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Original Article

Melatonin ameliorates hyperglycaemia-induced renal inflammation by inhibiting the activation of TLR4 and TGF-β1/Smad3 signalling pathway

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Abstract: Melatonin (MT), a hormone secreted from the pineal gland, has beneficial effects on the development of diabetic nephropathy (DN). In our study, we aimed to determine the effects of melatonin on renal inflammation and fibrosis in diabetic nephropathy. *In vivo*, we evaluated the blood and urine indices of metabolic and renal function, renal inflammation and renal fibrosis in db/db mice after melatonin treatment. Melatonin treatment significantly decreased urinary albumin excretion and reduced the morphological changes in kidney. Additionally, Melatonin reduced the levels of inflammatory factors in kidney such as interleukin-1β, monocyte chemotactic protein-1 and active nuclear factor-κB though toll-like receptor 4 signaling pathway (TLR4). Melatonin also reduced collagen type IV, fibronectin, transforming growth factor-β1 (TGF-β1) and decreased the phosphorylation of Smad3 in the renal tissue. These results indicated that melatonin reduced the inflammation and fibrosis in diabetic nephropathy though TLR4 and TGF-β1/Smad3 signaling pathway. *In vitro*, melatonin treatment reduced the cell proliferation, inflammatory factors releasing, TLR4 and TGF-β1/Smad3 signaling pathway. Our findings indicated that melatonin may provide a new perspective intervention to halt the hyperglycemia-induced inflammatory response of DN.

Keywords: Melatonin, inflammation, fibrosis, toll-like receptor 4, diabetic nephropathy

Introduction

In recent years, the incidence of diabetes has steadily increased year after year, putting tremendous pressure on public health. Diabetic nephropathy (DN) is a severe complication of diabetes and the most common cause of chronic kidney disease and end-stage renal disease [1, 2]. Chronic inflammation plays an important role in diabetic nephropathy [3, 4], and secretion of pro-inflammatory cytokines from macrophages leads to the development and pathogenesis of DN [5-7]. Secretion of monocyte chemoattractant protein 1 (MCP-1) from high glucose-induced mesangial cells promotes macrophage migration and activation [8, 9] and affects the proliferation of mesangial cells [10], subsequently eliciting kidney injury [11]. Min *et al*. have indicated that high glucose (HG)-induced mesangial cells secreted cytokines to increase adhesion and differentiation of monocytes [12].

Glomerular fibrosis is a major pathological feature of DN, manifesting mainly as an accumulation of extracellular matrix (ECM), a thickening of glomerular basement membrane, and glomerular capillary obliteration [13, 14]. Transforming growth factor beta 1 (TGF-β1) is an important profibrotic cytokine, which is significantly elevated in diabetes and can induce ECM production in mesangial cells [15]. The TGF-β1/Smad3 signalling is known to be an important pathway for activation of mesangial cells [16].

Evidence is accumulating to indicate that the toll-like receptors (TLRs) play an important role in the inflammatory response associated with DN [17, 18]. Lin *et al*. have indicated that TLR4 expression was significantly increased in the kidney of patients with diabetic nephropathy [19]. Upon binding ligand, the signalling pathway of TLR4 is activated though MyD88-dependent or MyD88-independent (adaptor protein myeloid differentiation factor 88) mechanisms, leading to the activation of NF-κB

(nuclear factor kappa-B p65), which contributes to the release of pro-inflammatory cytokines and chemokines [20]. High glucose is known to stimulate mesangial extracellular matrix accumulation and cell proliferation though the TGF-β1/Smad and NF-κB pathway [21-24].

Melatonin, also named N-acetyl-5-methoxytryptamine, is an important hormone secreted from the pineal gland, and it is known to be a powerful antioxidant [25, 26]. Xia *et al*. suggested that melatonin modulated TLR4-mediated inflammatory genes in LPS-stimulated RAW264.7 cells [27]. In addition, evidence has accumulated suggesting that melatonin treatment could improve DN and protect the function of the kidney by reducing urinary excretion or protecting podocytes [28, 29]. In our study, we hypothesized that melatonin has therapeutic potential in DN though preventing inflammation and fibrosis. We show that melatonin reduces inflammation and fibrosis in DN though TLR4 the TGF-β1/Smad3 signalling pathway.

Methods and materials

Antibodies and reagents

The TRIzol reagent was bought from Invitrogen (USA). Mannitol and glucose and were bought from Sigma (USA). TAK-242 and TLR4 inhibitor were obtained from Selleck (USA). Revert Aid Premium First Strand cDNA Synthesis and SYBR Green PCR master mix kits were from Vazyme (Nanjing, China). Anti-TLR4, anti-MyD88, anti-TRIF, anti-TGF-β1, anti-Smad3, and anti-phospho-Smad3 primary antibodies were from Abcam (Abcam, Cambridge, UK). Anti-phospho-IRF3, anti-IRF3, anti-NF-κBp p65, anti-NF-κB p65, anti-IκB, and anti-phospho-IκB primary antibodies were from Cell Signaling Technology (USA). Anti-actin, anti-FN, and anti-ColIV primary antibodies and anti-rabbit IgG and anti-mouse IgG secondary antibodies were from Wuhan Sanying Biotechnology Inc (Wuhan, China). CCK-8 kit was purchased from Vazyme (Vazyme Biotech Co, Nanjing, China). The Protein Assay Kit was from Beyotime Institute of Biotechnology (Jiangsu, China). The mouse IL-1β, mouse MCP-1, and TNF-α ELISA kits were obtained from Excell Bio (Shanghai, China). The mouse ColIV and mouse Fn ELISA kits were from RayBiotech (Guangzhou, China).

Animals

Nine-week-old male db/db mice and db/m mice (C57BLKS/J db/db) were purchased from the Experimental Animal Center of Nanjing Medical University. The animals were kept in the Experimental Animal Center of Anhui Medical University under optimum conditions (room temperature of 24±1°C, humidity 60%, alternating 12 h light and dark cycle). The animals had free access to water and food. The mice were randomly grouped as follows: (1) m (n=12); (2) db (n=12); (3) db+MT 50 (db/db mice were injected intravenously with MT 50 μg/kg. d, n=12); (4) db+MT 100 (db/db mice were injected intravenously with MT 100 μg/kg. d, n=12); (5) db+MT 200 (db/db mice were injected intravenously with MT 200 μg/kg. d, n=12). Mice within the first two groups were injected with PBS/DMSO intravenously. All animal protocols were approved by the Animal Research Ethics Committee of Anhui Medical University and the mice were sacrificed according to the recommendations of the NIH Guide to Care and Use of Laboratory Animals.

Sample collecting and blood monitoring

Blood glucose was monitored every four weeks. At 12 weeks after treatment, 24 h urine samples were collected, and the concentration of urinary proteins was detected using an ELISA kit. After 12 weeks of treatment, the animals were sacrificed, and the kidneys were harvested: the right kidneys were processed for western blots and qRT-PCR and the left kidneys were processed for histological examination.

Renal histology

Formalin-fixed paraffin-embedded renal tissues were cut into 3 μm thick slices and processed for periodic acid-Schiff (PAS) staining. Under a ×400 magnified field, 10 glomeruli and 10 tubulointerstitial areas of the cortex were observed. The percentage of mesangial matrix occupying glomerulus and indices for tubulointerstitial injury were evaluated by Image J.

Immunohistochemical analysis

The sections of renal tissues were deparaffinized and rehydrated through a graded series of ethanol concentrations to finish in distilled water. Endogenous peroxidase activation was

blocked with 3% H_2O_2 for 5 min and then the sections were washed in distilled water briefly. Sections were incubated with primary antibodies overnight at 4°C. The slices were washed and incubated with the secondary antibody for 20 min at 37°C. DAB was used as the chromogen and counterstaining was performed with haematoxylin. Sections incubated in PBS were used as negative controls. Ten glomeruli and ten tubulointerstitial lesions were selected randomly under high magnification, and Image-Pro-Plus 6.0 image analysis software was used to calculate the number of CD68-positive cells in glomeruli and tubulointerstitial areas and to quantitate TLR4 and p65 protein.

Cell culture

SV 40 MES 13, mouse mesangial cell line, was obtained from the Cell Bank of the Chinese Academy of Sciences. The cells were cultured in low glucose DMEM containing 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% FBS in a 5% CO_2 incubator at 37°C. The cells were divided into eight groups according to whether melatonin was added or not: 25 mM mannitol control group (abbreviated as MC), Normal group (abbreviated as NC), Melatonin 1000 μM group (abbreviated as MT), 25 mM glucose group (abbreviated as HG), HG+melatonin 10 μM group (abbreviated as HG+10 μM MT), HG+melatonin 100 μM group (abbreviated as HG+100 μM MT), HG+melatonin 1000 μM group (abbreviated as HG+1000 μM MT), HG+TLR4 inhibitor group (abbreviated as HG+TAK242).

Edu labelling

A Click-iT Edu Imaging Kit (with Alexa Fluor 594 label) was used to measure the proliferation of mesangial cells according to the manufacturer's instructions [31].

Cell viability assay

A CCK-8 kit was used to assess cell viability according to manufacturer's instruction. Briefly, the cells were seeded in 96-well plates and incubated with various concentrations of melatonin or DMSO for 24 h. Ten microliters CCK-8 solution was added to each well separately and the plates were incubated for another 4 h. Cell viability was determined by detecting the absorbance of the conversion dye at 490 nm using a microplate reader. The average optical density (OD) in the control cells was set as 100% viability, and treatment results expressed as a percentage of control.

Enzyme-linked immunosorbent assay (ELISA)

Culture medium from mesangial cell cultures was harvested and the supernatant collected after centrifugation. The levels of MCP-1, IL-1β, TNF-α, Fn, and ColIV in supernatants from mesangial cells cultures were determined by ELISA kit.

Western blotting

A total protein fraction obtained from homogenates of kidney tissues or mesangial cells was resolved by SDS-PAGE and electrotransferred to PVDF membranes. The membranes were blocked with 5% skimmed milk for 2 h at room temperature, and then incubated with primary antibody overnight at 4°C. The membranes were processed using antibodies against TLR4 (Abcam, IHC 1:100, WB 1:1000), MyD88 (Abcam, 1:800), TRIF (Abcam, 1:1000), p-IRF3 (CST, 1:1000), NF-κB p65 (CST, 1:1000), TGF-β1 (Abcam, 1:800), p-Smad3 (Abcam, 1:600), ColIV (Wuhan, Sanying, 1:500), and Fn (Wuhan, Sanying, 1:500). After being washed three times in PBST, the membranes were incubated with HRP-conjugated secondary antibody for 1 h. The membranes were developed using a chemiluminescence system and washed three times with PBST. Finally, the optical densities of developed bands were analysed by Image J and the relative ratio of proteins was quantified.

RNA extraction and qRT-PCR

Total RNA was extracted from kidney tissues and mesangial cells and processed for real time PCR as described in our previously reported [31]. cDNA was synthesized from Total RNA using the Revert Aid Premium First Strand cDNA Synthesis (Vazyme). qPCR was performed using the AceQ qPCR SYBR Green Master Mix (Vazyme). GAPDH was used as the endogenous reference gene: Forward GGTGAAGGTCGGTGTGAACG, and reverse CTCGCTCCTGGAAGATGGTG; TNF-α: forward GCTGAGCTCAAACCCTGGTA, and reverse CGGACTCCGCAAAGTCTAAG; MCP-1: forward TTGACCCGTAAATCTGAAGCTAAT, and reverse TCACAGTCCGAGTCACACTAGTTCAC; IL-1β: forward GCCTCGTGCTGTCGGACCCATAT, and reverse TCCTTTGAGGCCCAAGGCCACA; Fn: forward CTGACTGGCCTTACCAG-

Table 1. Change of the clinical and metabolic parameters (Mean ± SD, n=12)

Groups	Blood glucose (mmol·L^{-1})	Body weight (g)	Kidney weight (g)	UAER (μg·24 h^{-1})
db/m	6.83±1.45	23.82±1.10	0.20±0.04	17.30±2.43
db/db	33.33±3.81*	46.03±4.82*	0.30±0.04*	1107.1±109.31*
db/db+MT 50	32.24±4.65#	44.22±4.75#	0.25±0.06#	839.97±190.53#
db/db+MT 100	33.22±5.08#	44.92±4.06#	0.24±0.02#	574.01±120.22#
db/db+MT 200	32.30±3.57#	45.60±4.78#	0.23±0.04#	479.80±224.14#

The values are Mean ± SD, *P<0.05 versus values for db/m, #P<0.05 versus values of db/db.

AGG, and reverse GTTGTCATGGCACCATTCAG; ColIV: forward ACATCTCTGCCAGGACCAAG, and reverse GGCTGACATTCCACAATTCC. mRNA levels were normalized against the reference gene (GAPDH) and quantified using the $2^{-\Delta\Delta Ct}$ method.

Confocal microscopy analysis

Cells were plated on Petri dishes. Cell samples were fixed with 4% formaldehyde at room temperature for 30 min. After incubation for 2 h in blocking solution (5% BSA+0.2% Triton X-100), the cells were incubated with anti-Fn, anti-ColIV, or NF-κBp 65 primary antibody at 4°C overnight. After washing three times with PBS, the cells were incubated (in the dark) with Alexa Fluor 594 or Alexa Fluor 488 secondary antibody for 2 h at 37°C. After further washing, cells were incubated for 10 min in 40,60-diamidino-2-phenylindole (DAPI) to stain the nuclei, and the cells were observed with a LSM880 laser confocal microscope.

Statistical analysis

Statistical analysis was performed by using SPSS 16.0. The data were presented as mean ± standard deviation (SD). One-way ANOVA was performed to analyze difference among groups. P value less than 0.05 was considered as a significant difference.

Results

Effects of melatonin on clinical and metabolic parameters in diabetic mice

Compared to the db/m mice, significant changes in body weight, blood glucose, kidney weight and UAER (24 h urinary albumin excretion rate) were observed in the db/db mice (P<0.05). As shown in **Table 1**, the UAER level decreased significantly in db/db+MT (50, 100, 200 μg/kg) mice (P<0.05).

Effects of melatonin on renal histopathologic changes in kidneys of diabetic mice

Changes in renal histopathology were observed using formalin-fixed paraffin-embedded renal sections in a ×400 magnified field. The glomerular mesangial expansion and tubulointerstitial injury of db/db mice were severer than those of db/m mice (**Figure 1A-C**, P<0.05). However, the kidney histopathologic lesions were less severe after treatment with MT as observed in db+MT (50, 100, 200 μg/kg) mice (**Figure 1A-C**, P<0.05).

Effects of melatonin on inflammation in kidneys of diabetic mice

To study the effects of melatonin on inflammation in kidneys of diabetic mice, analysis of CD68 positive macrophage infiltration was followed by immunohistochemistry analysis. Compared to the control group, accumulation of CD68-positive macrophages was extensive in db/db mice (**Figure 1D-F**, P<0.05). The number of CD68-positive macrophages was decreased significantly in the db/db+MT group (**Figure 1D-F**, P<0.05). In addition, mRNA expression levels of TNF-α and MCP-1 in kidney were quantified by qRT-PCR. While TNF-α and MCP-1 mRNAs were expressed at a low level in the kidney of the control group, the expression levels of both mRNAs were significantly increased in the db/db mice group (P<0.05). TNF-α and MCP-1 mRNA expression levels in the kidney of the db/db+MT group were significantly downregulated compared with those of the db/db mice group (**Figure 1G**, P<0.05).

Effects of melatonin on TLR4 signalling pathway in kidneys of diabetic mice

The expression of TLR4 in the kidneys of mice were tested by Immunohistochemistry (**Figure 2A-C**), expression levels of TLR4 were significantly higher in glomeruli and tubulointersti-

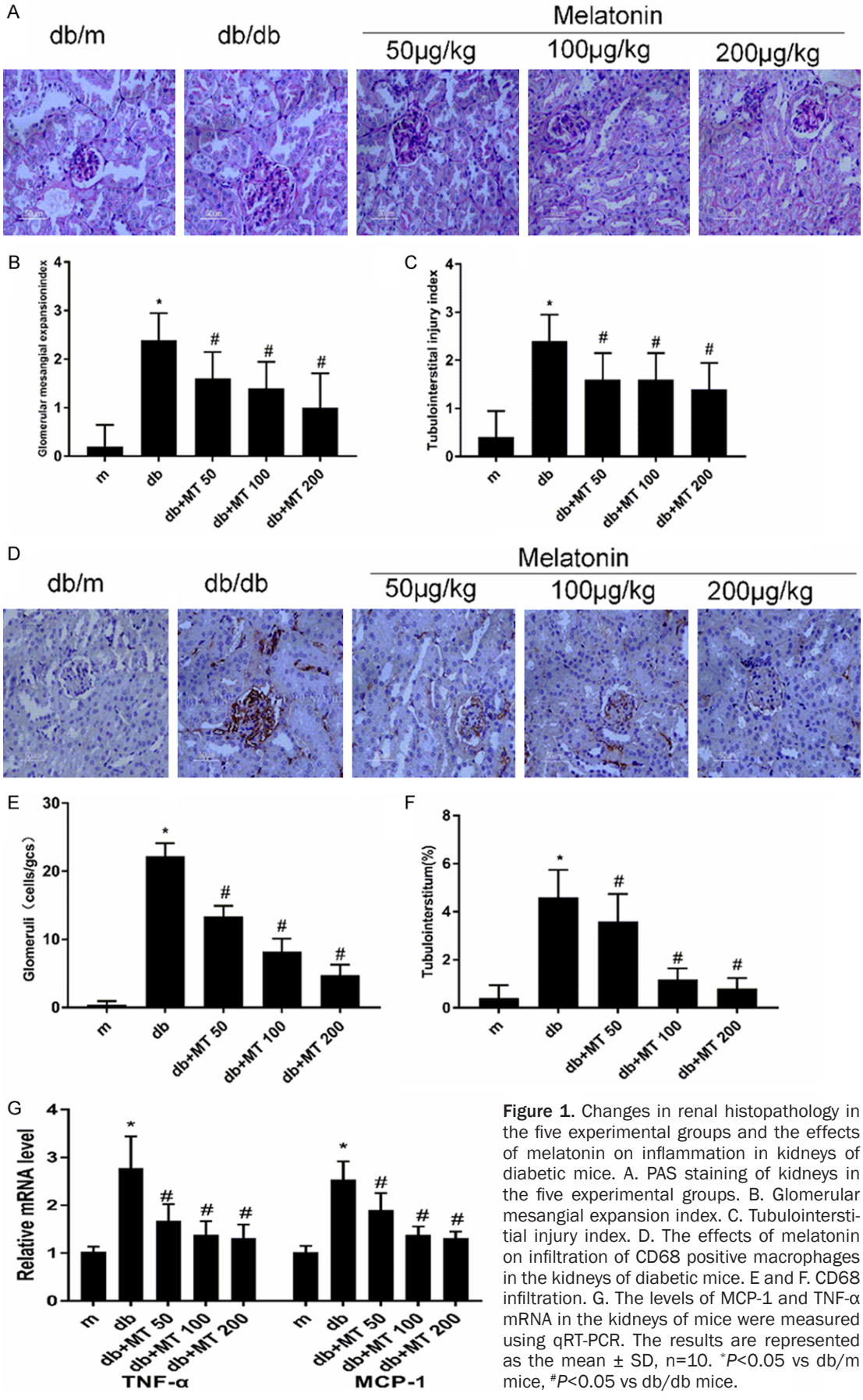

Figure 1. Changes in renal histopathology in the five experimental groups and the effects of melatonin on inflammation in kidneys of diabetic mice. A. PAS staining of kidneys in the five experimental groups. B. Glomerular mesangial expansion index. C. Tubulointerstitial injury index. D. The effects of melatonin on infiltration of CD68 positive macrophages in the kidneys of diabetic mice. E and F. CD68 infiltration. G. The levels of MCP-1 and TNF-α mRNA in the kidneys of mice were measured using qRT-PCR. The results are represented as the mean ± SD, n=10. $^{*}P<0.05$ vs db/m mice, $^{\#}P<0.05$ vs db/db mice.

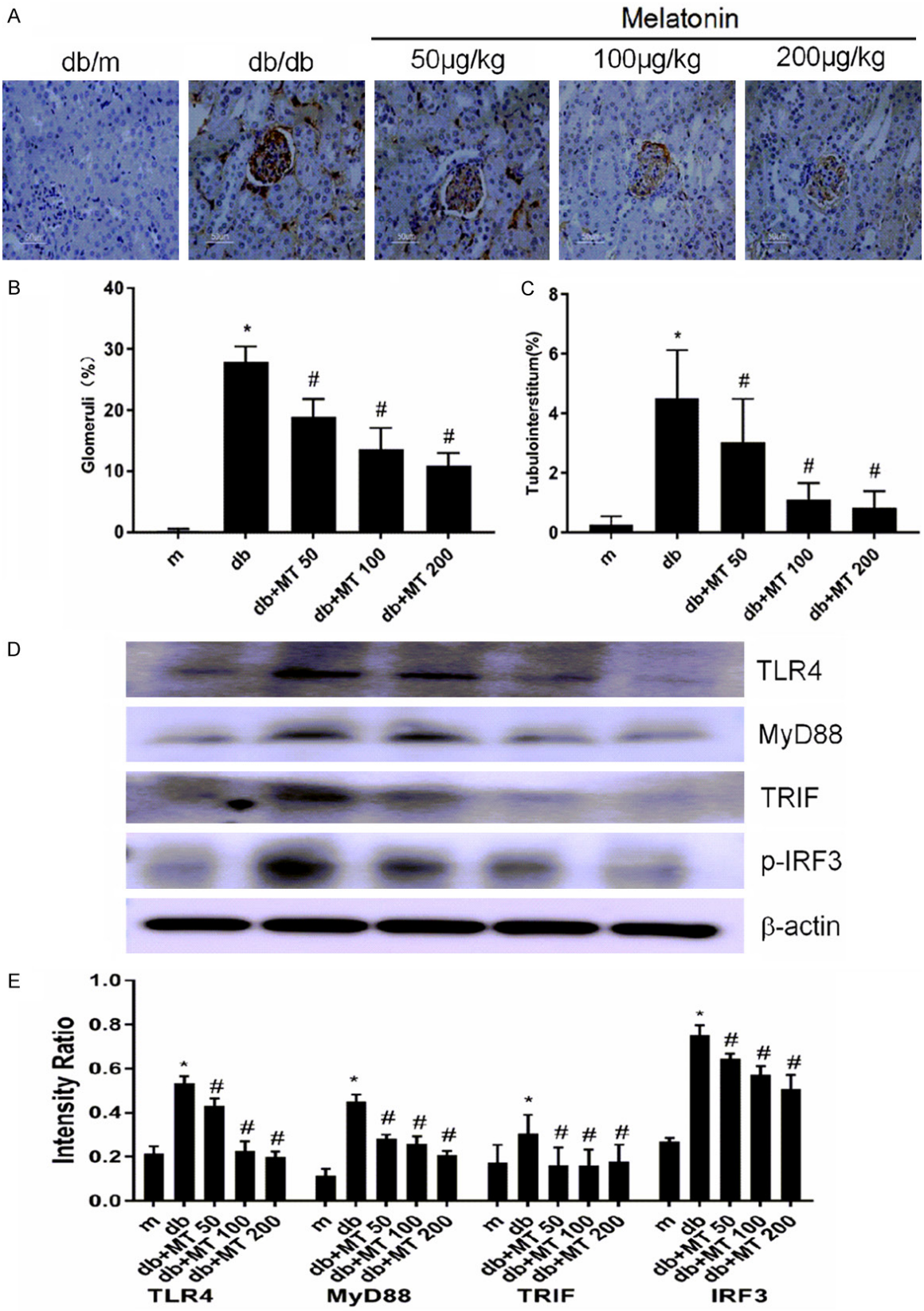

Figure 2. Effects of melatonin on the TLR4 signalling pathway in the kidneys of diabetic mice. A-C. Expression of TLR4 in the kidneys of mice. D and E. Expression of TLR4 signalling pathway proteins was analysed using western blotting. All data are expressed as the mean ± SD, IHC: n=10, WB: n=3. $^{*}P<0.05$ vs db/m mice, $^{\#}P<0.05$ vs db/db mice.

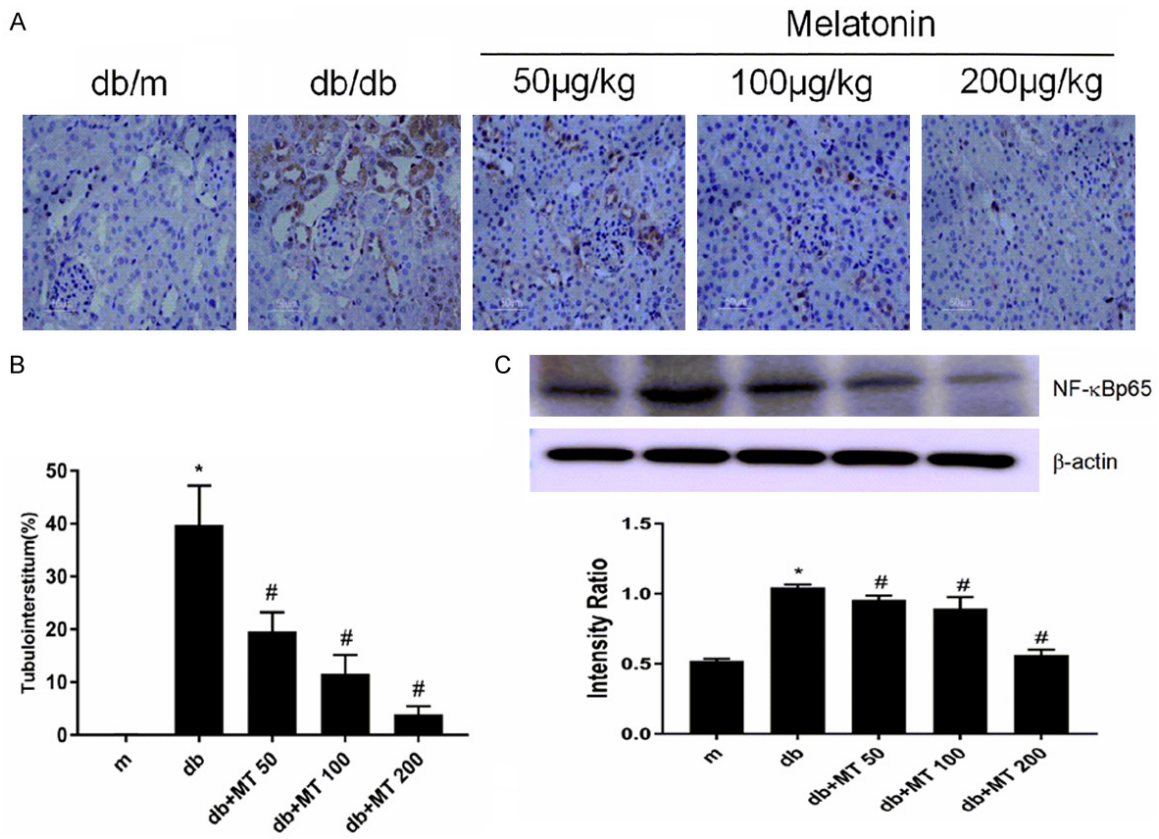

Figure 3. Effects of melatonin on NF-κB p65 in the kidneys of diabetic mice. A and B. NF-κB p65 immunostaining in the kidneys of mice. C. Western blot analysis of the expression of NF-κB p65. All data are expressed as the mean ± SD, IHC: n=10, WB: n=3. *P<0.05 vs db/m mice, #P<0.05 vs db/db mice.

tium of db/db mice than those of db/m mice (P<0.05) and melatonin treatment decreased the expression levels of TLR4 as compared with db/db mice (P<0.05). As shown in **Figure 2D**, **2E**, the expression levels of TLR4, MyD88, TRIF (TIR-domain-containing adapter-inducing interferon-β), and p-IRF3 proteins in the kidney of mice were detected by western blot. Expression levels of TLR4, MyD88, TRIF, and p-IRF3 were significantly higher in db/db mice than db/m mice (P<0.05). However, melatonin treatment decreased the expression levels of these proteins as compared with db/db mice (P<0.05).

Effects of melatonin on NF-κB p65 in kidneys of diabetic mice

The effects of melatonin on NF-κB p65 in kidneys of diabetic mice were investigated by immunohistochemistry and western blot analyses (**Figure 3A-C**). In the control group, staining for NF-κB p65 was rare. In contrast, NF-κB p65 was abundantly expressed in the kidney of diabetic mice (P<0.05). This overexpression was attenuated in db/db+MT (50, 100, 200 μg/kg) groups, indicating that melatonin treatment significantly reduces the expression of NF-κB p65 in diabetic mice (**Figure 3A-C**, P< 0.05).

Effects of melatonin on TGF-β1/Smad3 pathway in kidneys of diabetic mice

The expression of TGF-β1 and p-Smad3 in the kidney was detected by western blotting. As shown in **Figure 4A**, **4B**, levels of TGF-β1 and p-Smad3 protein were significantly higher in db/db mice compared with db/m mice (P< 0.05). Treatment with melatonin decreased TGF-β1 and p-Smad3 protein levels as compared with db/db mice (P<0.05).

Effects of melatonin on extracellular matrix production in kidneys of diabetic mice

To study the effects of melatonin on extracellular matrix production in kidneys of diabetic mice, ColIV and Fn were detected by western blot. In **Figure 4C**, **4D**, ColIV and Fn were higher in db/db mice than in db/m mice (P<0.05).

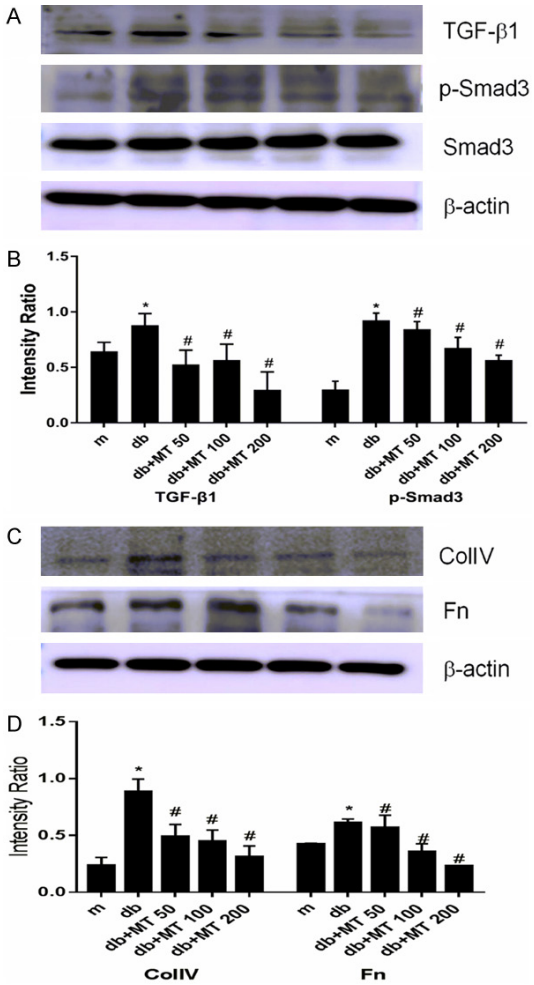

Figure 4. Effects of melatonin on the TGF-β1/Smad3 pathway. A and B. Western blot analysis of the expression of TGF-β1/Smad3 pathway proteins. C and D. Western blot analysis of the expression of ColIV and Fn in the kidneys. All data are expressed as the mean ± SD, n=3. *P<0.05 vs db/m mice, #P<0.05 vs db/db mice.

Treatment with melatonin decreased ColIV and Fn levels as compared with db/db mice (P<0.05).

Effects of melatonin on mesangial cells viability

To determine whether melatonin influences mesangial cell viability, mouse mesangial cells (SV 40 MES 13) were seeded in 96-well plates and treated with various concentrations of melatonin for 24 h. As shown in **Figure 5A**, while melatonin (10-10000 μM) had no detectable cytotoxic effect on cell viability, 10000 μM melatonin had an observable effect on the viability of mesangial cells (P<0.05).

Optimization of experiment conditions

As shown in **Figure 5B**, mesangial cells were treated with high glucose at different times. Western blot analysis showed that the expression of TLR4 was highest at 24 h (P<0.05). Melatonin at concentrations of 1000 μM significantly decreased TLR4 expression (**Figure 5C**, P<0.05).

Effects of melatonin on HG-induced secretion of inflammatory cytokines in mesangial cells

As shown in **Figure 5D**, the ELISA results show that the levels of TNF-α, IL-1β, and MCP-1 in HG-induced mesangial cells were markedly increased in the HG group (P<0.05). Treatment with melatonin reduced the levels of TNF-α, IL-1β, and MCP-1 compared to the HG group (P<0.05). In addition, TNF-α, IL-1β, and MCP-1 mRNA levels in the HG group were higher than those in untreated cells (P< 0.05), and melatonin treatment decreased mRNA expression levels of these cytokines (P<0.05).

Effects of melatonin on the TLR4 signalling pathway in HG-treated mesangial cells

To explore the effects of melatonin on the TLR4 signalling pathway in HG-treated mesangial cells, we performed western blot analysis of TLR4, MyD88, TRIF, and IRF3 protein expression. As shown in **Figure 6A**, the expression levels of these proteins were significantly higher in the HG group than in untreated cells (P<0.05). Treatment with melatonin decreased TLR4, MyD88, TRIF, and IRF3 expression levels compared with HG group (P<0.05).

Effects of melatonin on HG-induced proliferation in mesangial cells

To determine whether proliferation of mesangial cells was affected by melatonin, we performed 5-ethynyl-2'-deoxyuridine (EdU) staining. While proliferation was significantly increased in HG-induced mesangial cells, melatonin treatment decreased the HG-induced cell proliferation (**Figure 6B**).

Effects of melatonin on the TGF-β1/Smad3 signalling pathway in HG-treated mesangial cells

To investigate the effects of melatonin on the TGF-β1/Smad3 signalling pathway in HG-indu-

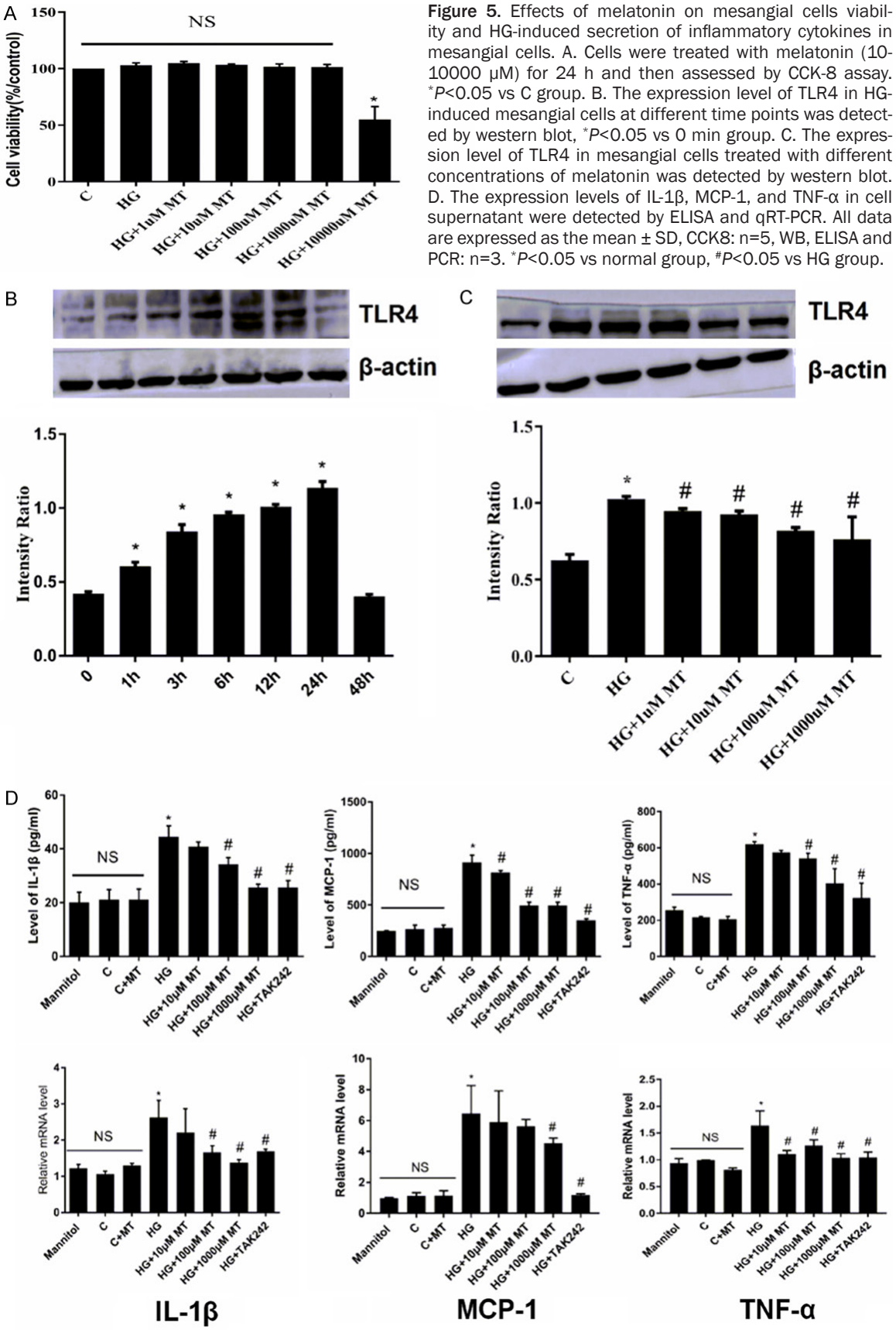

Figure 5. Effects of melatonin on mesangial cells viability and HG-induced secretion of inflammatory cytokines in mesangial cells. A. Cells were treated with melatonin (10-10000 μM) for 24 h and then assessed by CCK-8 assay. $^{*}P<0.05$ vs C group. B. The expression level of TLR4 in HG-induced mesangial cells at different time points was detected by western blot, $^{*}P<0.05$ vs 0 min group. C. The expression level of TLR4 in mesangial cells treated with different concentrations of melatonin was detected by western blot. D. The expression levels of IL-1β, MCP-1, and TNF-α in cell supernatant were detected by ELISA and qRT-PCR. All data are expressed as the mean ± SD, CCK8: n=5, WB, ELISA and PCR: n=3. $^{*}P<0.05$ vs normal group, $^{\#}P<0.05$ vs HG group.

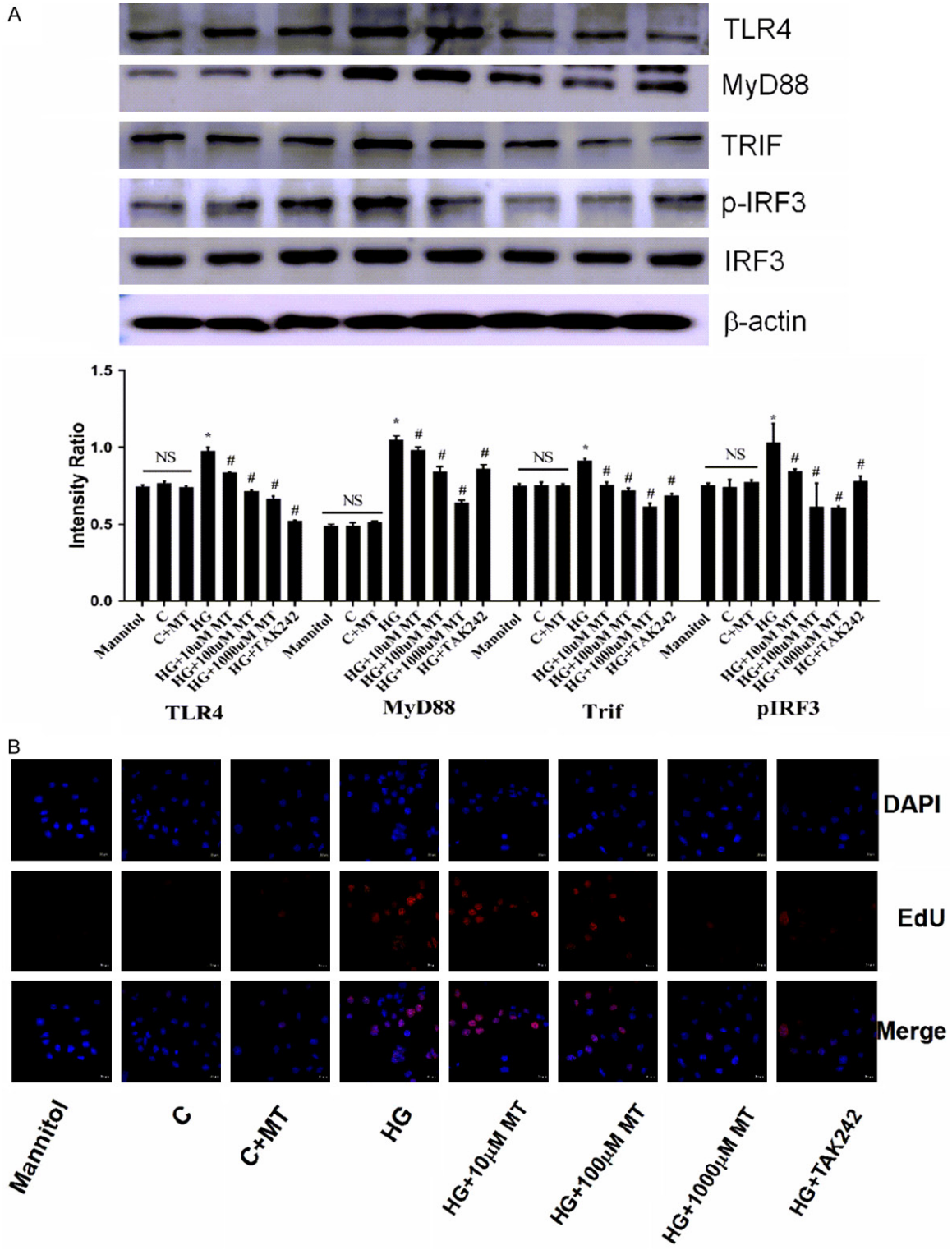

Figure 6. Effects of melatonin on the TLR4 signalling pathway and mesangial cell proliferation. A. The levels of TLR4, MyD88, TRIF, and p-TRF3 were detected by western blot analysis. B. Edu staining showed proliferation of HG-induced mesangial cells treated with melatonin. All data are expressed as the mean ± SD, n=3. $^{*}P<0.05$ vs normal group, $^{\#}P<0.05$ vs HG group.

ced mesangial cells, we performed western blot analysis on TGF-β1 and Smad3. As shown in **Figure 7**, the data revealed that melatonin treatment decreased TGF-β1 and Smad3 pro-

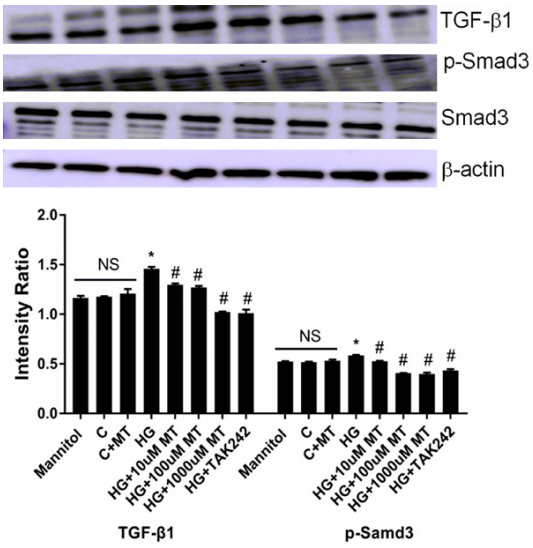

Figure 7. Effects of melatonin on the TGF-β1/Smad3 pathway. The levels of TGF-β1 and p-Smad3 were detected by western blot analysis. All data are expressed as the mean ± SD, n=3. *P<0.05 vs normal group, #P<0.05 vs HG group.

tein expression in HG-treated mesangial cells (P<0.05).

Effects of melatonin on HG-induced ECM production in mesangial cells

To determine the effects of melatonin on the induction of ECM components in HG-treated mesangial cells, expression of type IV collagen and fibronectin in mesangial cells was analysed by WB, ELISA and qRT-PCR. The results revealed that ColIV and Fn expression levels were increased in HG-treated mesangial cells compared with control group (P<0.05). Treatment with melatonin decreased ColIV and Fn expression levels in HG-treated mesangial cells (P<0.05). Moreover, immunofluorescence staining confirmed the observation that ColIV and Fn expression was decreased in HG-treated mesangial cells after melatonin treatment (**Figures 8** and **9**).

Effects of melatonin on the NF-κB signalling pathway in mesangial cells

The effects of melatonin on the NF-κB signalling pathway in mesangial cells were investigated by immunostaining and western blot analyses. The fluorescence intensity of p65 nuclear staining was significantly higher in HG-treated mesangial cells than in the untreated group (**Figure 10A**). Treatment with melatonin reduced nuclear translocation of p65 subunit as compared with the HG group (**Figure 10A**). At same time, we observed that melatonin inhibited the phosphorylation of I-κB, melatonin treatment decreased p-I-κB protein expression in HG-treated mesangial cells (**Figure 10B**, P< 0.05).

Discussion

DN is the most common diabetic microvascular complication of diabetes [1], but the precise mechanisms of development and of progression of DN remain unknown. Evidence is accumulating that inflammation is a major factor in the occurrence and development of DN in human and animal models [1, 4]. Several studies have reported levels of TNF-α and MCP-1 to be elevated in the urine of patients with type 2 diabetes, and that the level of TNF-α is positively correlated with urinary albumin excretion. A positive correlation between the level of MCP-1 and HbA1C, the urinary protein/creatinine ratio (ACR), and serum creatinine, and a negative correlation between the level of MCP-1 and glomerular filtration rate (eGFR) were also reported [32]. In addition, albuminuria is a leading risk factor for the progression of DN [33], and one of the major manifestations of renal involvement of incipient DN is an increased kidney/body weight ratio [34]. In our studies, we observed that melatonin treatment could decrease urinary albumin excretion and kidney/body weight ratio in db/db mice. Furthermore, renal injury (assessed by histopathology and expression of MCP-1, TNF-α, ColIV and Fn) was reduced in db/db mice following melatonin treatment. The results obtained *in vitro* using the mesangial cell model were consistent with the results of the *in vivo* study. Thus, melatonin treatment decreased these proteins in HG-induced mesangial cells. Taken together, these findings suggested that melatonin may play a protective role during the development of diabetic nephropathy. Moreover, remission in the experimental DN mice was not due to a hypoglycaemic effect. Next, we focused on potential mechanisms of melatonin intervention in the development of DN.

The role of TLR4 in innate immune responses has been previously demonstrated [35], and activation of TLR4 signalling pathway trigg-

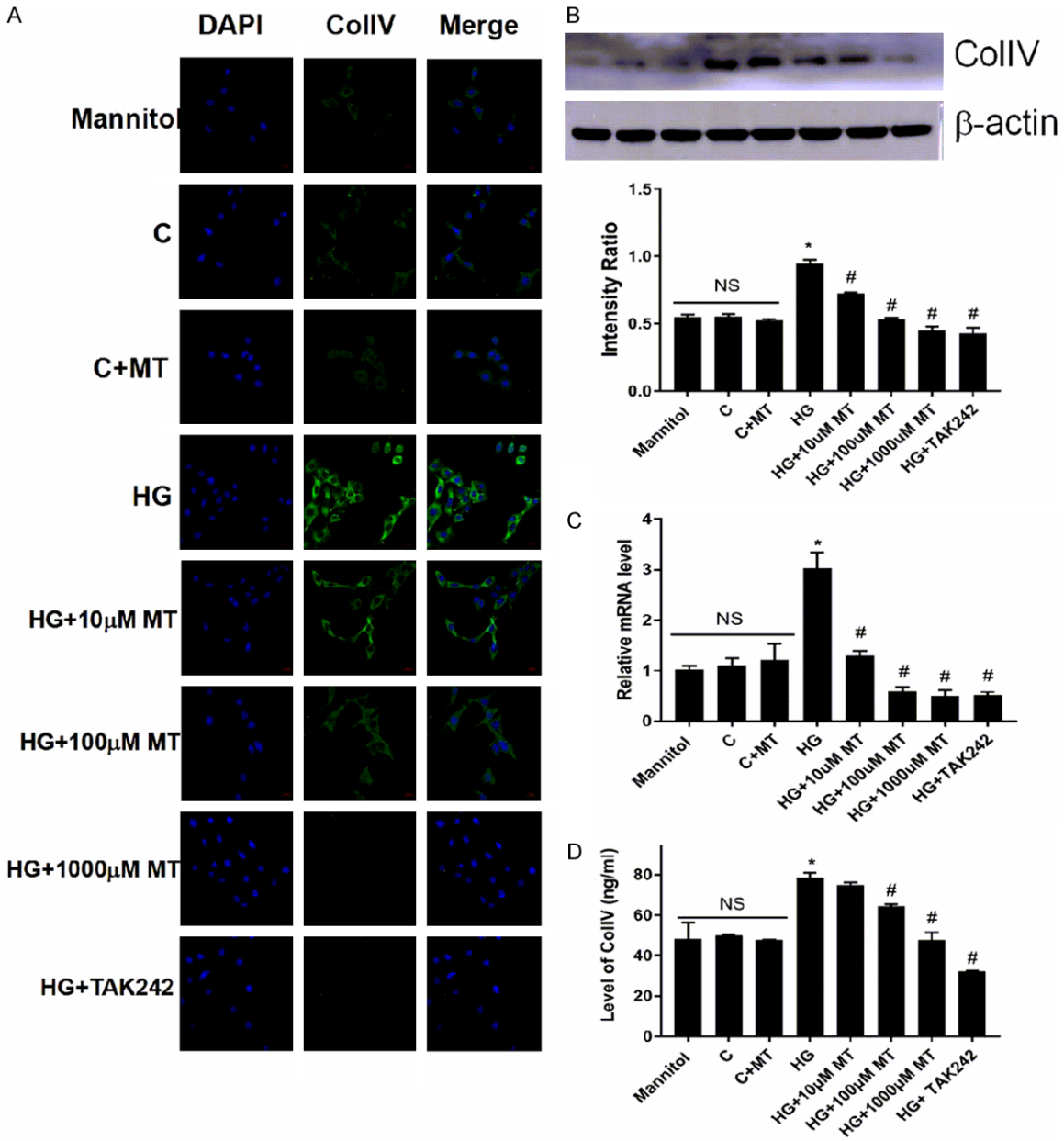

Figure 8. Effects of melatonin on HG-induced ColIV production in mesangial cells. A. ColIV expression in mesangial cells was detected by immunofluorescence. B. Western blot analysis of the expression of ColIV in mesangial cells. C. ColIV mRNA expression levels were detected by qRT-PCR. D. ColIV protein in cell supernatant was detected by ELISA. All data are expressed as the mean ± SD, n=3. $^{*}P<0.05$ vs normal group, $^{\#}P<0.05$ vs HG group.

ers transcription and release of proinflammatory cytokines, leading to renal dysfunction in acute and chronic kidney diseases [36, 37]. Moreover, evidence has been presented that the pro-inflammatory effects of TLR4 are associated with diabetes and diabetic complications [38]. Mohammad *et al*. [39] showed that expression of TLR2 and TLR4 increased in type 1 diabetic nonobese mice. The observed increase in TLR2/TLR4 triggered an increase in nuclear factor-κB activation (in response to TLR4 ligand binding), resulting in an increase in production of proinflammatory cytokines. Devaraj *et al*. [40] demonstrated that levels of MyD88, IRAK-1 protein phosphorylation, Trif, IRF3, and NF-κB activity were significantly reduced in TLR4 (-/-)+STZ mice compared to WT+STZ mice. In our present study, we observed

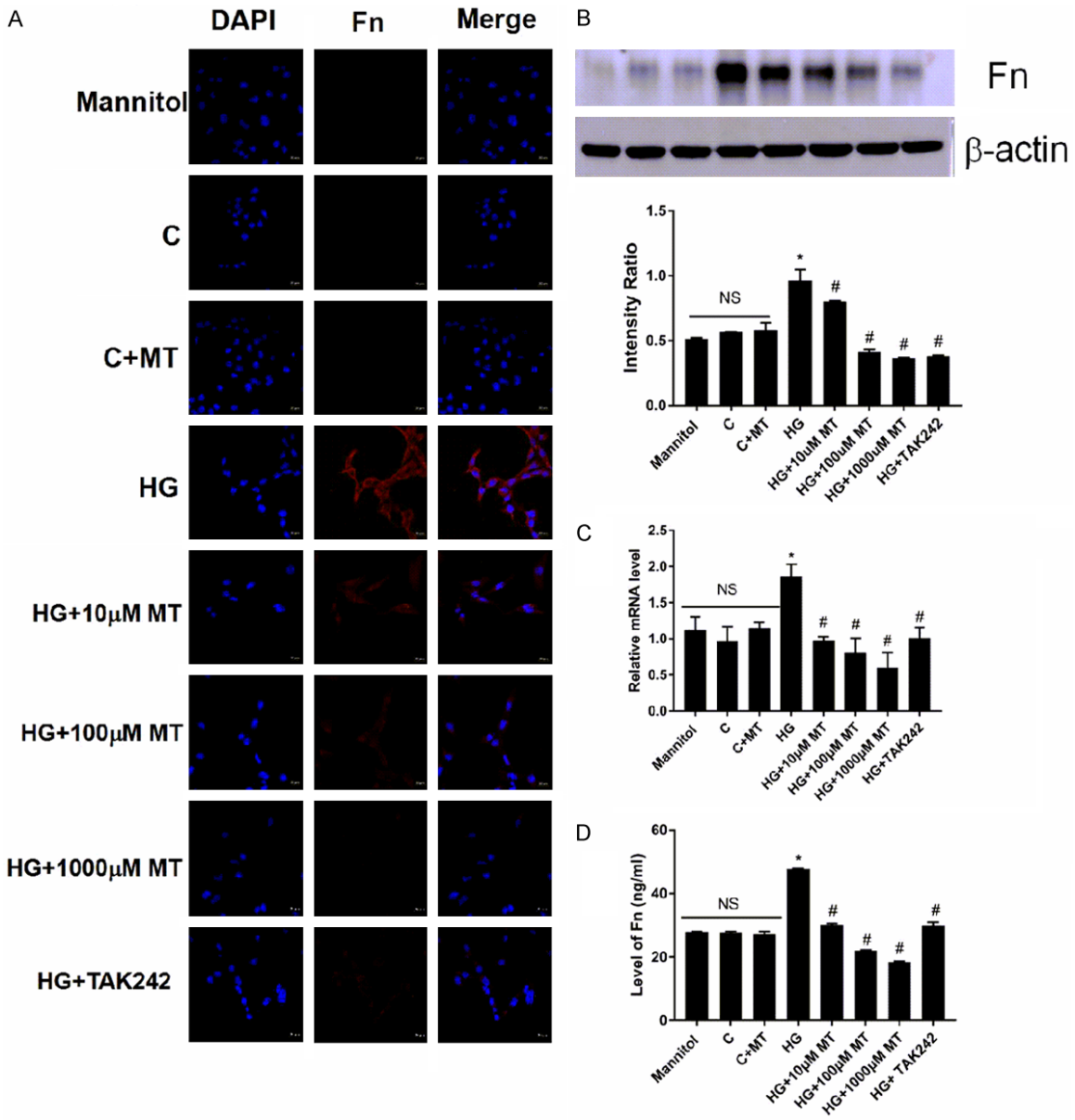

Figure 9. Effects of melatonin on HG-induced Fn production in mesangial cells. A. Fn expression in mesangial cells was detected by immunofluorescence. B. Western blot analysis of the expression of Fn in mesangial cells. C. Fn mRNA expression levels were detected by qRT-PCR. D. Fn protein in cell supernatant was detected by ELISA. All data are expressed as the mean ± SD, n=3. $^{*}P<0.05$ normal group, $^{\#}P<0.05$ vs HG group.

that melatonin significantly attenuated TLR4 and NF-κB expression. Moreover, melatonin significantly inhibited the nuclear translocation of NF-κB p65 subunit in HG-treated mesangial cells, which indicated that melatonin suppressed HG-mediated TLR4 and NF-κB activation (and downregulated HG-mediated inflammatory genes) though the TLR4 signalling pathway.

In diabetes, TGF-β1 plays an important role in the development of renal fibrosis, including in the accumulation of ECM protein and in tubulointerstitial and glomerular fibrosis [15, 41]. The TGF-β1/Smad3 signalling pathway is considered to be an important pathway in mesangial cells [16]. In diabetes, a variety of different factors may cause activation of the TGF-β1/Smad3 signalling pathway, triggering the deposition of collagen matrix and causing tubulointerstitial and glomerular fibrosis [42]. In our study, TGF-β1 was activated in the kidneys of db/db mice and in the HG-induced mesangial cells, and melatonin suppressed TGF-β1 ex-

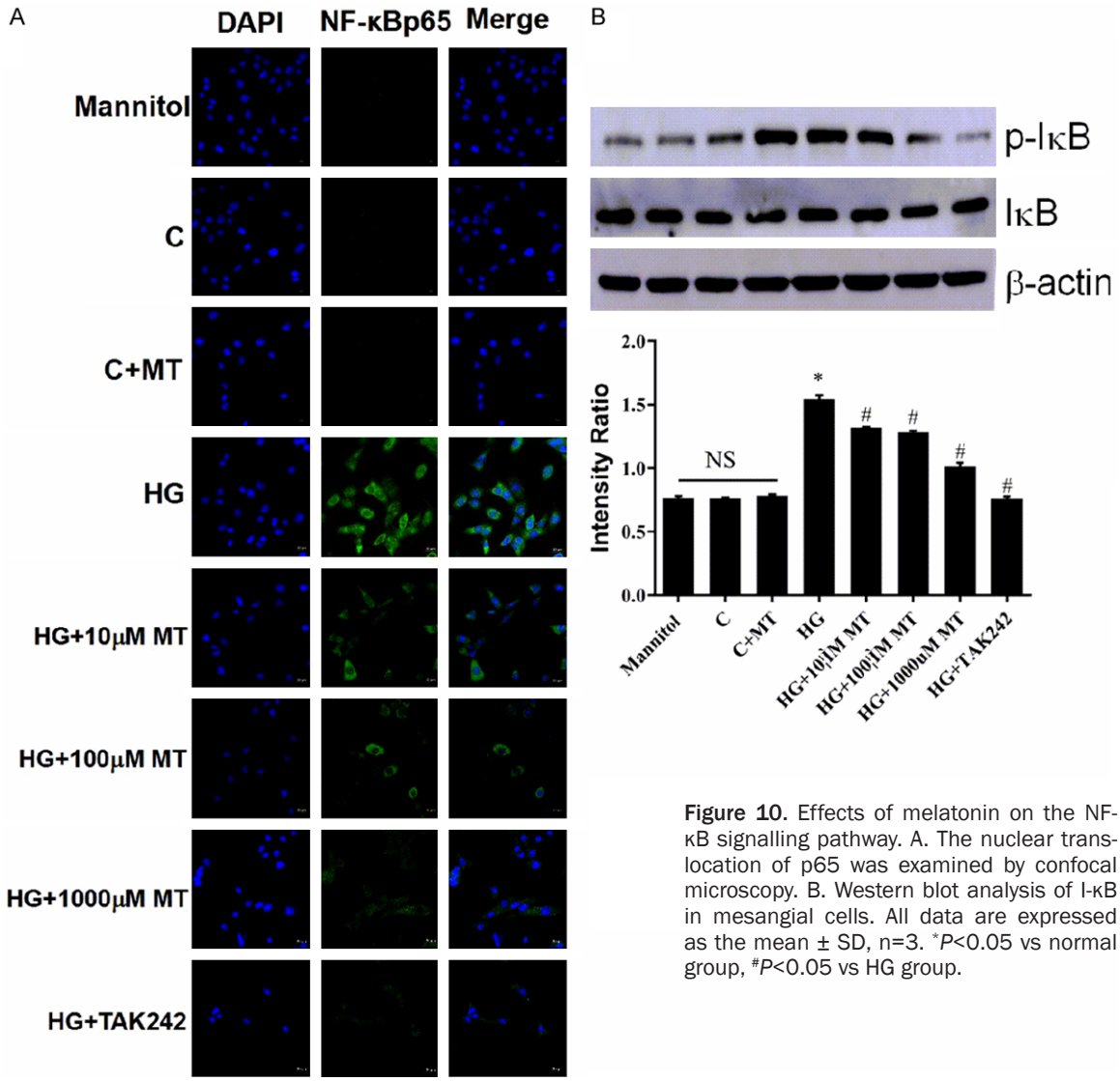

Figure 10. Effects of melatonin on the NF-κB signalling pathway. A. The nuclear translocation of p65 was examined by confocal microscopy. B. Western blot analysis of I-κB in mesangial cells. All data are expressed as the mean ± SD, n=3. $^{*}P<0.05$ vs normal group, $^{\#}P<0.05$ vs HG group.

pression in both systems. Moreover, melatonin decreased p-Smad3 expression in the kidneys of db/db mice. In addition, melatonin also significantly inhibited ColIV and Fn expression in kidneys of db/db mice and also in HG-induced mesangial cells. Together, these results suggest that melatonin negatively regulates the TGF-β1/Smad3 pathway to control renal fibrosis in DN.

Conclusion

Melatonin, a hormone that regulates the wake-sleep cycle, can inhibit renal inflammation and fibrosis in DN by inhibiting the TLR4 and TGF-β1/Smad3 signalling pathways. Thus, melatonin may be a promising therapeutic target for diabetic nephropathy. Future research is warranted to explain how melatonin interacts with TLR4 and the TGF-β1 pathway.

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Disclosure of conflict of interest

None.

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