Effects of Dapagliflozin on Renal Interstitial Fibrosis in Diabetic Rats through Smad3, TIMP1 and MMP24 Pathway

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Objective: This research was designed to probe into the effects of Dapagliflozin on renal interstitial fibrosis in diabetic rats through Smad3, TIMP1 and MMP24 pathway. Methods: Rats were bought to establish models, and then intervened by Dapagliflozin. Human mesangial cell lines (HMCs) stimulated by high glucose were purchased, and the Smad3, TIMP1 and MMP24 levels in rats after modeling and Dapagliflozin intervention were detected. The Smad3, TIMP1 and MMP24 protein expression in kidney tissue was examined after the rats were killed, and the expression in an intervention group (IG) and a blank group (BG) were analyzed. The cells were divided into three groups: Dapagliflozin intervention (Group 1), TGF-β1/Smad3 pathway inhibitor SIS3 intervention (Group 2) and no intervention (Group 3). The TIMP1 and MMP24 levels were assessed. Results: The Smad3 and MMP24 levels in group A were higher than those in other two groups ($p < 0.05$), while those of TIMP1 were lower ($p < 0.05$). Compared with pre-intervention, the Smad3 and MMP24 levels in groups A and B decreased ($p < 0.05$), while those of TIMP1 increased ($p < 0.05$). The Smad3 and MMP24 protein levels in groups A and B were higher than those in other two groups ($p < 0.05$), while those of TIMP1 was lower ($p < 0.05$). Compared with the BG, the Smad3 and MMP24 expression in the IG was lower ($p < 0.05$) and that of TIMP1 was higher ($p < 0.05$). The TIMP1 expression in Group 3 was lower ($p < 0.05$) and that of MMP24 was higher than those in Groups 1 and 2 ($p < 0.05$). Conclusion: Dapagliflozin can treat diabetic renal interstitial fibrosis by inhibiting TGF-β1/Smad3 signaling pathway, decreasing MMP24 and increasing TIMP1.

Keywords: Dapagliflozin, Smad3, TIMP1, MMP24, renal interstitial fibrosis in diabetic rats

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Diabetic nephropathy is one of the most important diabetic microvascular complications [1], which is a crucial reason for chronic and end-stage kidney diseases [2]. At the moment, the exact pathogenesis is unknown. It is generally believed that diabetic patients are in a state of hyperglycemia for a long time, which leads to microangiopathy, thus bringing about increased renal vascular pressure and diabetic nephropathy [3]. In the late stage, due to the serious change of glomerulus, some glomerulosclerosis eventually developed into renal interstitial fibrosis [4], which became a serious pathological feature threatening the life and health of diabetic nephropathy patients [5]. Dapagliflozin, an inhibitor of sodium-glucose cotransporter 2, has been one of the commonly used drugs for treating type 2 diabetes clinically [6]. Moreover, it also has a marked inhibitory effect on the development of diabetic renal interstitial fibrosis [7]. A number of research reports have confirmed that Dapagliflozin has obvious improvement effect on fibrosis [8], but its specific mechanism is still vague.

According to many related references, Smad3, as the first signal molecule of TGF-β1 signal transmission, is the most important way of TGF-β1 signal transmission [9]. TGF-β1, as a key factor exerting biological effects, has been proved to be involved in the pathological changes of diabetes and the occurrence of renal interstitial fibrosis [10]. Tissue inhibitor of metalloproteinases 1 (TIMP1) is a familiar glycoprotein in tissues, which can determine the normal operation of matrix metalloproteinases (MMPs) and maintain

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the stable state of extracellular matrix together [11]. In previous studies, we found that Dapagliflozin could conduct through TGF-β 1 signaling pathway, and regulate the changes of TIMP1 and MMP24, thereby affecting kidney disease and insulin resistance [12]. Thus, we suspect that Dapagliflozin may affect diabetic renal interstitial fibrosis through Smad3, TIMP1 and MMP24. In order to confirm our conjecture, this experiment will establish a diabetic renal interstitial fibrosis rat model, analyze the conditions of Smad3, TIMP1 and MMP24 in rats under the intervention of Dapagliflozin, and provide a reliable theoretical basis for future clinical use.

MATERIALS AND METHODS

Data Of Rats

Sixty clean grade 12-week-old male SD rats, weighing 200-230 g, were bought from Beijing Vital River Laboratory Animal Technology Co., Ltd., and the certificate number was SCXK (Beijing) 2016-0011. The experiment was carried out after one week of normal feeding. Rats were divided into three groups: A, B and C, with 20 rats in each group. Diabetic renal interstitial fibrosis rats (Group A), diabetic rats (Group B) and normal rats (Group C) were established.

Rat Modeling

Group A rats were fed with high carbon water, fat and protein, and injected with streptozotocin (60 mg/kg) intraperitoneally. The injection dose was adjusted according to the symptoms of rats, and the model was established for 12 weeks. One week after modeling, 24-hour urine volume and protein were detected, and the model was successful when it was diagnosed as diabetic renal interstitial fibrosis. Those in group B were only fed with high fat, and blood glucose was measured one week later. FBG > 15.0 mmol/L was regarded as a successful model, and the model was established for 12 weeks. Rats in normal group and Group C were injected with the same dose of citric acid buffer.

Dapagliflozin Intervention

After successful modeling, rats in the three groups were given Dapagliflozin. Totally 10 mg/(kg·d) was dissolved in normal saline (1 mg/mL) once a day. After the test, the rats were were anaesthetized and decapitated, and the kidney tissues were collected for subsequent test.

Cell Data And Culture

High glucose stimulated human mesangial cell lines (HMCs) were acquired from ATCC cell bank. The cells were cultivated in the medium with Dapagliflozin (intervention group, IG) and that without Dapagliflozin (blank group, BG). When the cells grew to 80%, they were passaged for subsequent detection.

Outcome Measures

The Smad3, TIMP1 and MMP24 levels in rats of Groups A, B and C and those after Dapagliflozin intervention were detected (according to ELISA kit instructions). The kidney tissues were collected after the rats were killed, and the levels of Smad3, TIMP1 and MMP24 protein in the tissues and those in the IG and the BG were tested (detected by Western blot (WB)). Rescue experiment: The cells were divided into three groups: Dapagliflozin intervention (Group 1), TGF-β 1/Smad3 pathway inhibitor SIS3 intervention (Group 2) and no intervention (Group 3). The TIMP1 and MMP24 levels were tested by WB.

STATISTICAL METHODS

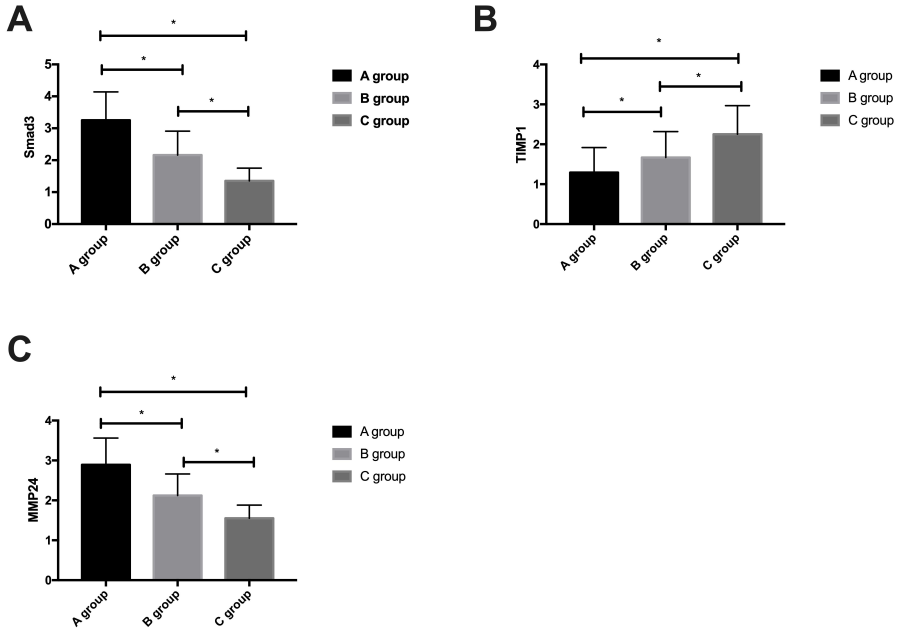
The data were processed and analyzed by SPSS22.0. The calculated average value of experimental results was recorded in the form of (mean±standard deviation). The inter-group comparison was analyzed via independent-samples T test, the multi-group comparison was assessed by one-way analysis of variance (ANOVA) and LSD back testing, and multiple time points was compared by repeated measures ANOVA and bonferroni back testing. There is statistical difference when $P < 0.05$.

RESULTS

Expression Of Smad3, Timp1 And Mmp24 In Three Groups Of Rats

The Smad3, TIMP1 and MMP24 levels in diabetic rats with renal interstitial fibrosis (Group A), diabetic rats (Group B) and normal rats (Group C) were tested by ELISA. It manifested that the Smad3 and MMP24 levels in Group A were higher than those in Group B ($p < 0.05$), the levels in Group B were higher than those in Group C ($p < 0.05$), while the TIMP1 levels in Group A were lower than those in Group B ($p < 0.05$), and those in group B were lower than those in Group C ($p < 0.05$) (Figure 1).

Fig. 1 Expression of Smad3, TIMP1 and MMP24 in three groups of rats

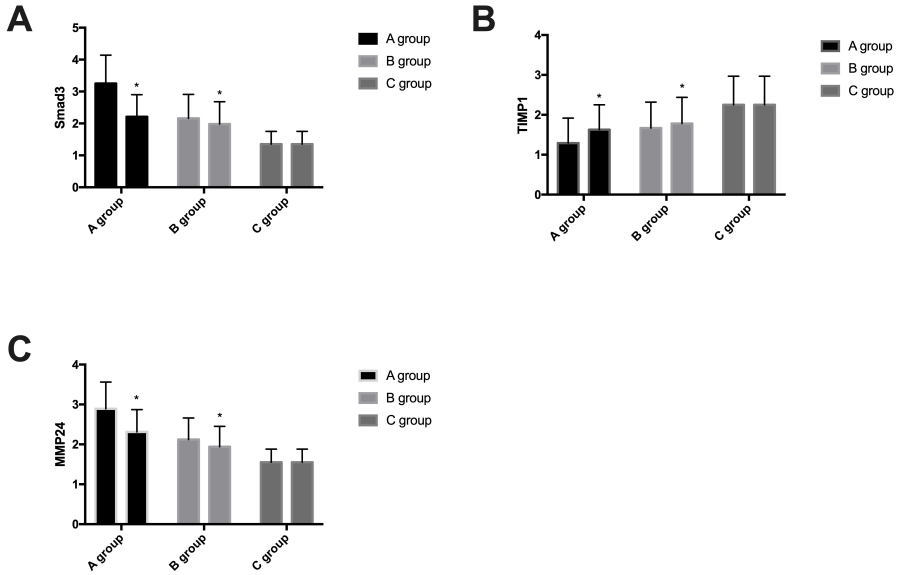


Smad3 levels in three groups of rats;
TIMP1 levels in three groups of rats;
MMP24 levels in three groups of rats.
* means $p < 0.05$.

Expression Of Smad3, Timp1 And Mmp24 In Three Groups Of Rats After Dapagliflozin Intervention

The Smad3, TIMP1 and MMP24 levels in diabetic rats with renal interstitial fibrosis (Group A), diabetic rats (Group B) and normal rats (Group C) were tested. It showed that the the Smad3 and MMP24 levels in Groups A and B decreased compared with those before intervention ($p < 0.05$), while those of TIMP1 increased ($p < 0.05$) (Figure 2).

Fig. 2 Expression of Smad3, TIMP1 and MMP24 in three groups of rats after Dapagliflozin intervention

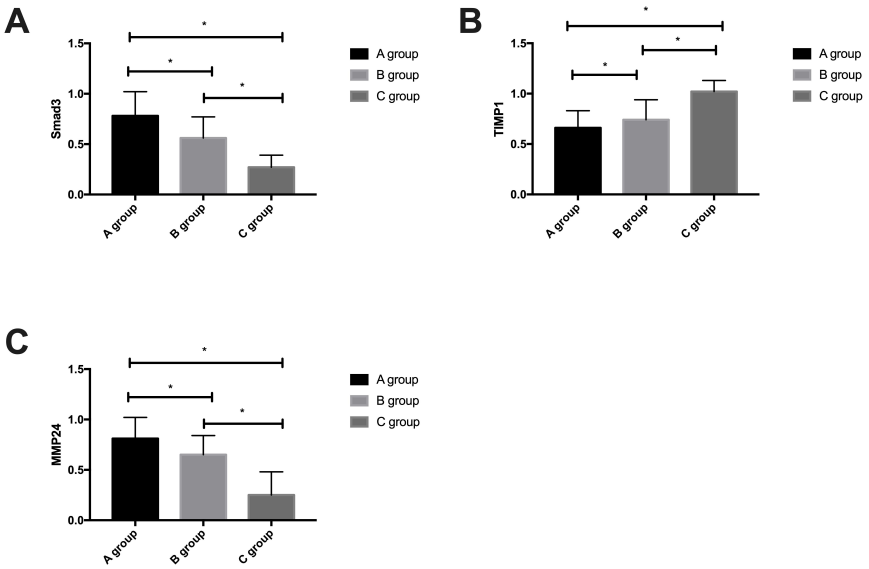


Smad3 levels in three groups of rats after Dapagliflozin intervention;
TIMP1 levels in three groups of rats after Dapagliflozin intervention;
MMP24 levels of rats in three groups after Dapagliflozin intervention.
* means $p < 0.05$.

Expression Of Smad3, Timp1 And Mmp24 Protein In Tissues

The Smad3, TIMP1 and MMP24 protein levels in kidney tissue of rats were detected by WB. It revealed that the Smad3 and MMP24 protein levels in Groups A and B were higher than those in group B ($p < 0.05$), and those in Group B was higher than that in Group C ($p < 0.05$), while the TIMP1 levels in Group A were lower than that in Group B ($p < 0.05$), and those in Group B were lower than those in Group C ($p < 0.05$) (Figure 3).

Fig. 3 Expression of Smad3, TIMP1 and MMP24 protein in tissues

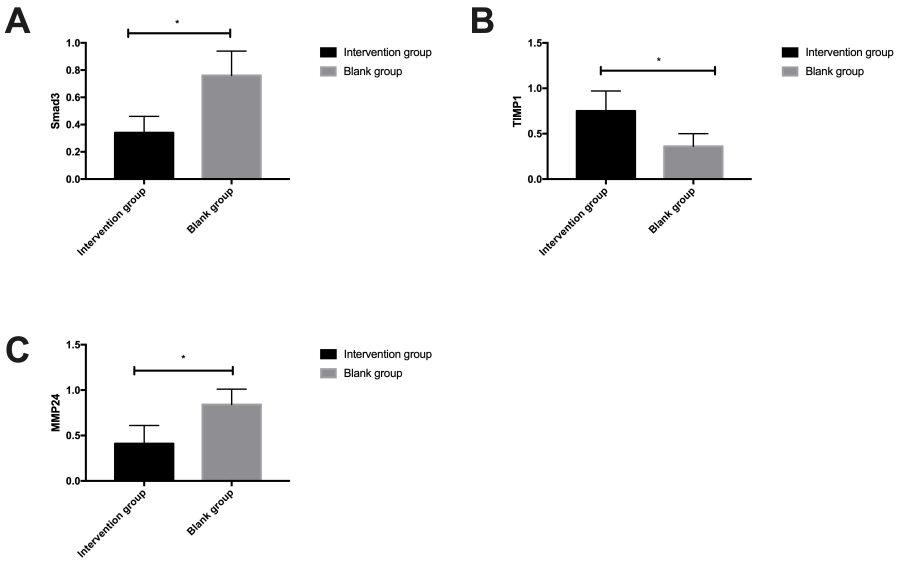


Smad3 protein expression in rat kidney tissue; TIMP1 protein expression in rat kidney tissue; MMP24 protein expression in rat kidney tissue. * means $p < 0.05$.

Expression Of Smad3, Timp1 And Mmp24 In Ig And Bg

The Smad3, TIMP1 and MMP24 levels in the cells of the medium containing Dapagliflozin (IG) and that without Dapagliflozin (BG) were tested by WB. It showed that the expression of Smad3 and MMP24 in the IG was lower than that in the BG ($p < 0.05$), while that of TIMP1 in the IG was higher than that in the BG ($p < 0.05$) (Figure 4).

Fig. 4 Expression of Smad3, TIMP1 and MMP24 in IG and BG

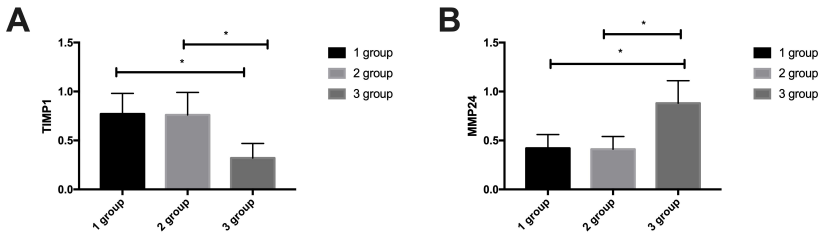


Smad3 expression in IG and BG;
TIMP1 expression in IG and BG;
MMP24 expression in IG and BG.
* denotes $p < 0.05$.

Effects Of Tgf- B 1/Smad 3 Pathway Inhibitor

The cells were divided into three groups: Group 1 was intervened by Dapagliflozin, Group 2 was intervened by SIS3, an inhibitor of TGF- β 1/Smad3 pathway, and Group 3 did not intervened. The TIMP1 and MMP24 expression was tested by WB. It manifested that there was no difference in the expression of TIMP1 and MMP24 between Groups 1 and 2 ($p > 0.05$), but that in Group 3 was the lowest ($p < 0.05$), and that of MMP24 was higher than the other two groups ($p < 0.05$) (Figure 5).

Fig. 5 Rescue experiment



TIMP1 expression in three groups;
MMP24 expression in three groups.
* means $p < 0.05$.

DISCUSSION

Diabetic nephropathy affects about 40% of patients with types 1 and 2 diabetes. It's mainly caused by cardiovascular causes, resulting in a higher risk of death [13]. As one of the high-risk manifestations of diabetic nephropathy, renal interstitial fibrosis is a precursor to the development of nephritis, renal failure and even renal cancer [14]. Hence, the treatment of diabetic renal fibrosis is the key to prevent and treat malignant diseases [15]. Dapagliflozin, as one of the effective drugs for treating diabetes, also has a certain improvement effect on renal fibrosis [16]. But the specific drug mechanism is still vague. The relationship between Dapagliflozin and Smad3, TIMP1, MMP24 was analyzed. This research may reveal the preliminary pharmacological mechanism of Dapagliflozin, and provide a reliable theoretical basis for future clinical use.

Firstly, we established animal models of diabetic and renal fibrosis rats, and detected the expression of Smad3, TIMP1 and MMP24. We found that Smad3 and MMP24 increased while TIMP1 decreased in diabetes. However, in rats with renal fibrosis, the changes were more marked. While using WB to detect Smad3, TIMP1 and MMP24 in rat kidney tissue again, the results are consistent with the above situation, which can verify the accuracy of our experimental results. The expression differences of Smad3, TIMP1 and MMP24 suggest that Smad3, TIMP1 and MMP24 are involved in the development of diabetes and also play a vital role in the process of renal fibrosis. Smad3 is activated by TGF- β , activin I receptor and orphan receptor ALK-7, which can directly bind to DNA and inhibit the activation and proliferation of T cells [17]. It has been suggested that the occurrence of diabetic renal fibrosis can be prevented by inhibiting Smad3. The mechanism is that inhibiting Smad3 can decrease the transcription of lncRNA Erbb4-IR and increase the transcription of miR-29b, thus protecting the kidney of mice from progressive kidney injury [18]. This view can also support our experimental results from the side, and confirm that the increase of Smad3 reflects the aggravation of diabetic renal fibrosis. Looking at previous studies, we also discovered that Smad3 regulated cytoskeletal protein, which affected cell damage induced by high glucose [19]. It might be one of the mechanisms of Smad3 participating in the development of diabetic renal fibrosis. In the process of diabetes, Smad3 is activated in high sugar environment, which negatively regulates cell growth, aggravates cell stress and hypoxia reaction, and causes a series of inflammatory changes and injuries, thus causing the occurrence of renal fibrosis [20]. In related studies, it was also found that Smad increased in nephritis and was proved to be the mediator of fibrosis reaction [21, 22], which also preliminarily confirmed our conjecture. TIMP1, just as its name implies, mainly inhibits metalloproteinases. It has been proved to play an important role in liver fibrosis [23], which can reduce tissue cell fibrosis by inhibiting the proliferation of fibroblasts [24]. The main way of TIMP1 in the process of inhibiting fibrosis is through MMPs [25]. TIMP1 can form a complex with activated interstitial collagenase and stromelysin and MMPs, and reduce the degradation of MMPs on cell matrix [26]. The results of MMP24 detection in this research also accord with the above situation, which can testify

the role of TIMP1 and MMP24 in promoting the development of renal fibrosis.

Therefore, in order to understand the role of Dapagliflozin, we analyzed the expression changes of Smad3, TIMP1 and MMP24 in rats under the intervention of Dapagliflozin. Similarly, through the two detection methods of ELISA and WB, the results manifested that Smad3 and MMP24 were inhibited, while TIMP1 was increased under the intervention of Dapagliflozin. Combined with our above analysis, we can see that the treatment of diabetic renal fibrosis by Dapagliflozin can improve the development process of renal fibrosis by inhibiting Smad3 expression and reducing cell immune damage, and then the concentration of TIMP1 increases and inhibits the cell necrosis caused by MMP24. Dapagliflozin is an inhibitor of sodium-glucose cotransporter 2 (SGLT2), and previous studies have shown that TGF-β increases the SGLT2 expression through phosphorylated smad3 [27]. On the basis of this, we can speculate that Dapagliflozin can reduce the signal transduction of TGF-β to smad3 by inhibiting SGLT2, thus inhibiting the smad3 expression. Thus, in order to confirm our conjecture, we compared the expression of Smad3, TIMP1 and MMP24 between the intervention of Dapagliflozin and TGF-β1/Smad3 pathway inhibitor SIS3. It signified that there was no difference in Smad3, TIMP1 and MMP24 between them, which suggested that the intervention effect of Dapagliflozin was consistent with that of SIS3. It can be preliminarily confirmed that the intervention pathway of Dapagliflozin in the above conjecture is to inhibit the TGF-β1/Smad3 pathway.

In this research, we did not analyze and detect other members of Smad3, TIMP1 and MMP24 family. Because the relationship between classical representative genes such as MMP-3 and MMP-5 and renal fibrosis has been confirmed, and there is no novelty in scientific research after repeated verification. While others, such as Smad2, have little research on the relationship with renal fibrosis. So it is impossible to determine whether there may be a potential connection between them. Based on the research, we will make a supplementary analysis of our undetected gene proteins as soon as possible to obtain more perfect experimental results.

To sum up, Dapagliflozin can treat diabetic renal interstitial fibrosis by inhibiting TGF-β1/Smad3 signaling pathway, decreasing MMP24 and increasing TIMP1.

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