

## Original Research Article

# Tanshinone IIA suppresses fibrosis induced by high glucose conditions in HK-2 cells via inhibition of extracellular matrix deposition, reduction of oxidative stress, and inhibition of epithelial to mesenchymal transition

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### Abstract

**Purpose:** To investigate the anti-fibrotic effects of tanshinone IIA (TS) on renal tubular epithelial cells (HK-2 cells) under high glucose conditions and their related molecular mechanism(s) of action.

**Methods:** After treatment with TS (6 µg/mL) for 24 h, the morphology of HK-2 cells stimulated by high glucose was observed under the microscope. Additionally, potential mechanisms related to the anti-fibrosis effects of TS were evaluated using western blotting assay and quantitative real time PCR (qRT-PCR), including transforming growth factor (TGF) β1, α-smooth muscle actin (α-SMA), heme oxygenase 1 (HO-1), laminin (LN), fibronectin (FN), and E-cadherin (E-cad).

**Results:** A high-glucose culture environment induced fibrosis of HK-2 cells, as indicated by changes in cell morphology. The anti-fibrotic effects of TS were mainly associated with a decrease in the expression levels of TGF-β1, α-SMA and LN, while the expression of E-cad increased. These results also revealed that TS increased the expressions of HO-1.

**Conclusion:** The findings suggest that TS suppresses fibrosis caused by high glucose in HK-2 cells by inhibiting extracellular matrix deposition and epithelial-mesenchymal transition and by reducing oxidative stress. Further investigations are needed to evaluate the clinical application of this compound in diabetic nephropathy.

**Keywords:** Tanshinone IIA, Diabetic nephropathy, HK-2 cells, Fibrosis

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## INTRODUCTION

Diabetic nephropathy (DN) is a chronic microvascular complication of diabetes mellitus (DM). Approximately one-third of diabetic

patients eventually develop DN, however, the related pathogenesis has not yet been clarified. DN lesions commonly include damage to the glomerulus, renal blood vessels, and renal tubules. Early glomerular is glomerulosclerosis

includes glomerular hypertrophy, basement membrane thickening, and extracellular matrix (ECM) accumulation [1]. It has been reported that epithelial-mesenchymal transition (EMT) is a key factor in transforming renal tubular epithelial (HK-2) cells into fibroblasts [2].  $\alpha$ -Smooth muscle actin ( $\alpha$ -SMA) which is a significant bio-marker of interstitial cells would be significantly up-regulated, and it is commonly regulated by the signaling pathways of transforming growth factor (TGF)  $\beta$ /Smad and wnt/ $\beta$ -catenin [3]. Therefore,  $\alpha$ -SMA and TGF- $\beta$ 1 have beneficial effects on understanding the progress of EMT. Furthermore, oxidative stress can also be considered as a central link in the pathogenesis of DN, and heme oxygenase-1(HO-1) is a sensitive indicator of oxidative stress.

Accumulating scientific reports have revealed lots of extracts or compounds from natural herbal medicines that have medicinal properties for the prevention or treatment of diabetes mellitus and its complications [4,5]. Tanshinone IIA (TS), a medicinal component of *Radix Salviae Miltiorrhizae*, has a protective effect on the heart [6], is mainly used for the treatment of cardiovascular and cerebrovascular diseases. Currently, TS has been found to act as an antioxidant, reducing the injury of ischemia and reperfusion [7,8]. Interestingly, it has also been reported that TS show protective effects on diabetic nephropathy [5]; however, the molecular mechanisms involved are still unclear. Thus, in this study, we aimed to explore the anti-fibrotic effects of TS on HK-2 cells (under high glucose conditions) and the molecular mechanisms involved, providing evidence for further research regarding TS in DN treatments.

## EXPERIMENTAL

### Chemicals and reagents

TS was obtained from Zrbiorise (Shanghai, China); MEM- $\alpha$  was from Gibco (Grand Island, USA); FBS was from HyClone (California, USA); MTT and BCA kits were from Beyotime (Shanghai, China); glucose was from Sailboat Co. Ltd. (Tianjin, China); the TIANScriptII RT Kit and SYBR Green were from Tiangen Biotech Co. Ltd. (Beijing, China); antibodies against TGF $\beta$ 1 (ab119558),  $\alpha$ -SMA (ab32575), HO-1 (ab204524), E-cad (ab197751), FN (ab32419) and LN (ab11575) were from Abcam Biotechnology (Cambridge, UK).

### Cell culture

The 5 - 10<sup>th</sup> generation cells of HK-2 cells (Pinosin Biotech Co. Ltd, Wuhan, China) were

cultured in MEM- $\alpha$  added with 10 % FBS, penicillin and streptomycin (37 °C, 5 % CO<sub>2</sub> and 95 % air).

### Cytotoxicity assays of TS

Digested cell suspensions were put into 96-well plates, and edge holes were filled with sterile PBS. TS solution at different concentrations (1, 3, 5, 8, 10, 20, and 40  $\mu$ g/mL) was then added to the 96-well cell culture plates, after which the MTT solution was added. Finally, the absorbance of each well was determined at OD 490 nm to calculate cell viability. The IC<sub>50</sub> of TS at 6  $\mu$ g/mL was selected because of its low cytotoxicity on HK-2 cells.

### Fibrosis in HK-2 cells caused by high glucose

Non-fibrotic HK-2 cells without glucose but treated with the different concentrations of TS listed above were considered mock cells, while cells exposed to glucose alone were used as control cells. HK-2 cells treated with both glucose and 6  $\mu$ g/mL of TS were considered TS-treated cells. After rinsing the cells, a solution of 30 mmol/L glucose was added to the culture plate and cultured for 24 h as control groups. After treating with TS (6  $\mu$ g/mL), cells were observed under a microscope.

### qRT-PCR assay

Total RNA was extracted with the TRIzol reagent. Reverse transcription into cDNA was performed using the TIANScriptII RT Kit. RT-qPCR was conducted using the Applied Biosystems (ABI) real-time PCR system (CA, USA) to determine the mRNA expression of TGF- $\beta$ 1, HO-1,  $\alpha$ -SMA, FN, LN, and E-cad in HK-2 cells. The procedure was performed at 95 °C for 10 s, at 58 °C for 30 s, and at 72 °C for 30 s (40 cycles).

### Western blotting assay

Cell proteins were collected, and the protein contents were determined using the BCA kits. Based on protein quantification, proteins were added to the protein gel electrophoresis sample buffer, mixed gently, denatured at 95 °C for 10 min, and immediately inserted into ice. The proteins were then transferred onto PVDF membranes (Millipore, USA) by electroblotting. The PVDF membranes were blocked with a blocking solution, after which they were cultured overnight at 4°C with antibodies against TGF $\beta$ 1 (dilution 1:1000),  $\alpha$ -SMA (dilution 1:1000), HO-1 (dilution 1:1200), E-cad (dilution 1:1500), FN (dilution 1:1000), and LN (dilution 1:800).

Subsequently, the proteins were incubated with a secondary antibody at a dilution of 1:3000. Grayscale values were obtained using Image J software and the levels of relative proteins were evaluated by comparing the gray value of the given proteins with the levels of  $\beta$ -actin.

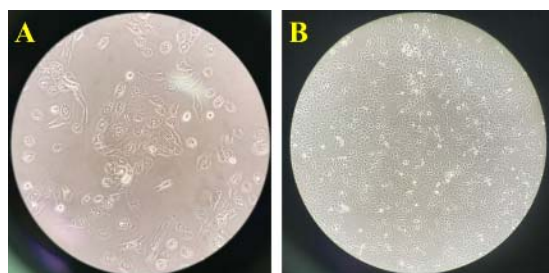
### Statistical analysis

Data were expressed as mean  $\pm$  SD. The statistical differences were carried out by the ANOVA followed by LSD using SPSS 17.0 software for windows.  $P < 0.05$  was considered as statistically significant.

## RESULTS

### Fibrosis is induced by high glucose environment in HK-2 cells

Regarding the morphology of HK-2 cells, we observed round or polygonal cells that showed a "paving stone" (Figure 1 A). In HK-2 cells stimulated with high glucose levels, we observed that renal tubular epithelial cells lost their intrinsic morphology, resembling a long spindle-like fibroblast, and that the nucleus also showed a spindle-like shape (Figure 1 B).



**Figure 1:** Morphology of (A) Mock HK-2 cells and (B) HK-2 cells stimulated with glucose

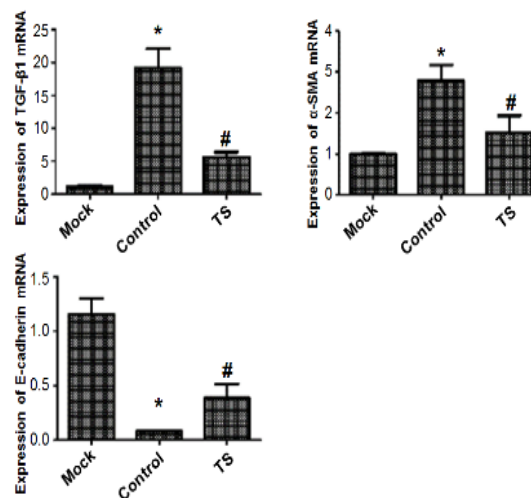
### Effects of TS on TGF- $\beta$ 1 and phenotypic transformation

Compared to control cells, TS-treated cells showed reduced levels of TGF- $\beta$ 1,  $\alpha$ -SMA. Similarly, the western blotting results (Figure 3) showed that, in TS-treated cells, the expression levels of TGF- $\beta$ 1 and  $\alpha$ -SMA reduced, but levels of E-cad increased ( $p < 0.05$ ).

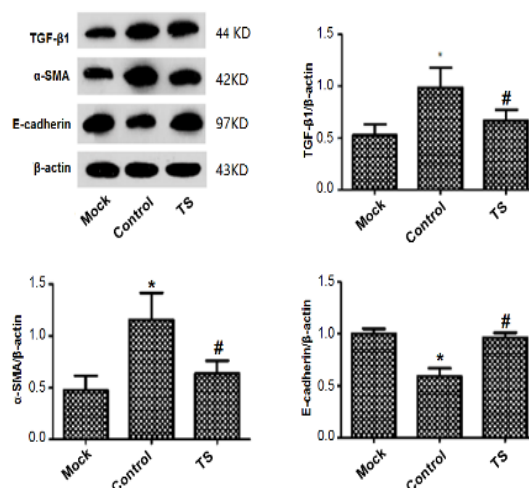
### Effect of TS on ECM

Furthermore, the RT-qPCR assay was used to evaluate the effect of TS on the expressions of FN and LN mRNA. As can be seen from Figure 4, the expression levels of FN and LN mRNA in TS-treated cells significantly decreased ( $p < 0.05$ ). Similarly, the western blotting showed that

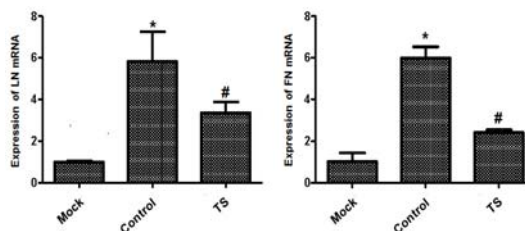
the expression of LN in TS-treated cells also decreased ( $p < 0.05$ ; Figure 5).



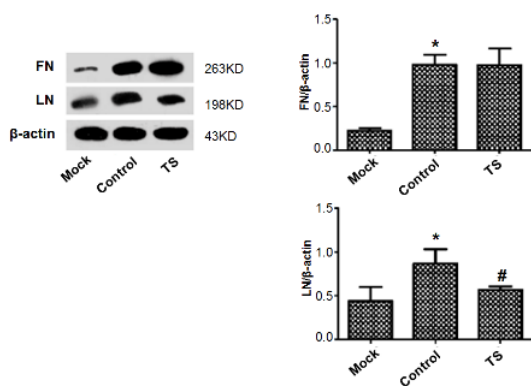
**Figure 2:** The mRNA expression of TGF- $\beta$ 1,  $\alpha$ -SMA and E-cad in HK-2 cells. \* $P < 0.05$  vs. mock group; # $p < 0.05$  vs. TS group



**Figure 3:** The protein expression of TGF- $\beta$ 1,  $\alpha$ -SMA and E-cad in HK-2 cells. \* $P < 0.05$  vs. mock group; # $p < 0.05$  vs. TS group



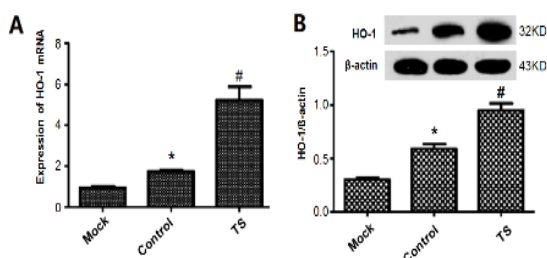
**Figure 4:** The mRNA expression of LN and FN in HK-2 cells. \* $P < 0.05$  vs. mock group; # $p < 0.05$  vs. TS group



**Figure 5:** Protein expression of LN and FN in HK-2 cells. \* $P < 0.05$  vs. mock group; # $p < 0.05$  vs. TS group

**Effects of TS on HO-1 expression**

These results exhibited that the expression level of HO-1 increased after treatment with high glucose levels, but showed even higher levels in TS-treated cells ( $p < 0.05$ ; Figure 6). Furthermore, the western blotting results showed the same results ( $p < 0.05$ ).



**Figure 6:** The mRNA (A) and protein (B) expression of HO-1 in HK-2 cells. \* $P < 0.05$  vs. mock group; # $p < 0.05$  vs. TS group

**DISCUSSION**

In the progression of DN to ESRD, symptoms such as the decrease in glomerular filtration rate (GFR) and fibrosis of glomeruli, tubules, and interstitium are particularly important [9,10]; hence, reducing or blocking the development of fibrosis is essential in the treatment of DN.

Transforming growth factor-β1 is essential in renal fibrosis as it is a key cytokine in glomerulosclerosis [11]. TGF-β1 is also a key growth factor in the process of fibrosis, and the accumulation of ECM, where the expression level of TGF-β1 in glomerular mesangial cells was enhanced under high glucose conditions [12,13]. The necessity of Smad3 for TGF-β1-induced EMT has been confirmed [14]. Under stress conditions, renal tubular epithelial cells undergo transdifferentiation of cell phenotypes,

transdifferentiating from epithelial cells to myofibroblasts in order to adapt to changes in their microenvironment. α-SMA is a marker of this phenotypic transdifferentiation [15].

There are two Smad3 reaction regions in the α-SMA gene promoter. The expression of α-SMA protein converts the cells into myofibroblasts and synthesizes large amounts of ECM components [16], such as fibronectin (FN) and laminin (LN) [17]. Moreover, TGFβ1 receptors formed by TGFβ1 can activate smad2/3, eventually causing a decrease in E-cadherin and an increase in α-SMA [18]. Epithelial cadherin (E-cadherin) reduces renal damage and transdifferentiates into interstitial fibroblasts. The adhesion function between cells is impaired and EMT occurs, causing RIF [19].

Previous studies have revealed that antioxidant treatment has become essential for the treatment of DM and its complications, with many natural agents offering promising effects on DM and its complications based on their antioxidative properties [20,21]. Nath *et al* [22] demonstrated that HO-1 can promote the production of CO and bilirubin after catalyzing hemoglobin, which has a protective effect on renal ischemia-reperfusion injury.

Kim *et al* [23] found that α7 nicotinic acetylcholine receptors in proximal tubular cells induced the expression of HO-1, protecting cells from renal injury. Pan *et al* [24] also found that HO-1 expression is important in protecting kidneys in preventing cisplatin-induced nephrotoxicity in mice. Our results showed that after administering high glucose levels for 2 h, TGFβ1 and α-SMA increased and E-cadherin decreased, indicating that EMT occurred. After kidney injury, related pathways such as TGFβ1/Smads are activated, α-SMA is expressed, and myofibroblasts are produced, resulting in a large amount of ECM. In addition, the expression of HO-1 increased after intervention with high glucose.

The results of the TS-treated groups showed that after treatment, TGFβ1, α-SMA, and LN expression decreased, while E-cad expression increased, indicating that the alterations in EMT caused by high glucose levels improved: the expression of α-SMA was inhibited, the formation of mesenchymal cells was prevented, and EMT was inhibited. The decrease in LN indicates a decrease in ECM. The expression of HO-1 increased and the number of HO-1 in TS-treated cells increased in comparison to control cells. However, the expression of FN did not decrease

significantly after intervention with TS, thus further experiments are needed.

## CONCLUSION

These results indicate that Tanshinone IIA inhibits the expression of TGF $\beta$ 1 and  $\alpha$ -SMA, thus affecting the occurrence of EMT, enhances the expression of HO-1, and delays DN progression. This lends some support for the use of Tanshinone IIA as a potential treatment for delaying DN progression.

## DECLARATIONS

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### 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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