1</sup>·d<sup>-1</sup> group; D: MT 10 mg·kg<sup>-1</sup>·d<sup>-1</sup> group; E: MT 20 mg·kg<sup>-1</sup>·d<sup>-1</sup> group. Magnification 200×.



**Figure 3.** TGF-β1 protein immunostaining in the parietal peritoneum in each group. A: C group; B: M group; C: MT 5 mg·kg<sup>-1</sup>·d<sup>-1</sup> group; D: MT 10 mg·kg<sup>-1</sup>·d<sup>-1</sup> group; E: MT 20 mg·kg<sup>-1</sup>·d<sup>-1</sup> group. Magnification 400×.

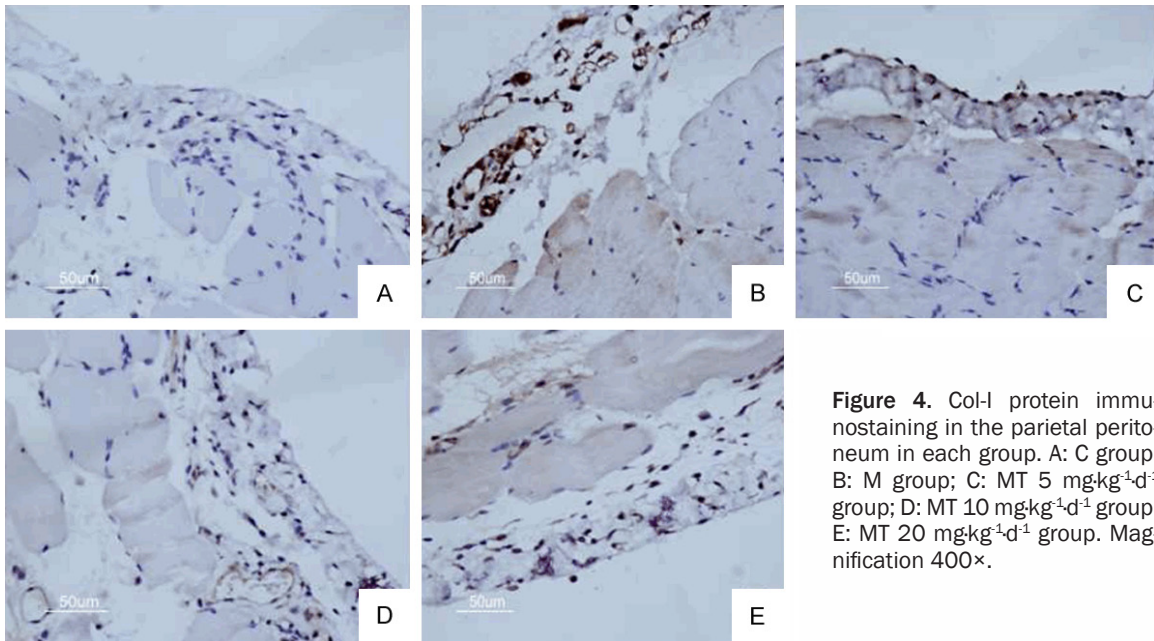
ed that the expression of the TGF-β1 and p-Smad 2/3 proteins in the M group were higher than they were in the C group ( $P=0.003$ ,  $P=0.001$ ,  $P<0.05$ ), and the expression of Smad 7 protein was lower ( $P=0.001$ ,  $P<0.05$ ). Compared with the M group, the expression of TGF-β1 and p-Smad 2/3 protein in MT 5 mg·kg<sup>-1</sup>·d<sup>-1</sup> ( $P=0.015$ ,  $P=0.000$ ,  $P<0.05$ ), 10 mg·kg<sup>-1</sup>·d<sup>-1</sup> ( $P=0.001$ ,  $P=0.000$ ,  $P<0.05$ ), 20 mg·kg<sup>-1</sup>·d<sup>-1</sup> ( $P=0.000$ ,  $P=0.011$ ,  $P<0.05$ ) intervention groups decreased, and the expression of Smad 7 pro-

tein increased in the MT 10 mg·kg<sup>-1</sup>·d<sup>-1</sup> ( $P=0.001$ ,  $P<0.05$ ), 20 mg·kg<sup>-1</sup>·d<sup>-1</sup> ( $P=0.001$ ,  $P<0.05$ ) intervention groups.

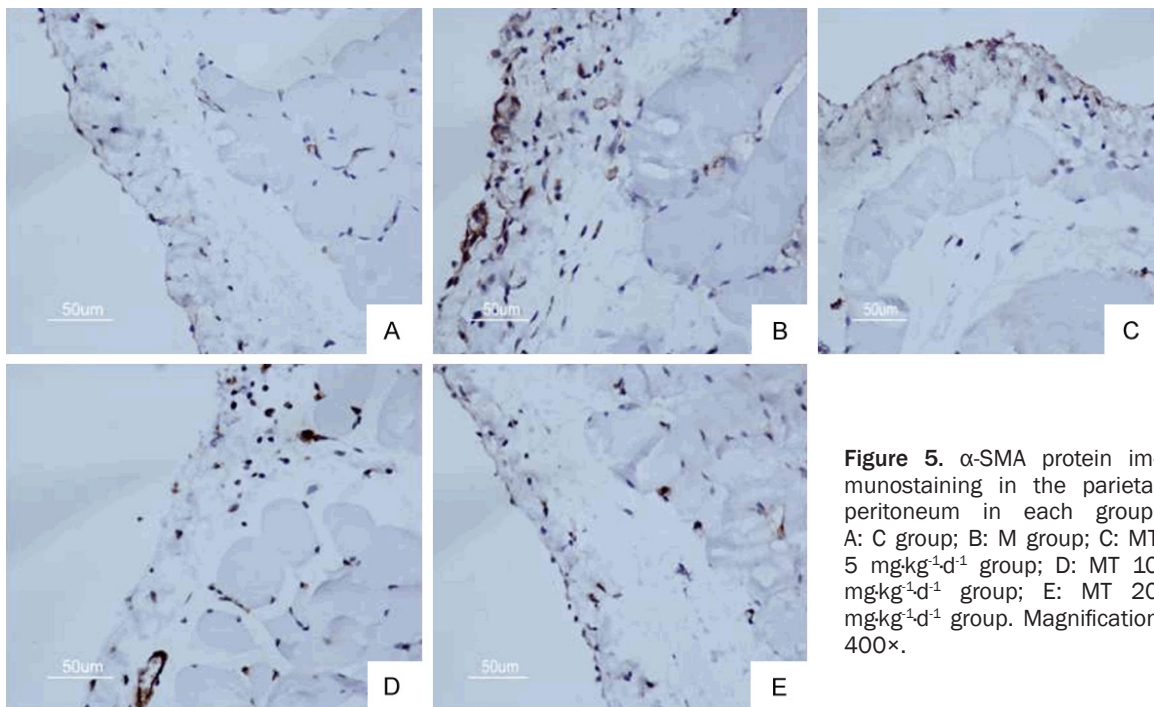
### Discussion

For PD patients, the peritoneal membrane is exposed to the peritoneal dialysis fluid, and the local production of inflammatory cytokines is induced by the high concentrations of glucose and glucose degradation products (GDPs)

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**Figure 4.** Col-I protein immunostaining in the parietal peritoneum in each group. A: C group; B: M group; C: MT 5 mg·kg<sup>-1</sup>·d<sup>-1</sup> group; D: MT 10 mg·kg<sup>-1</sup>·d<sup>-1</sup> group; E: MT 20 mg·kg<sup>-1</sup>·d<sup>-1</sup> group. Magnification 400×.

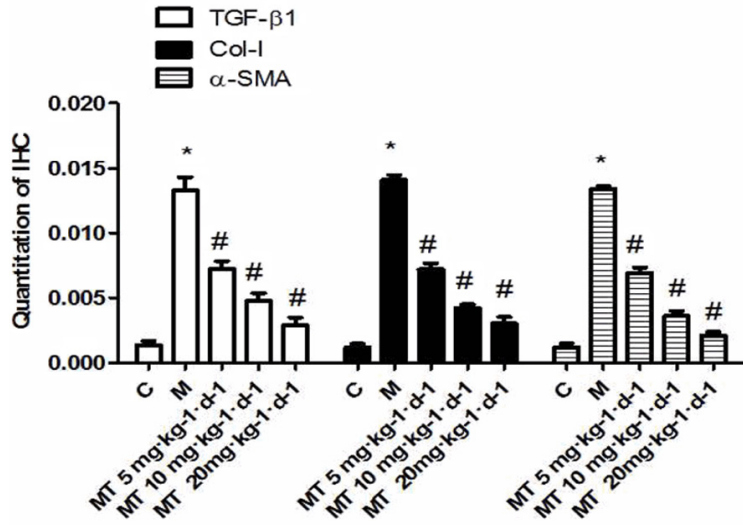


**Figure 5.** α-SMA protein immunostaining in the parietal peritoneum in each group. A: C group; B: M group; C: MT 5 mg·kg<sup>-1</sup>·d<sup>-1</sup> group; D: MT 10 mg·kg<sup>-1</sup>·d<sup>-1</sup> group; E: MT 20 mg·kg<sup>-1</sup>·d<sup>-1</sup> group. Magnification 400×.

of the conventional PD solutions which promote chronic inflammation [7]. The most important features of peritoneal fibrosis are the loss of the mesothelial cell, the thickening of the submesothelial layer, and angiogenesis [5], as we observed through the pathological and morphological changes in our study.

Studies have shown that peritoneal mesothelial cells epithelial-mesenchymal transition

(EMT) is the initial and reversible link of peritoneal fibrosis [17, 18]. Related experiments show that high concentrations of glucose can stimulate TGF-β1, inhibit the expression of E-cadherin, and increase the expression of α-SMA [19, 20]. Peritoneal EMT promotes the progress of peritoneal fibrosis, which is considered to be induced by TGF-β1 [21], a key mediator of fibrogenesis [8]. Meanwhile, the pivotal role of the TGF-β/Smad signaling pathway in



**Figure 6.** Quantitative analyses of the TGF-β1, Col-I and α-SMA immunohistochemical staining. The results are represented as the mean ± SD. \*P<0.05 vs C group, #P<0.05 vs M group.

traperitoneal injection of 4.25% PD solution by 100 ml·kg<sup>-1</sup>·d<sup>-1</sup> for 4 weeks. Compared with the model group, peritoneal thickness thinning, less subcutaneous matrix deposition, and less inflammatory cell infiltration were observed in the MT intervention groups. The results of the peritoneal function analysis showed that the PD efficacy in the model group was lower than it was in the control group, but that was improved in the melatonin intervention groups. The above results show that melatonin could play a role in improving peritoneal function.

the pathogenesis of peritoneal changes induced by PD has been demonstrated [22]. In fibrosis, TGF-β1 binds to its receptor and activates TGF-β1 receptor I-kinase, inducing the phosphorylation of Smad 2 and Smad 3. Phosphorylated Smad 2 and Smad 3 are from the Smad complex with Smad 4, which translocates into the nucleus [23]. Friedman, et al. showed that TGF-β1 could promote the transcription of collagen type I and III, which enhances liver fibrosis through the Smad 3 signaling pathway [24].

Melatonin has been demonstrated to play anti-pulmonary fibrosis and anti-hepatic fibrosis roles in some experimental models [14, 25]. Furthermore, melatonin attenuated fibrotic responses in lung and kidney in a sepsis-induced injury model by suppressing the TGF-β1/Smad signaling pathway [26, 27]. Also, melatonin suppresses fibrotic responses on nicotine-induced vasculopathy through reductions in TGF-β1 [28]. In several animal models of CKD involving experimental hypertension, diabetes mellitus, and various models of nephrotoxicity, melatonin reduced the oxidative burden, attenuated the chronic inflammation and limited fibrosis [29].

However, at present melatonin has not been used in the prevention and treatment of PD-related peritoneal fibrosis. In this study, a peritoneal fibrosis model was established by an in-

TGF-β1 can significantly stimulate the upregulation and activation of Smad 2/3, promote

the phenotypic changes and increase extracellular matrix deposition. The protein Smad 7 negatively regulates these pathways. In our study, the expression of the TGF-β1 and p-Smad 2/3 proteins in the model group were increased compared with the control group, but the expression of the Smad 7 protein was lower. Compared with the model group, the expression of TGF-β1 and the p-Smad 2/3 proteins in the melatonin intervention groups were decreased, while the expression of the Smad 7 protein was increased. It showed that melatonin may exert its anti-fibrosis effect by down-regulating the TGF-β1/Smad pathway.

In conclusion, the present study shows that melatonin can inhibit peritoneal fibrosis and improve peritoneal structure and function. It might inhibit fibrosis by down-regulating the expression of TGF-β1, α-SMA and inhibiting the synthesis of Col-I. Melatonin may exert its anti-fibrosis effect by down-regulating the TGF-β1/Smad pathway.

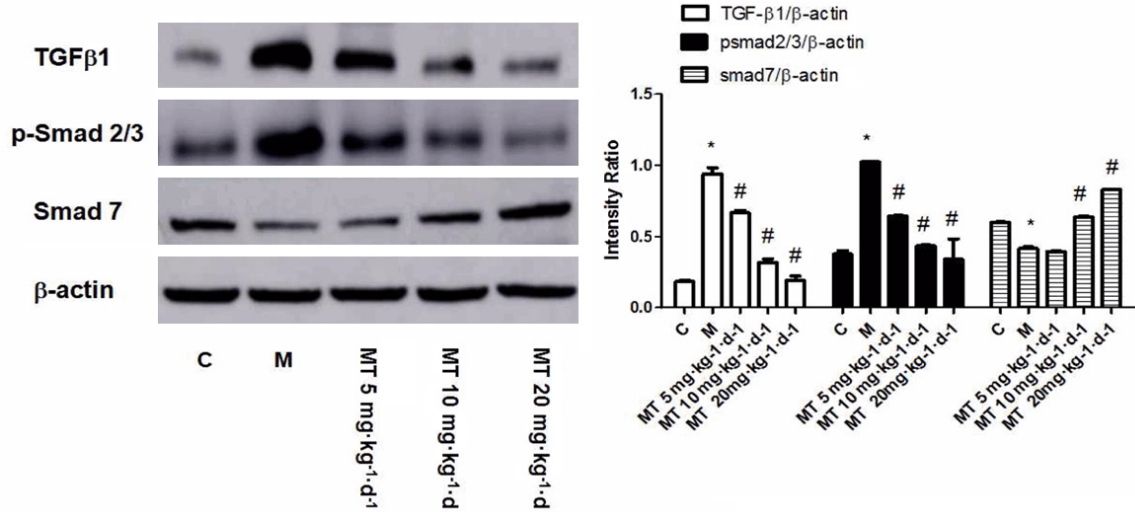
**Acknowledgements**

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**Disclosure of conflict of interest**

None.

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**Figure 7.** The protein levels of TGF-β1, p-Smad 2/3, and Smad 7 were assessed by Western blot analysis. The values are represented as Mean ± SD of at least three repeated experiments. \* $P < 0.05$  vs C group, # $P < 0.05$  vs M group.

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