Original Article
Effects of melatonin on diabetic nephropathy rats via Wnt/β-catenin signaling pathway and TGF-β-Smad signaling pathway

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Abstract: Objective: This research aimed to explore the protective effect of melatonin on diabetic nephropathy (DN) rats induced by streptozotocin (STZ) and its related signaling pathways. Methods: 100 SPF male Sprague-Dawley rats were divided into four groups: low dose melatonin group, medium dose melatonin group and high dose melatonin group. Rats were 35 mg/kg STZ once to establish a DN model, and control rats were given the corresponding dose of normal saline. A renal function test was used to measure urine protein (UP), blood urea nitrogen (BUN) and serum creatinine (Scr). Pathological changes of renal tissues were obtained by HE staining and Masson staining. Oxidative stress-related indicators were measured in a STZ-induced DN rat. Western blot was used to measure target proteins in renal tissues. Results: The levels of UP, BUN and Scr in the model group were significantly higher than control group ($P<0.05$). After administration of melatonin, each administration group was significantly decreased compared to the model group. Pathological changes of renal tissues in the high dose group were the closest to the control group. After administration of melatonin, activities of SOD, CAT and GSH-Px were significantly increased in the medium dose group and the high dose group ($P<0.05$), while the activity of MDA was significantly decreased ($P<0.05$). The expression of Wnt4 and β-catenin in the model group were higher than the control group ($P<0.01$). When melatonin was given, the expression of Wnt4 and β-catenin in the medium dose group and the high dose group were significantly lower than the model group. Levels of TGF-β1, p-Samd2 and p-Samd3 in the control group were lower than the model group ($P<0.05$), and were decreased in the medium dose group and the high dose group. Conclusions: Melatonin improves renal function, relieves oxidative stress, and protects the renal tissue via the Wnt/β-catenin signaling pathway and the TGF-β1-Smad2/3 signaling pathway in STZ-induced DN rats.

Keywords: Melatonin, diabetic nephropathy, streptozotocin, oxidative stress, Wnt/β-catenin signaling pathway, TGF-β1-Smad2/3 signaling pathway

Introduction

Diabetic nephropathy (DN) has been implicated in the development of diabetic microangiopathy in the initial stage of diabetic diseases [1], and is a leading cause of end-stage renal disease in the developed world [2]. Oxidative stress has been implicated in the pathophysiology of DN, and may provide a significant diagnostic reference for DN in diabetes patients [3, 4]. The Wnt/β-catenin signaling pathway participates in the rhein amelioration of kidney injuries in DN mice, and wnt/β-catenin may be a new potential therapy method in the treatment of DN in the future [5, 6]. The TGF-β-Smad signaling pathway was involved in the pathogenesis

of DN, and may be a useful therapeutic approach to prevent DN [7, 8].

Melatonin is a ubiquitous molecule, widely distributed in nature, with functional activity occurring in unicellular organisms, plants, fungi and animals [9]. Melatonin as an antioxidant reduces oxidative stress in several ways: direct detoxification of reactive oxygen and reactive nitrogen species, and indirectly by stimulating antioxidant enzymes while suppressing the activity of pro-oxidant enzymes [10]. The Keap1-Nrf2-ARE pathway may mediate the actions of melatonin on radical metabolizing/producing enzymes, and beyond its direct free radical scavenging and indirect antioxidant effects, it may

enhance its ability to limit oxidative stress with a variety of physiological and metabolic advantages [11]. Previous studies showed that melatonin might be helpful in reducing diabetes-induced renal damage in streptozotocin (STZ)-induced DN rats [12]. The combination of Losartan Potassium and melatonin had the most potent effect on treating the deleterious action of diabetes on DN kidneys [13].

The aim of this study was to explore effect of melatonin on renal function, pathological changes, oxidative stress, the wnt/β-catenin signaling pathway and the TGF-β1-Smad2/3 signaling pathway on renal tissue of STZ-induced DN rats.

Materials and methods

Animals

100 SPF male Sprague-Dawley rats weighing 180 ± 10 g were purchased from Guangdong Medical Experimental Animal Center. Rats were fed in individual cages (n=5) under constant temperature (25°C). Humidity was controlled at 50% ± 5%; the feeding room was ventilated and dry, and kept quiet, with 12 h day-night cycles. Rats were free to drink water and eat food. Rats were used for the experiment after a week's adaptation. The experiment was approved by the ethics committee of The Third Hospital of Hebei Medical University.

Establishment of animal model, grouping and administration

15 rats were taken as control group. The other rats were given 35 mg/kg STZ (Sangon Biotech Co. Ltd., Shanghai, China) once to establish a DN model, and the control rats were given the corresponding dose of normal saline. The rats' fasting blood glucose was measured 72 hours after the STZ administration, and the establishment of the DN model was successful with the blood glucose level >16.7 mmol/L and positive urine protein after 2 weeks. Sixty successful DN rats were randomly divided into four groups: the model group, the low dose melatonin (Shanghai Institute of Biochemistry, Shanghai, China) group (5 mg/kg), the medium dose melatonin group (15 mg/kg) and the high dose melatonin group (30 mg/kg). Melatonin was dissolved in physiological saline containing 2% ethanol and administered continuously for 30 days.

Renal function test

30 days after the administration, the urine of each rat in each group was collected using metabolic cages. The supernatant was collected by centrifugation (3500 r/min, 4°C, 10 min), and the level of urine protein (UP) was measured. After the last administration, the rats were fasting but free to consume water overnight. The rats in each group were anesthetized with chloral hydrate and blood samples were collected via the femoral artery. After being placed for 1 h at room temperature, the supernatant was taken by centrifugation (3500 r/min, 4°C, 10 min). Levels of blood urea nitrogen (BUN) and serum creatinine (Scr) were measured. Levels of urine protein, creatinine and urea nitrogen were measured using the kit, and the specific test was performed according to the kit's instructions.

HE staining and Masson staining of renal tissue

After the rats were anesthetized with chloral hydrate, the right kidney was removed and fixed in 4% paraformaldehyde for 12 h. The rats were embedded in paraffin and cut into slices. The slices were stained with HE and observed under a light microscope. The Masson staining of renal tissue slices was done with hematoxylin for 60 s, washed with rinse solution for 60 s, stained with ponceau acid red dye magenta for 60 s, and then washed with a rinse solution for 60 s. After color separation of phosphomolybdic acid for 7 min, the staining solution was removed and stained with bright green-blue for 5 min, and washed with absolute ethanol 3 times; neutral resin was used for mounting with anhydrous ethanol evaporated to dry; and then the slices were observed under a lighted microscope.

Immunohistochemistry analysis

The kidneys were routinely paraffin-embedded and sliced, and each slice was deparaffinized by using the SP three-step method. After the PBS solution was washed, the antigen was heat-repaired and the blocking process was completed. After the blocking was completed, the primary antibody containing Wnt4 (ab189-
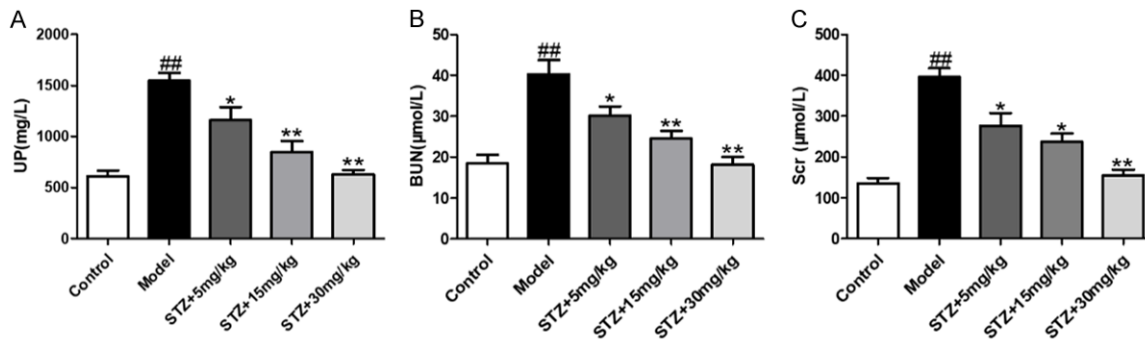
Figure 1. Effects of melatonin on UP, BUN and Scr in STZ-induced DN rats. A: The levels of UP in different groups; B: The levels of BUN in different groups; C: The levels of Scr in different groups. #P<0.05, ##P<0.01, compared with the control group. *P<0.05; **P<0.01, compared with the model group.
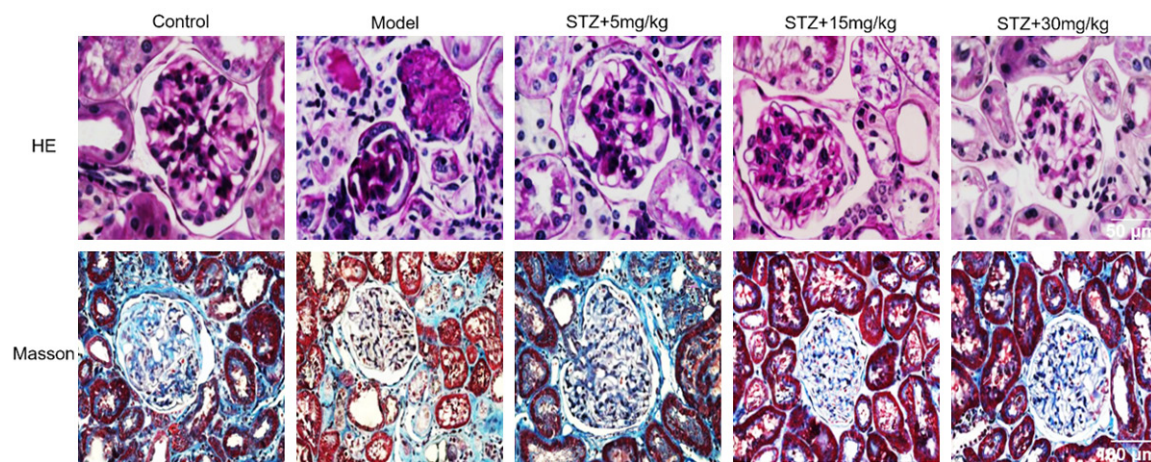

Figure 2. Pathological changes of renal tissues in STZ-induced DN rats determined by HE and Masson assays.

037, Abcam, MA, USA) and β-catenin (ab32572, Abcam, MA, USA) was added (incubated at 4°C. overnight), and the secondary antibody was incubated for 2 h. Subsequently, under the microscope, DAB developing, hematoxylin counterstaining, dehydration, transparency, and mounting were performed. After mounting, the immunohistochemical staining was observed under a microscope. Data was analyzed by using the Image-Pro Plus version 6.0 image processing software. The iImmunohistochemistry was performed according to the immunohistochemistry kit's instructions.

Activities of SOD, CAT, GSH-Px and level of MDA in renal tissue

Rats were anesthetized with chloral hydrate and the left renal tissue was removed. The homogenate medium was used for tissue homogenization by adding 10% of the tissue homogenate and centrifuged at 3500 rpm/min

at 4°C for 10 min. The supernatant was used to measure activities of SOD, CAT, GSH-Px and the content of MDA. Another part of the supernatant was used to measure protein concentration by using the BCA method. All operations are performed according to kit instructions.

Western blot assay

The rats were anesthetized with chloral hydrate and the left renal tissue was removed. The tissue protein lysate (V:V=10:1) was added after being thoroughly ground with liquid nitrogen and then a broad spectrum of phosphatase inhibitors (1:100) and PMSF (1:100) was added. After 2 h of incubation in an ice bath, the supernatant was centrifuged (4°C, 3500 rpm, 10 min), and the protein concentration was determined using the BCA method. The appropriate loading volume was calculated and the appropriate volume loading buffer was added, then denaturation was performed in a boiling water
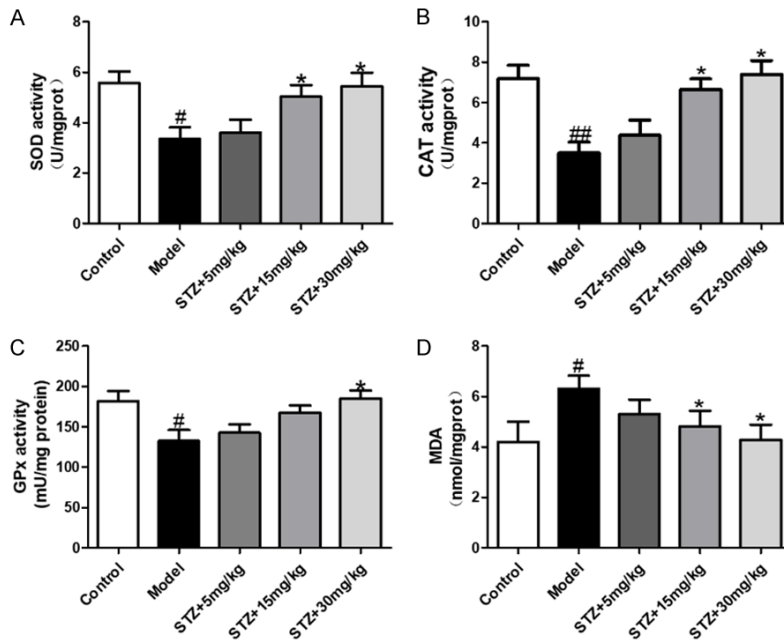
Figure 3. Effects of melatonin on oxidative stress-related indicators in STZ-induced DN rats. A: SOD activity in different groups; B: CAT activity in different groups; C: GPx activity in different groups; D: MDA activity in different groups. #P<0.05, ##P<0.01, compared with the control group. *P<0.05; **P<0.01, compared with the model group.

Results

Effects of melatonin on UP, BUN and Scr in STZ-induced DN rat

The levels of UP, BUN and Scr were significantly increased in the model group compared with the control group (*P*<0.05). After the administration of melatonin, each administration group was significantly decreased, and the effect of the improvement in the high dose group was the best (*P*<0.01) (**Figure 1**), indicating that melatonin had a significant protective effect on STZ-induced DN rats.

Pathological changes of renal tissues in STZ-induced DN rat

bath for 10 min. Respectively, SDS-PAGE electrophoresis and transfer membrane were performed. Primary antibodies containing Wnt4 (ab169592, Abcam, MA, USA), β-catenin (ab32-572, Abcam, MA, USA), β-actin (ab8226, Abcam, MA, USA), p-EGFR (ab5650, Abcam, MA, USA), EGFR (ab52894, Abcam, MA, USA), TGFβ (ab64715, Abcam, MA, USA), Smad 2 (ab40855, Abcam, MA, USA), p-Smad 2 (ab188334, Abcam, MA, USA), Smad 3 (ab28379, Abcam, MA, USA) and p-Smad 3 (ab92698, Abcam, MA, USA) were added to incubate after being blocked with 5% fetal bovine serum (BSA) for 2 h, then incubated with primary antibody overnight (4°C), washed twice with TBST for 2 h, and then washed twice with TBST. B-actin was used as an internal reference. A gel imaging system was used for imaging, and Image Studio software was used analyze the relative gray value of the target protein.

Statistics analysis

Data were analyzed by SPSS 19.0. The experimental data were expressed as x̄ ± s. One-way ANOVA and t-tests were used for the different groups. *P*<0.05 was considered significant difference.

The pathological observation of the HE staining is shown in **Figure 2**. The glomerular structure in rats in the normal group was intact, abnormal changes were not seen in the matrix and mesangium, and the tubular structure was intact. The renal tissue in the rats in the model group showed obvious necrosis with glomerular atrophy, the brush edges disappeared in the proximal tubule epithelial cells, a large number of tubular epithelial cells showed degeneration, and a large number of inflammatory cells could be seen in the renal interstitium infiltration, indicating that STZ induced DN rats had renal parenchymal lesions. The lesion degree of rats in the administration group was significantly reduced, the degree of glomerular basement membrane thickening was significantly improved, and the degree of glomerular mesangial matrix hyperplasia was significantly alleviated. The high dose group was the closest to the control group.

The Masson staining results were shown in **Figure 2**. The trace collagen was visible in the glomerular basement membrane and the tubulointerstitium in the rats in the normal group. A large number of blue-stained collagen fibers was observed in the glomeruli and tubules of
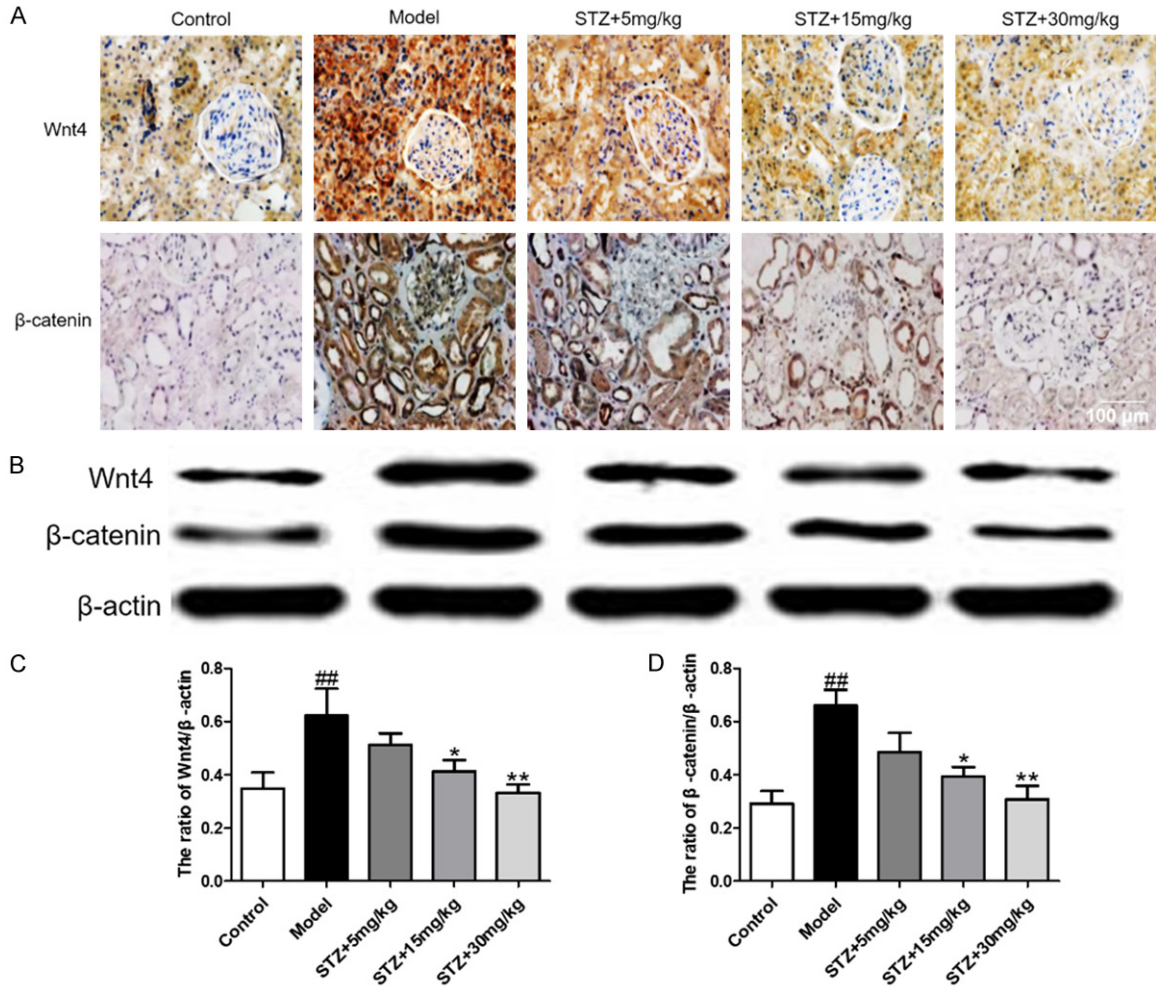
Figure 4. Effects of melatonin on Wnt/β-catenin signaling pathway in STZ-induced DN rats. A: Immunohistochemistry of Wnt4 and β-catenin in different groups; B: The Western blot results of Wnt4 and β-catenin; C: The analysis of Wnt4; D: The analysis of β-catenin. #$P<0.05$, ##$P<0.01$, compared with the control group. *$P<0.05$; **$P<0.01$, compared with the model group.

the model group, which were significantly increased compared to the blank group, indicating that collagen fibers were precipitated. When melatonin was given, the collagen fibers in the glomeruli and tubulointerstitium of each administration group were significantly lower than those of the model group, indicating that melatonin could relieve renal tissue fibrosis in STZ-induced DN rats.

Effects of melatonin on oxidative stress-related indicators in STZ-induced DN rat

Compared with the blank group, the activities of SOD, CAT and GSH-Px were significantly decreased in the model group ($P<0.05$), and activity of MDA was significantly increased ($P<0.05$), indicating that the renal tissue of STZ-induced

DN rats was in a state of peroxidative stress. When melatonin was given, the activities of SOD, CAT and GSH-Px were significantly increased in the medium and high dose groups ($P<0.05$), while the activity of MDA was significantly decreased ($P<0.05$), indicating that melatonin had significant improvement effect on oxidative stress in STZ-induced DN rat (**Figure 3**).

Effects of melatonin on the Wnt/β-catenin signaling pathway in STZ-induced DN rats

The results of the immunohistochemistry showed that compared with the control group, the expression of Wnt4 and β-catenin in the model group was significantly increased in the tubules and the glomeruli ($P<0.01$). When melatonin
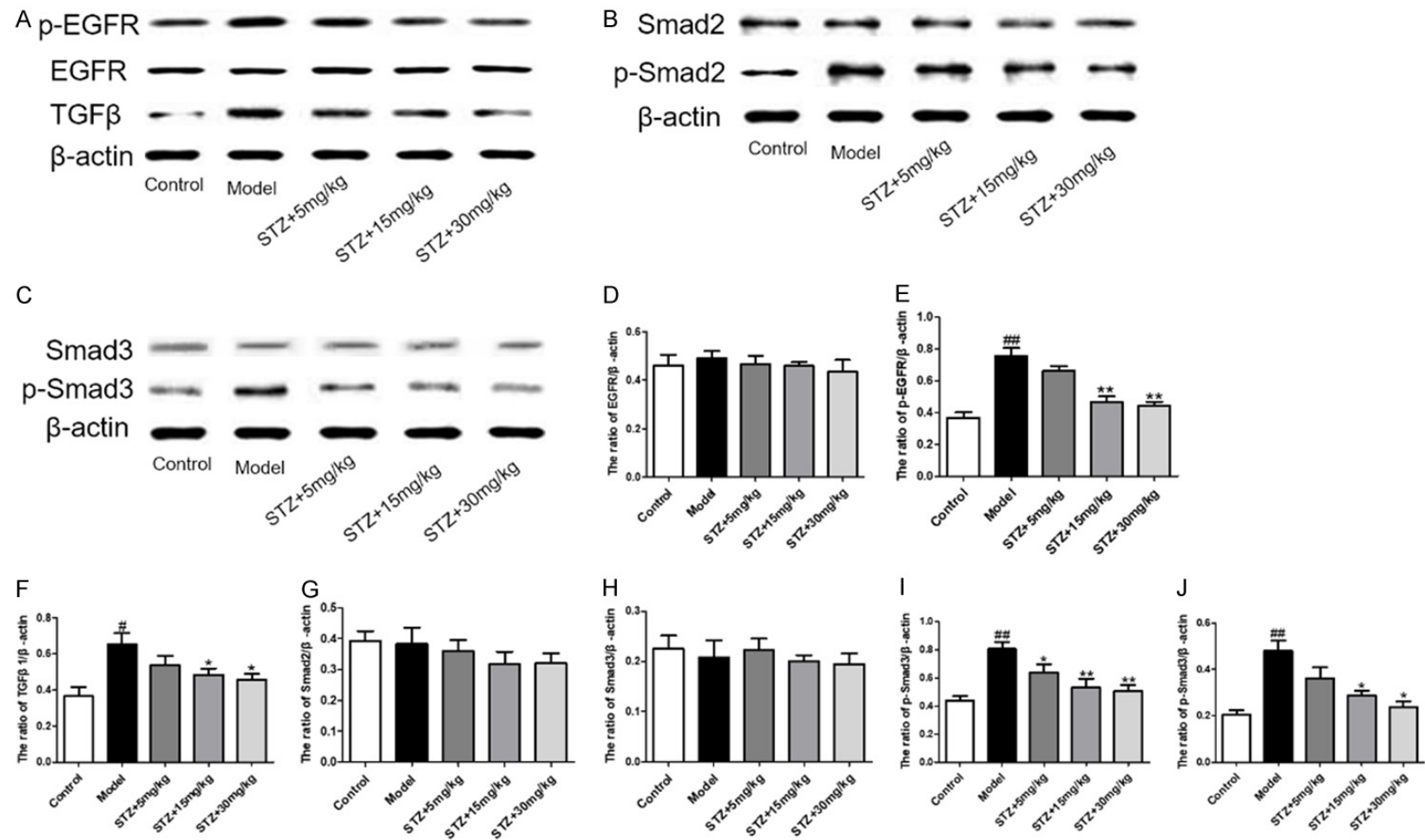
Figure 5. Effects of melatonin on TGF-β1-Smad2/3 signaling pathway in STZ-induced DN rats. A: The Western blot of p-EGFR, EGFR and TGFβ; B: The Western blot of Smad2 and p-Smad2; C: The Western blot of Smad3 and p-Smad3; D: The analysis of EGFR; E: The analysis of p-EGFR; F: The analysis of TGFβ; G: The analysis of Smad2; H: The analysis of p-Smad2; I: The analysis of Smad3; J: The analysis of p-Smad3. #P<0.05, ##P<0.01, compared with the control group. *P<0.05; **P<0.01, compared with the model group.

was given, their expression was decreased, the high-dose group most significantly ($P<0.01$). Western blot was used for quantitative analysis, and the results were consistent with the results of the immunohistochemistry. Compared with the control group, the expression of Wnt4 and β-catenin in the model group were increased significantly ($P<0.01$). When melatonin was given, the expression of Wnt4 and β-catenin in the medium dose group and the high dose group were significantly lower than the model group, indicating that melatonin could act on the Wnt/β-catenin signaling pathway to protect the renal tissue (**Figure 4**).

Effects of melatonin on TGF-β1-Smad2/3 signaling pathway in STZ-induced DN rat

The results showed that, compared with the blank group, the expression of EGFR did not change significantly in the model group and in each administration group. But compared with the control group, the expression of p-EGFR was significantly increased in the model group ($P<0.05$), and its content was significantly reduced when melatonin was given. In addition, levels of TGF-β1, p-Samd2 and p-Samd3 were also significantly increased in the model group ($P<0.05$). When melatonin was given, levels of TGF-β1, p-Samd2 and p-Samd3 were decreased in the medium dose group and in the high dose group. There were no significant changes in levels of Smad2 and Smad3 among the different groups (**Figure 5**). The results showed that melatonin could regulate the TGF-β1-Smad2/3 signaling pathway to improve renal tissue fibrin production.

Discussions

Melatonin is an effective free radical scavenger and antioxidant and can scavenge the highly toxic hydroxyl radical, the peroxynitrite anion, and possibly the peroxyl radical [14]. Melatonin plays an important role in many disease processes, such as repressing oral squamous cell carcinoma metastasis by inhibiting tumor-associated neutrophils [15], showing supportive effects on oxidants and anastomotic healing during intestinal ischemia/reperfusion injury [16], and inhibiting the proliferation of breast cancer cells induced by bisphenol A via the targeting of estrogen receptor-related pathways [17]. In this study, melatonin was used to treat STZ-induced DN rats, indicating that melatonin

could protect renal tissue and relieve renal tissue fibrosis, and the effect was positively related with dose.

DN is one of the major microvascular complications in diabetes [18]. DN induces renal and harmful fibrosis in diabetes patients. Level changes of UP, BUN and Scr were the main indicators used to evaluate renal function. In previous studies, levels of UP, BUN and Scr were decreased in DN rats compared to groups treated with melatonin and certain drugs [13]. These results were consistent with our study. In this study, after the administration of melatonin, levels of UP, BUN and Scr in different melatonin groups were significantly decreased, and the effect of the improvement in the high dose group was the best. All these results showed that melatonin had a significant protective effect on renal tissue in STZ-induced DN rats.

Oxidative stress has a great role in diabetes and diabetes induced organ damage, and endoplasmic reticulum stress is involved in the onset of diabetic nephropathy [19]. An increased understanding of the role of oxidative stress in DN has led to the exploration of a number of therapeutic strategies, the success of which has so far been limited [20]. Conversely, sodium nitroprusside intoxication caused a decrease of the activity of enzymes in the tested organs in all intervals, while administration of melatonin and sodium nitroprusside resulted in increased activities of SOD, CAT and GSH-Px in all the organs after 3 h and 6 h, providing new data to add to the study of antioxidant properties of melatonin and sodium nitroprusside-induced oxidative stress with regard to time-dependent properties in different types of tissues [21]. In this study, the changes of oxidative stress-related indicators were similar to those reported in previous studies, and the activities of SOD, CAT and GSH-Px were significantly decreased in model group and were increased after administration of melatonin, indicating that melatonin could improve the oxidative stress of renal tissue in STZ-induced DN rats.

The Wnt/β-catenin signaling pathway is signaled by Wnt family of secreted glycolipoproteins via the transcriptional coactivator β-catenin, which controls embryonic development and adult homeostasis [22]. Results demonstrated that HG induced up-regulation of ubiq-

uitin carboxy-terminal hydrolase 1 (UCH-L1) and activation of the Wnt/β-catenin signaling pathway in podocytes of DN, indicating that Wnt/β-catenin/UCH-L1 may be a new potential therapy method in the treatment of DN [6]. Proteinuria, renal dysfunction, and dyslipidemias were closely associated with the abnormal activation of the Wnt signaling pathway in the mouse model of DN, and the mechanism that Chaihuang-Yishen granules regulate proteinuria, renal function, and blood lipids may be associated with inhibition of the abnormally activated Wnt signaling pathway [23]. The Wnt/β-catenin signaling pathway was involved in the RH ameliorating of kidney injuries in DN mice [5]. In our study, the expression of Wnt4 and β-catenin in the model group was significantly higher than the control group in the tubules and glomeruli. When melatonin was given, the expression of Wnt4 and β-catenin in the medium dose group and the high dose group were significantly decreased compared to the model group. All these results showed that melatonin could protect the renal tissue via the Wnt/β-catenin signaling pathway.

TGF-β1 is a key cytokine that leads to fibrosis, which promotes glomerular sclerosis and tubulointerstitial fibrosis in diabetic nephropathy by increasing the formation of the extracellular matrix. The activation of EGFR mediates the activation of the TGF-β1-Smad2/3 signaling pathway. In this study, the expression of EGFR did not change significantly in the model group or in each administration group, but the expression of p-EGFR was significantly increased in the model group, and its content was significantly reduced when melatonin was given. Silencing Smad2 and Smad3 efficiently blocked the effect of TGF-β2 on cell proliferation, migration, and extracellular matrix production. Smad2 and Smad3 are both key in the TGF-β2 signaling pathway [24]. Many studies have shown certain drugs that act on renal tissue via the TGF-β1-Smad2/3 signaling pathway. Inhibition of renal fibrosis in Chaihuang-Yishen granule-treated diabetic rats was associated with inhibition of TGF-β1/Smad3 signaling as demonstrated by up-regulation of Smad7 but down-regulation of TGF-β1, TGF-β receptors, activation of Smad3, and expression of miRNA-21 [25]. Qiwei Granules can block the TGF-β1/Smads signaling pathway and delay the progression of renal interstitial fibrosis by down-

regulating the expression of TGF-β1, p-Smad2, p-Smad3 and ILK and up-regulating the expression of Smad7 [26]. Asiatic acid inhibited liver fibrosis by blocking TGF-β/Smad signaling in vivo and in vitro [27]. When melatonin was given, levels of TGF-β1, p-Samd2 and p-Samd3 were decreased in the medium and high dose groups in our study. These results indicate that melatonin could improve renal tissue fibrin production by regulating the TGF-β1-Smad2/3 signaling pathway.

In conclusion, different doses of melatonin were used to treat STZ-induced DN rats in this study, and results indicated that melatonin could improve renal function and the oxidative stress of renal tissue, and protect the renal tissue via the Wnt/β-catenin signaling pathway and the TGF-β1-Smad2/3 signaling pathway. However, this experimental study was limited to an animal model, so further study needs to be performed for clinical patients.

Disclosure of conflict of interest

None.

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