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Original Research Article

Tanshinone IIA mitigates peritoneal fibrosis by inhibiting EMT via regulation of TGF-β/smad pathway

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Abstract

Purpose: *To explore the effects of tanshinone IIA (T-IIA) on Dianeal-N PD-4 (PDF)-induced expression of fibrogenic cytokines in human peritoneal mesothelial cells (HPMCs), and to elucidate the mechanisms of action involved.*
Methods: *Seven groups of HPMCs were used in the study: control group, PDF group, T-IIA group, LY364947 group, and 2 transforming growth factor-β (TGF-β) groups (TGF-β+ 50 μM T-IIA and TGF-β+ 100 μM T- IIA). The expression levels of mRNA and protein of TGF-β, smad2, smad7, α-smooth muscle actin(α-SMA), fibronectin, collagen I, E-cadherin, N-cadherin, matrix metalloprotein-2(MMP-2), and MMP-9 in the various groups were determined by reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting as appropriate.*
Results: *The expressions of α-SMA, fibronectin, collagen I, TGF-β and smad2 were significantly up-regulated in HPMCs by PDF treatment, but smad7 was down-regulated, relative to the control group ($p < 0.01$).These PDF-induced effects were reversed by T-IIA ($p < 0.05$). Inhibition of TGF-β/smad pathway by LY364947 treatment led to significant decrease in the expressions of fibrosis-related proteins, when compared with PDF group ($p < 0.05$). TGF-β treatment also produced numerous spindle-shaped HPMCs characteristic of epithelial-mesenchymal transition (EMT). However, this morphological transition was alleviated, and the expression levels of EMT-related proteins were significantly down-regulated by exposure to the two doses of T-IIA ($p < 0.05$).*
Conclusion: *Tanshinone IIA inhibits EMT in HPMCs by regulating TGF-β/smad pathway, thus mitigating peritoneal fibrosis. Therefore, T-IIA has promising potential as a new drug for the treatment of peritoneal dialysis (PD)-induced fibrosis.*

Keywords: *Peritoneal dialysis, Peritoneal fibrosis, Tanshinone IIA, Epithelial-mesenchymal transition*

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INTRODUCTION

End-stage renal disease (ESRD) is a major public health issue all over the world [1]. Peritoneal dialysis (PD) is a widely used renal replacement treatments for ESRD. However, about 3 – 36 % of PD patients have experienced peritoneal ultrafiltration failure within 1 - 4 y after PD [2]. The major cause of peritoneal ultrafiltration failure is peritoneal fibrosis:

repeated PD increases the risk of peritoneal fibrosis, resulting in peritoneal ultrafiltration failure which eventually forces patients to abandon the treatment [2,3]. Therefore, peritoneal fibrosis is a critical constraint in the development of PD. Thus, it is important to investigate the mechanism of peritoneal fibrosis and develop new therapeutic targets for the disease as a way of enhancing the development of PD.

Peritoneal fibrosis is characterized by absence of peritoneal mesothelial cell layer and increase in mesenchymal layer [3,4]. Factors such as peritoneal dialysis fluid biocompatibility, peritonitis, and cytokines are implicated in the etiology of peritoneal fibrosis [3,4]. Multiple mechanisms contribute to the incidence and progression of the disease. These include fibrosis-promoting factor, oxidative stress and peritoneal EMT of mesothelial cells [3,4]. However, EMT is the main mechanism involved in peritoneal fibrosis [4].

EMT is a biological process in which mature epithelial cells are transformed into cells with mesenchymal phenotype [5]. During this process, epithelial cells usually lose their adhesion ability and rearrange their cytoskeleton, leading to a decline in peritoneal function [5]. It has been reported that many signal transduction pathways may lead to EMT in peritoneal mesothelial cells. These are ROS / MMP-9 pathway [6], PI3k / AKT pathway [7], and transforming growth factor (TGF-β) signaling pathway [8]. Accumulating evidence have consistently indicated that TGF-β1 is the marker that induces EMT in peritoneal mesothelial cells, and that the TGF-β1 / smad3 signaling pathway is a key factor involved in EMT [8-10]. Although a lot of studies have been carried out on peritoneal mesothelial cell EMT, the mechanisms involved in the process have not been fully elucidated. It has been reported that T-IIA, an active component of the Chinese herb *Danshen*, inhibits fibrosis in various tissues [11-13]. In previous animal studies, it was shown that T-IIA significantly inhibited high glucose-based, PDF-induced peritoneal fibrosis [14]. The present study was aimed at investigating the effects of T-IIA on PDF-induced expression of fibrogenic cytokines in cultured HPMCs, and the underlying mechanism(s).

EXPERIMENTAL

Chemicals and reagents

Tanshinone IIA (Figure 1) was obtained from Carephar (Nanjing, China). Dianeal-n PD-4 with 4.25 % glucose (PDF) was obtained from Baxter (Guangzhou, China). All the antibodies were purchased from Abcam (USA). Normal Australian fetal bovine serum (FBS), high-glucose DMEM medium, and 0.25 % EDTA-Trypsin were purchased from GIBCO (NY, USA). Trizol reagent was product of Invitrogen (California, USA), and real-time PCR reaction kit was from Takara (Dalian, China).

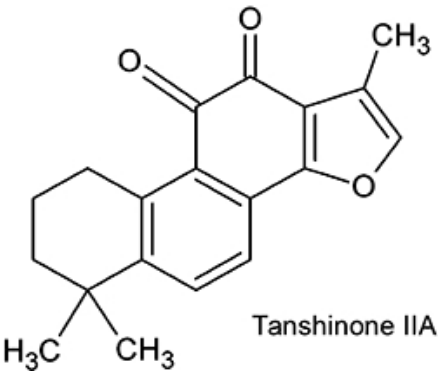

Figure 1: Chemical structure of Tanshinone IIA

Cell culture

Human peritoneal mesothelial cells (HPMCs) were obtained from ATCC (VA, USA). The cells were cultured in a high-glucose DMEM medium containing 10 % FBS in a 5 % CO_2 humidified incubator at 37 °C. The third-generation cells were used for subsequent experiments. When grown to sub-fusion state, the cells were incubated in serum-free culture medium for 24 h to synchronize cell growth, and then assigned to 7 groups: the control group, PDF group, PDF+50 μM T-IIA group, PDF + 5 μM TGF-β inhibitor (LY364947) group, 5 ng/mL TGF-β group, 5 ng/mL TGF-β + 50 μM T - IIA group and 5 ng/mL TGF-β + 100 μM T - IIA group. The cells were then used for the following experiments after another 72 h culture in their respective treatment states.

Real-time quantitative PCR

Trizol reagent (Invitrogen, USA) was used to extract the total RNA from the cells, cDNA was synthesized using commercially available reverse transcription kit (Takara, China). RNA (1ug) was reverse-transcribed into cDNA, which was then subjected to PCR amplification. The PCR reactions were performed using ABI 7500 Real-Time PCR System (ABI, USA). The amplification conditions were as follows: 95 °C for 30 s; 95 °C for 5 s, 60 °C for 34 s (40 cycles). The gene specific primer pairs for TGF-β, smad2, smad7, α-SMA, fibronectin, collagen I, E-cadherin, N-cadherin, MMP-2, MMP-9, and

GADPH are listed in Table 1. All the mRNA expression levels were normalized to that of GADPH using the $2^{-\Delta\Delta Ct}$ method. All the determinations were conducted in triplicate.

Western blot analysis

The cells were harvested and lysed in RIPA buffer (Sigma-Aldrich, USA). BCA assay (Thermo Fisher Scientific, USA) was applied to determine the protein concentrations. Extracted total protein (30µg) was separated by 10 % SDS-PAGE, electro-transferred onto a polyvinylidene fluoride (PVDF) membrane, and sealed with skim milk at 4 ^{o}C overnight. Then, the membrane was incubated with primary antibodies (Abcam, USA) against TGF-β, smad2, smad7, α-SMA, fibronectin, collagen I, E-cadherin, N-cadherin, MMP-2, MMP-9, tubulin, and GADPH for 4 hours at room temperature. The membrane was washed 3 times with Tris-buffered saline containing 0.1 % Tween-20 (TBS-T), and incubated with horseradish peroxidase (HRP) conjugated to goat anti-mouse secondary antibody (1:2000; Abcam, USA) at room temperature for two hours. It was then washed thrice in TBS-T. Tubulin and β-actin were used as internal references. Bands were developed using an electro-chemiluminescence (ECL) kit (Thermo Fisher Scientific, USA).

Statistical analysis

Analyses were performed using SPSS v.13.0 (SPSS Inc., Chicago, IL). The results are presented as mean ± SD. Differences between groups were assessed by one-way ANOVA. Statistical significance was assumed at $p < 0.05$ (two-tailed).

RESULTS

T-IIA exerted protective effect against PDF-induced fibrosis in HPMCs

To study the effect of T-IIA on PDF-induced fibrosis, the expression levels of fibrosis-related proteins in HPMCs were determined. RT-PCR analysis (Figure 2 A) showed that the mRNA expression levels of α-SMA, fibronectin, and collagen I were significantly up-regulated in HPMCs when treated with PDF ($p < 0.01$, relative to the control group). Consistent with the mRNA expression, the protein expression levels of these proteins were also significantly up-regulated when compared with the control group ($p < 0.05$; Figures 2B and Figure 2C). Moreover, in the PDF+IIA group, T-IIA treatment significantly decreased the expressions of α-SMA, fibronectin, and collagen I with respect to mRNA (Figure 2A) and protein (Figures 2B and Figure 2C). This indicates that T-IIA exerts protective effect on the HPMCS. Interestingly, PDF treatment significantly up-regulated the expressions of TGF-β and smad2, and down-regulated the expression of smad7 ($p < 0.01$, relative to the control group; Figure 2). These effects brought about by PDF were reversed by T-IIA treatment ($p < 0.05$, when compared to the PDF group; Figure 2).

T-IIA treatment inhibited fibrosis in HPMCs by regulation of the TGF-β/smad pathway

As shown in Figure 3, LY364947 treatment significantly inhibited the expression of TGF-β ($p < 0.05$, versus the PDF group), and down-regulated the expressions of smad2, and smad7.

Table 1: Primers used for real-time PCR analysis of mRNA

Target gene	Primer sequence
GAPDH	Forward (5'-3') TGAACGGGAAGCTCACTGG
	Reverse (5'-3') TCCACCACCCTGTTGCTGTA
α-SMA	Forward (5'-3') ATTGTGCACACCATTGGGA
	Reverse (5'-3') GATGGTTGTGTAGGGGTTGG
E-cadherin	Forward (5'-3') GATGGTTGTGTAGGGGTTGG
	Reverse (5'-3') AGGCTGTGCCTTCCTACAGA
FN	Forward (5'-3') CAGTGGGAGACCTCGAGAAG
	Reverse (5'-3') GTCCCTCGGAACATCAGAAA
Collagen 1	Forward (5'-3') TCCTGCGTGTACCCCACTCA
	Reverse (5'-3') ACCAGACATGCCTCTTGTCCTT
TGF-β1	Forward (5'-3') CACGTGGAGCTGTGCCAGAA
	Reverse (5'-3') GAACCCGTTGATGTCCACTT
MMP-2	Forward (5'-3') ATGACAGCTGCACCACTGAG
	Reverse (5'-3') ATTTGTTGCCCAGGAAAGTG
MMP-9	Forward (5'-3') AGGGCACATCCTATGACAGC
	Reverse (5'-3') ATTTGTTGCCCAGGAAAGTG

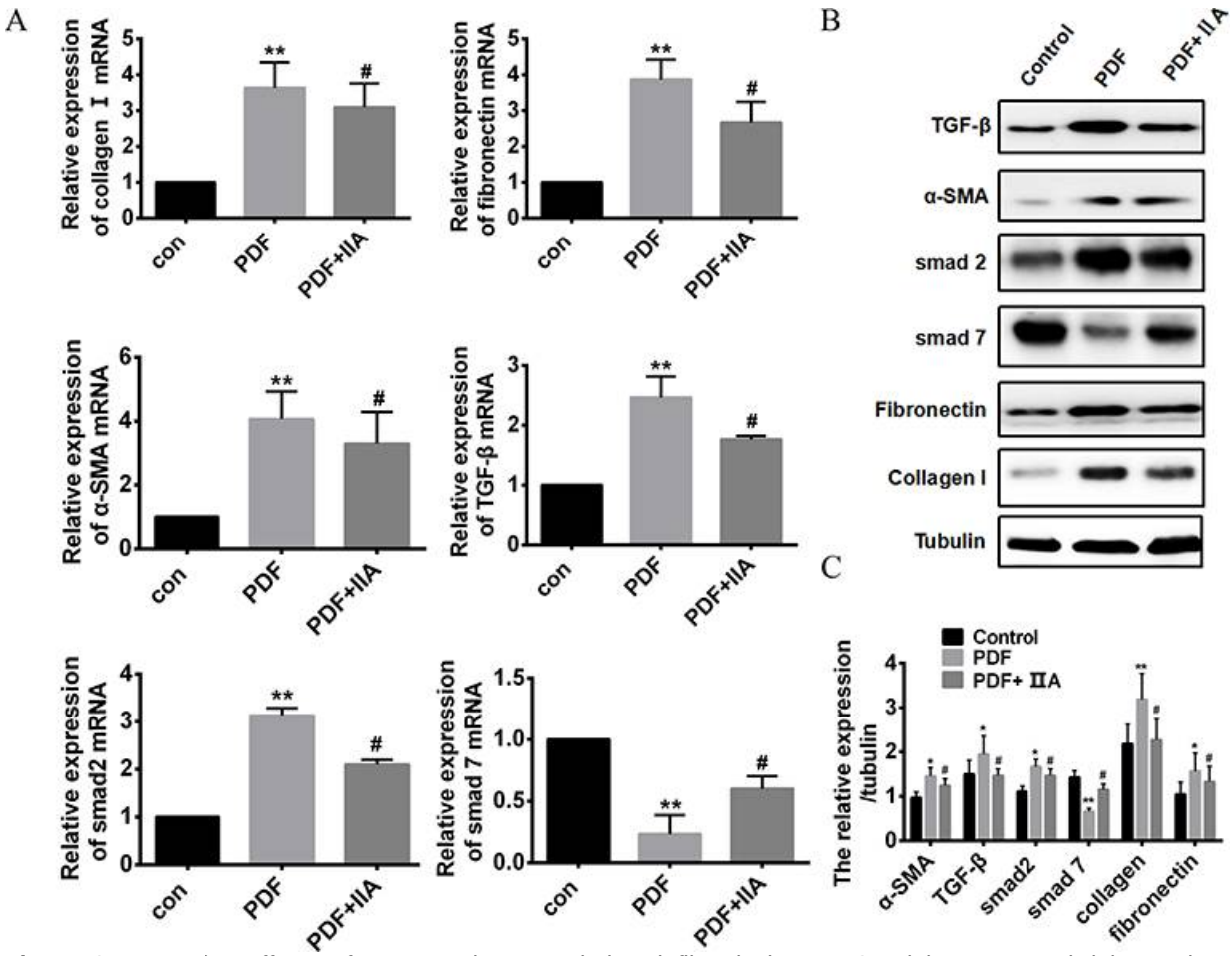

Figure 2: Protective effects of T-IIA against PDF-induced fibrosis in HPMCs. (A) mRNA and (B) protein expression levels of fibrogenic cytokines and TGF-β/smad pathway-related proteins in HPMCs in different treatment groups determined by RT-PCR and Western blot. (C) Quantitative analysis of the developed protein bands. *, $p < 0.05$, **, $p < 0.01$, versus the control group; #$p < 0.05$, ##$p < 0.01$, versus PDF group

Due to the inhibition of TGF-β/smad pathway, in expression of mRNA (Figure 3A) and protein (Figures 3B & 3C) levels, the expressions of the fibrosis-related proteins were significantly decreased when compared with the PDF group ($p < 0.05$, Figure 3).

T-IIA treatment inhibited EMT of HPMCs

Characteristically, mesenchymal cells have highly proliferative ability, with spindle-shape and well-spread morphology [15]. As shown in Figure 3A, TGF-β treatment led to appearance of numerous spindle-shaped HPMCs, which indicates that the HPMCs underwent EMT. However, this morphological transition was alleviated by exposure of the HPMCs to different concentrations of T-IIA. Moreover, RT-PCR (Figure 4B) and Western blot (Figures 4C and Figure 4D) results showed that the expressions of N-cadherin, MMP-2, and MMP-9 were significantly decreased, compared with the TGF-β group, while E-cadherin expression was significantly up-regulated ($p < 0.05$). These results indicate that T- IIA treatment inhibited the EMT process induced in HPMCs by TGF-β.

DISCUSSION

Peritoneal dialysis (PD) is an important alternative treatment for ESRD. In PD, the peritoneum is used as a semipermeable membrane to remove metabolic wastes and excess water in the body according to dispersion and penetration principles [2,3]. It has always been thought that the activation of fibroblasts inherent in peritoneal tissue and the infiltration of inflammatory cells are the most important factors that cause structural and functional changes in peritoneal tissue [16]. However, recent studies have found that the long-term stimulation by non-biocompatible peritoneal dialysis solution can lead to the EMT of peritoneal mesothelial cells [2,3,16]. EMT is a highly regulated process that has a vital role in embryonic development, tumor formation and fibrosis in some chronic inflammatory diseases [3,4]. Peritoneal mesothelial cell EMT is one of the key factors responsible for gradual decreases in peritoneal functions in peritoneal dialysis patients [3,4].

TGF-β1 is an important fibrosis-promoting factor in several tissues and organs, including peritoneal tissue; its role is to phosphorylate the signaling protein smad2 /3 [17]. Smad protein is a downstream signal regulatory protein of the TGF-β1 receptor complex, and it transfers TGF-β signal from the cell membrane to the nucleus [17,18]. Many of the biological function of TGF-β are mediated by smads signaling pathway, and smad2 / 3 falls into the type of receptor-modulating smads, the phosphorylation of which acts as the most important step and sign of smad pathway activation [17,18]. Therefore, the expression level of intracellular p-smad2 /3 is an index of the degree of activation of the pathway.

Excessive expression of smad7 inhibits the binding of smad2 and smad3 molecules to activated type I receptors as well as their phosphorylation, thereby exerting a negative regulating effect on TGF-β signal transduction [19]. Down-regulation of the expression of smad7 protein is associated with fibrosis, while the up-regulation of its expression reverses the occurrence and progression of fibrosis [17,19]. This is consistent with the results obtained in this study. PDF treatment significantly up-regulated the expression of TGF-β and smad2, and down-regulated the expression of smad7, but this trend was reversed by T-IIA. This finding indicates that T-IIA inhibits fibrosis in HPMCs by regulation of the TGF-β/smad pathway.

TGF-β1 is also a key regulator of EMT [8]. It can induce transition in HPMCs *in vitro* and *in vivo* [20,21]. The transition of epithelial cells to mesenchymal cells involves loosening of epithelial cells, loss of microvilli, decreased expressions of epithelial cell markers like E-cadherin and Z0-1, and increased expression of fibroblast markers (e.g. a-SMA). It also involves cytoskeleton re-modelling, intercellular matrix degradation and changes in adhesion between the cells and matrix, resulting in enhanced ability of cell migration and invasion [5].

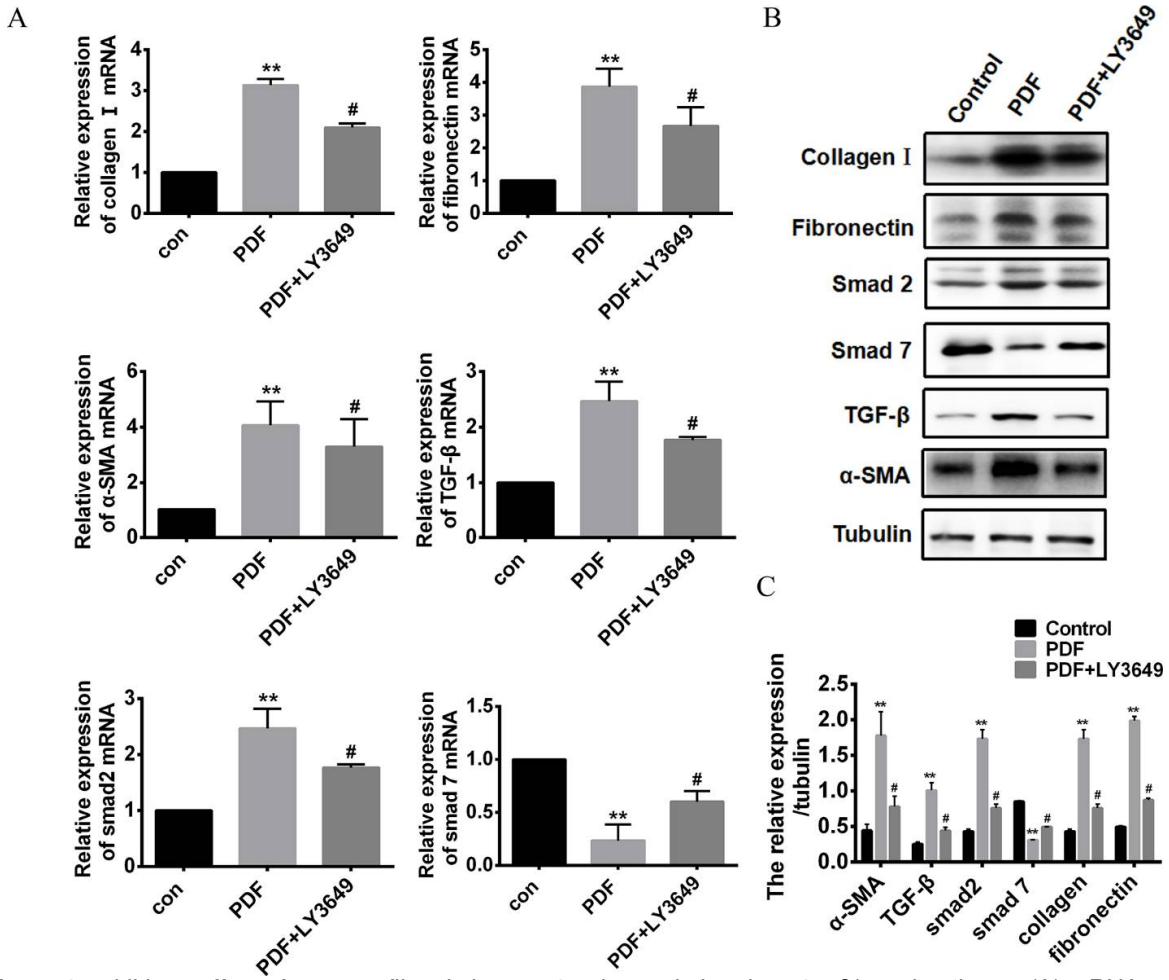

Figure 3: Inhibitory effect of T-IIA on fibrosis in HPMCs via regulating the TGF-β/smad pathway. (A) mRNA and (B) protein expression levels of fibrogenic cytokines and TGF-β/smad pathway-related proteins in HPMCs in different treatment groups determined by RT-PCR and Western blot. (C) Quantitative analysis of the developed protein bands. **p* < 0.05, ***p* < 0.01, versus the control group; #*p* < 0.05, ##*p* < 0.01, versus PDF group

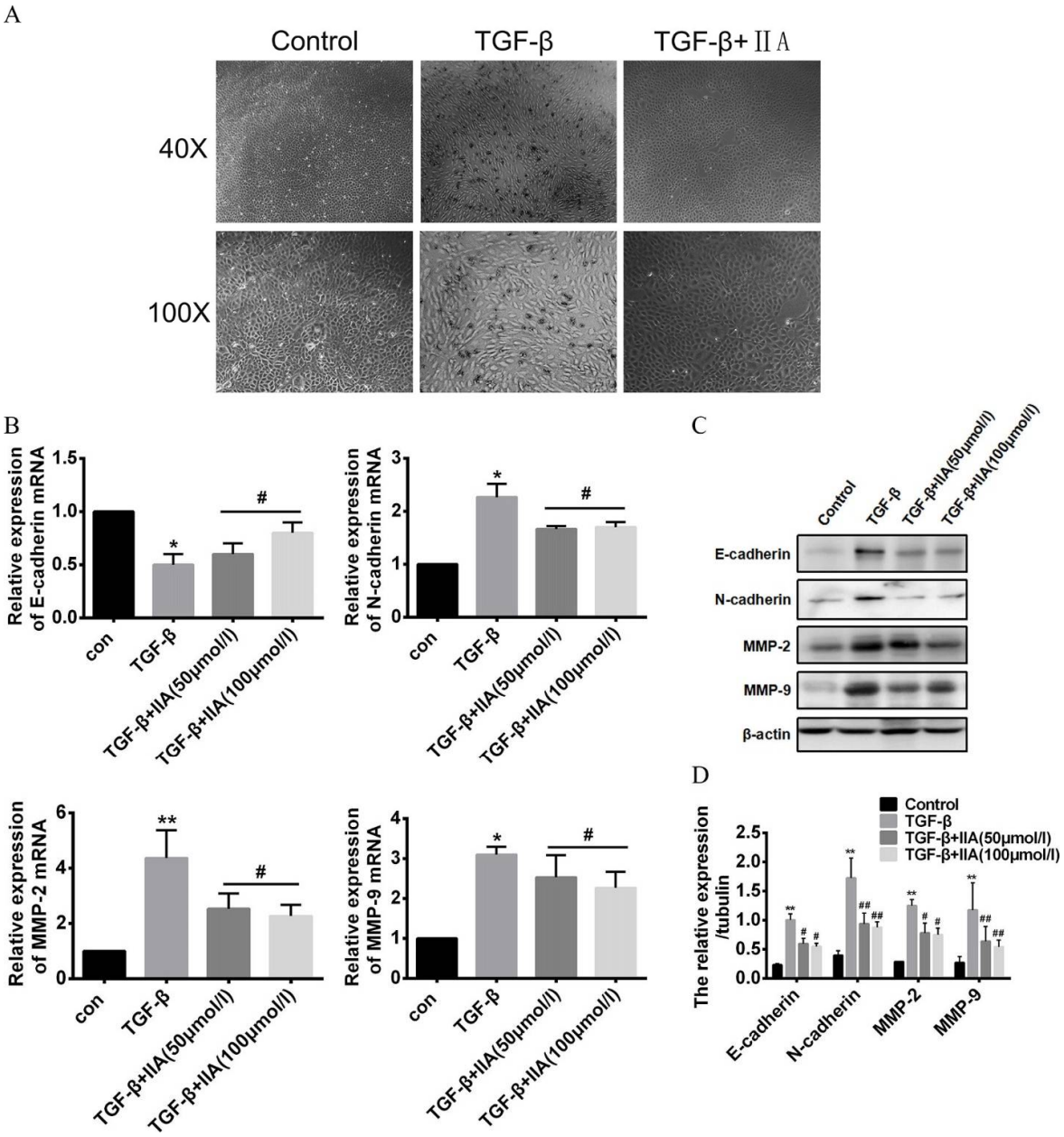

Figure 4: Inhibitory effect of T-IIA on EMT in HPMCs. (A) Morphology of HPMCs under different treatments. (B) mRNA and (C) protein expression levels of EMT-related proteins (E-cadherin, N-cadherin, MMP-2, and MMP-9) in HPMCs in different treatment groups determined by RT-PCR and Western blot. (D) Quantitative analysis of the developed protein bands; *p < 0.05, **p < 0.01, versus the control group; #p < 0.05, ##p < 0.01, versus TGF-β group.

Yanez-Mo *et al* [22] discovered that peritoneal mesothelial cells exhibited EMT shortly after PD, which manifested in loss of epithelial cell morphology, down-regulated expression of cytokeratin and E-cadherin, and up-regulated expression of a2 integrin. They also found that peritoneal mesothelial cells cultured *in vitro* in PD effluent showed many morphological changes, including epithelial cell-likeness, fibroblast-likeness and a mixture of both features.

The intercellular adhesion molecule-1 (ICAM-1) was highly expressed in these different forms of cells, suggesting that the fibroblast-like cells were derived from peritoneal mesothelial cells. A study of 35 patients treated with standard PD for 2 years, found that 74 % of them experienced mesothelial cell layer loss, 46 % experienced peritoneal fibrosis, and 17 % had evidence of *in situ* EMT [23]. Studies have shown that TGF-β1 mediates EMT through Smad pathway [17,19]. The target gene regulating TGF-β1 in EMT is dependent on the transcriptional regulation of smad2 and smad3, while smad7 interdicts its combination with TGF-β type I receptor by

inhibiting the phosphorylation of activated Smad protein [17,19].

Overexpression of TGF-β1 in rodents can cause EMT of peritoneal mesothelial cells [24]. This has been demonstrated by using adenovirus to introduce TGF-β1 into the peritoneum so as to overexpress TGF-β1, thereby inducing EMT of HPMCs and peritoneal fibrosis [25]. Bone morphogenetic protein reverses HPMC transition by inhibiting smad2/3 and MAPKs resulting from hyperglycemia-activated TGF-β1. In the present study, it was discovered that PDF significantly enhanced the expressions of mRNAs for TGF-β1 and smad2, and suppressed the mRNA expression of smad7. However, the addition of T-II A led to significant down-regulation of the expression of mRNAs for TGF-β1 and smad2, and significant up-regulation of the expression of smad7 mRNA. This clearly suggests that T-II A inhibits the TGF-β1 signal transduction pathway; and by implication, it also inhibits EMT. The results of this study indicate that TGF-β1/smad3 signaling transduction pathway plays an important role in EMT during peritoneal fibrosis, and that T-II A has promising potential as an effective drug for the prevention of tissue fibrosis.

CONCLUSION

T-IIA treatment inhibits EMT of HPMCs via regulation of TGF-β/smad pathway, thereby preventing PD-induced peritoneal fibrosis. Thus T-IIA offers tremendous potential for the development of a new drug for the treatment of PD-linked peritoneal fibrosis.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors

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