

Original Article

The effects of sodium tanshinone Ila sulfonate pretreatment on high glucose-induced expression of fractalkine and apoptosis in human umbilical vein endothelial cells

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Received November 2, 2014; Accepted November 13, 2014; Epub April 15, 2015; Published April 30, 2015

Abstract: The development of diabetes mellitus (DM) and its complications is a chronic inflammatory response process, chemokines and their receptors play an important role in this course of events. The aim of this study is to observe the effects of sodium tanshinone Ila sulfonate (STS) on high glucose-induced fractalkine (FKN) level, and investigate possible mechanisms of STS works. HUVECs cells were employed to explore the effects of STS on FKN protein. TUNEL assay was used to detect the apoptosis rate of HUVECs. Immunohistochemistry was utilized to detect the β -actin and P-GSK-3 β (Ser9) protein expression. Immunofluorescence was employed to detect FKN protein expression. Real-time RT-PCR was used to examine β -actin, GSK3 β and FKN mRNA expression. The results indicated that the STS treatment could significantly decrease the apoptosis rate caused by high-glucose ($P < 0.05$). STS improves β -catenin and p-GSK-3 β (Ser9) expression, and inhibits FKN levels induced by high glucose. STS inhibited GSK-3 β and FKN mRNA induced by high glucose. In conclusion, STS may play the role of anti-inflammatory by regulate canonical Wnt pathway to inhibit the expression of FKN induced by high glucose.

Keywords: Diabetes mellitus, fractalkine, sodium tanshinone Ila sulfonate, apoptosis

Introduction

The development of diabetes mellitus (DM) and its complications is a chronic inflammatory response process, chemokines and their receptors play an important role in this course of events [1, 2]. Fractalkine (FKN), as a newly discovered chemokines, which is classified to CX3C motif have a significant relationship with the incidence of diabetic nephropathy and coronary heart disease [3]. Moreover, Wnt signaling pathway play a considerable role in cells metabolism, especially in metabolism dysfunction caused by DM [4], the expression of endothelial cells Wnt1 and p-Glycogen synthase kinase (GSK)-3 β could be decreased induced by high glucose exposure [5]. Studies have found that high glucose could induce human umbilical vein endothelial cells (HUVECs) expressed FKN [6], our preliminary study confirmed and further found that high glucose-induced HUVEC expressed FKN and Wnt signal-

ing pathway may involved in these process [7]. In addition, sodium tanshinone Ila sulfonate (STS) have the ability of anti-inflammatory [8], but whether STS affected HUVECs to express FKN and Wnt signaling pathway involved in this process is still not been reported. In this research, we observed that the effects of STS pretreatment on high glucose-induced expression of FKN in HUVECs, and investigate the possible mechanisms of STS works.

Materials and methods

HUVECs culture and reagents

HUVECs were obtained from the cell bank of Xiangya School of Medicine, Central South University. Cells were cultured in Dulbecco's modified eagle medium (DMEM)/low glucose containing 10% fetal bovine serum (Zhejiang Tianhang Biological Technology Co., Ltd. China), 2 mmol/L L-glutamine, grown on tissue culture

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Table 1. Primers and annealing temperature

Gene	Primers	Fragment Length	Annealing temperature
β-actin	Sense primer: 5' CTG GGA CGA CAT GGA GAA AA 3'	564 bp	59.4 °C
	Antisense primer: 5' AAG GAA GGC TGG AAG AGT GC 3'		
GSK3B	Sense primer: 5' CCT CAA ATT AAG GCA CAT CC 3'	76 bp	56 °C
	Antisense primer: 5' CAC GGT CTC CAG TAT TAG CAT C 3'		
FKN	Sense primer: 5' CCG AAG GAG CAA TGG GTC AAGG 3'	376 bp	56 °C
	Antisense primer: 5' CAT CCT GAG CCT TTG GCG TCG 3'		

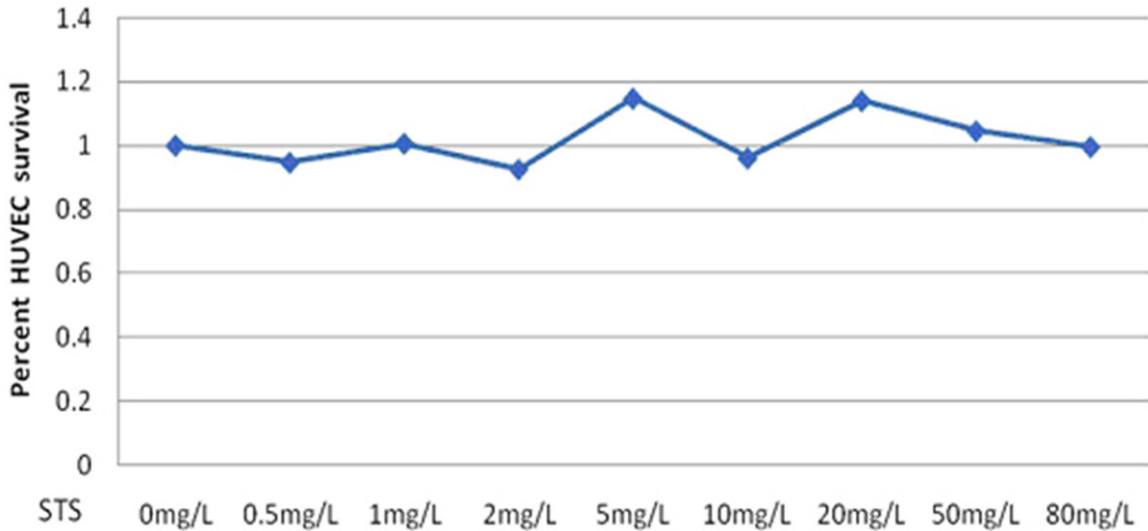


Figure 1. The effects of STS on viability of HUVECs at different concentrations by MTT.

dish at 37°C in 5% CO₂ with 100% humidity. Every 2 days, removing the non-adherent cells by washing with phosphate-buffered saline (PBS). Cells were passaged when at 80-90% confluence.

Group for experiment

The HUVECs in logarithmic growth phase were planted in good condition on the 24-well plate in a density of 1×10^4 per hole and 6-well plate in a density of 4×10^4 per hole and divided into five groups: control (C) group, high glucose (HG) group, STS group, HG + STS group and HG + STS + LiCl group. MTT confirmed that the most appropriate concentration to established high glucose model is 33.3 mmol/L glucose, and that the most appropriate concentration for pretreatment of LiCl and STS is 10 mmol/L and 5 mg/L respectively. The HUVECs in different group were cultured with different medium and pretreated by different Intervention separately, The HUVECs were cultured in DMEM with 5.5 mmol/L glucose (C group), in DMEM with 33.3 mmol/L glucose (HG group), in DMEM with 5.5

mmol/L glucose and 5mg/L STS (STS group), in DMEM with 33.3 mmol/L glucose and 5 mg/L STS (HG + STS group), in DMEM with 33.3 mmol/L glucose and 5 mg/L STS as well as 10 mmol/L LiCl (HG + STS + LiCl group). According to the groups above, after plant the HUVECs which cultured with DMEM (5.5 mmol/L glucose) for 10 h, added 5 mg/L STS and 10 mmol/L LiCl to pretreatment for 2 h and then after used 33.3 mmol/L glucose to pretreatment for 48 h, cells were 70%-80% confluent.

TUNEL to detect the apoptosis rate

Following the instructions from the manufacturer, Camera immediately after experiment. The nucleus with brown particles is apoptotic cells; and nucleus with blue is normal cells. Lets 1000 cells as a unit to calculate the apoptosis rate.

Western blot

HUVECs were homogenized in ice-cold RIPA lysis buffer (Sigma, USA) containing a cocktail

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Table 2. Effects of STS on apoptosis of HUVECs ($x \pm s$)

Groups	Apoptosis rate (%)
Normal	3.5 ± 0.7
HG	12.9 ± 0.6*
STS	3.3 ± 0.5 [◇] ◆
HG + STS	8.2 ± 0.6*◆
HG + STS + LiCl	5.2 ± 0.5*◆△

HG: high glucose; STS: sodium tanshinone IIA sulfonate; LiCl: thium chloride; HUVECs: human umbilical vein endothelial cells. * $P < 0.05$ VS N; [◇] $P > 0.05$ VS N; ◆ $P < 0.05$ VS HG; △ $P < 0.05$ VS HG + STS (n = 7).

of protease inhibitors (Roch, Switzerland). Denatured total cell lysates were run in SDS-PAGE, transferred to nitrocellulose membranes and blocked overnight at 4°C in 5% milk. The blocked membranes were incubated overnight at 4°C with rabbit anti-human GSK-3β (Ser9) monoclonal antibody (1:1000; EPITOMICS, Inc), rabbit anti-human β-catenin monoclonal antibody (1:2000; EPITOMICS, Inc), goat anti-human FKN polyclonal antibody (1:2000; Santa Cruz biotechnology, inc) followed by 1 hour incubation at room temperature with an HRP-conjugated secondary antibody (Santa Cruz, USA, 1:1000). Immuno-detection was performed using the ECL + reagents (Amersham Biosciences, UK). The blots were scanned and the pixel count and intensity of each band was quantified using the Scion image software (Scion, MD). Signals were normalized against β-actin and the results were expressed as a percentage of the negative control signal.

Immunohistochemistry for detection of β-actin, P-GSK-3β (Ser9) protein

We used SABC reagent to staining and operated according to manufacturer's instruction (WUHAN BOSTER BIO-ENGINEERING CO, LTD, CHINA). Immunohistochemistry was performed with specific primary antibodies: rabbit anti-human GSK-3β (Ser9) monoclonal antibody (1:100; EPITOMICS, Inc), rabbit anti-human β-catenin monoclonal antibody (1:250; EPITOMICS, Inc), goat anti-human FKN (1:200; Santa Cruz biotechnology, inc). Each slice were randomly selected seven high magnification view (× 200).

Immunofluorescence for detection of FKN protein expression level

Following the instructions from the manufacturer (rabbit anti-goat FITC (1:50) Zhongshan

golden bridge biotechnology CO, LTD, Beijing, China). After the staining, samples were viewed with an inverted fluorescent microscope and further demonstrated by laser scanning confocal microscope. Each slice were randomly selected seven high magnification view (× 200), using Image-Pro Plus 6.0 software to analysis the result, the average optical density value as the expression level of cells protein.

Real-time RT-PCR for detection of β-actin, GSK3β and FKN mRNA expression level

Plant the cells on the 6-well plate at a density of 4×10^4 per hole, each hole contains 3 ml Medium. Cells were cultured in non-serum medium for 24 h. After cultured in DMEM/LG for 24 h. And then divided them into different groups, give corresponding treatment as before. Total RNA from different groups of HUVECs were isolated using Trizol reagent (TAKARA BIOTECHNOLOGY (DALIAN) CO., LTD) according to manufacturer's instruction. Oligonucleotide primers and probes were designed according to Gene Bank by Shanghai Biological Engineering Co. The primer sequences and reaction conditions are listed in supplementary **Table 1**. The reaction mixtures were heated at 94°C for 4 minutes, followed by 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. Subsequently, PCR products were electrophoresed through 1.5% agarose gel, and then used Gel/fluorescence image analysis system for scanning.

Statistics analysis

Significant differences between groups were determined by Student's t test. The mean and standard deviations of the result between groups was used and P value < 0.05 was taken as statistically significant. Analyses were performed with the SPSS 20.0 statistical software package.

Results

The effects of STS at different concentrations on the proliferation of HUVECs

There is no significant injury on HUVECs when the STS concentration was between 0 to 80 mg/L. Moreover, it has a determinate role of promoting the proliferation of HUVECs when STS at the concentration of 5 mg/L and 20 mg/L. According to the principle of minimum

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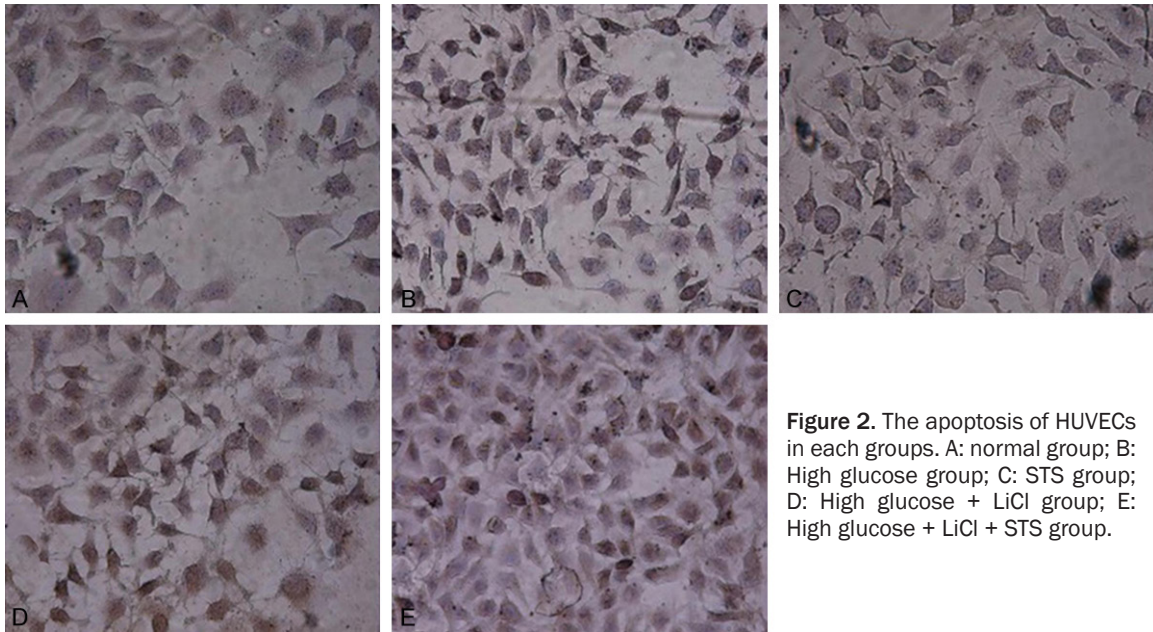


Figure 2. The apoptosis of HUVECs in each groups. A: normal group; B: High glucose group; C: STS group; D: High glucose + LiCl group; E: High glucose + LiCl + STS group.

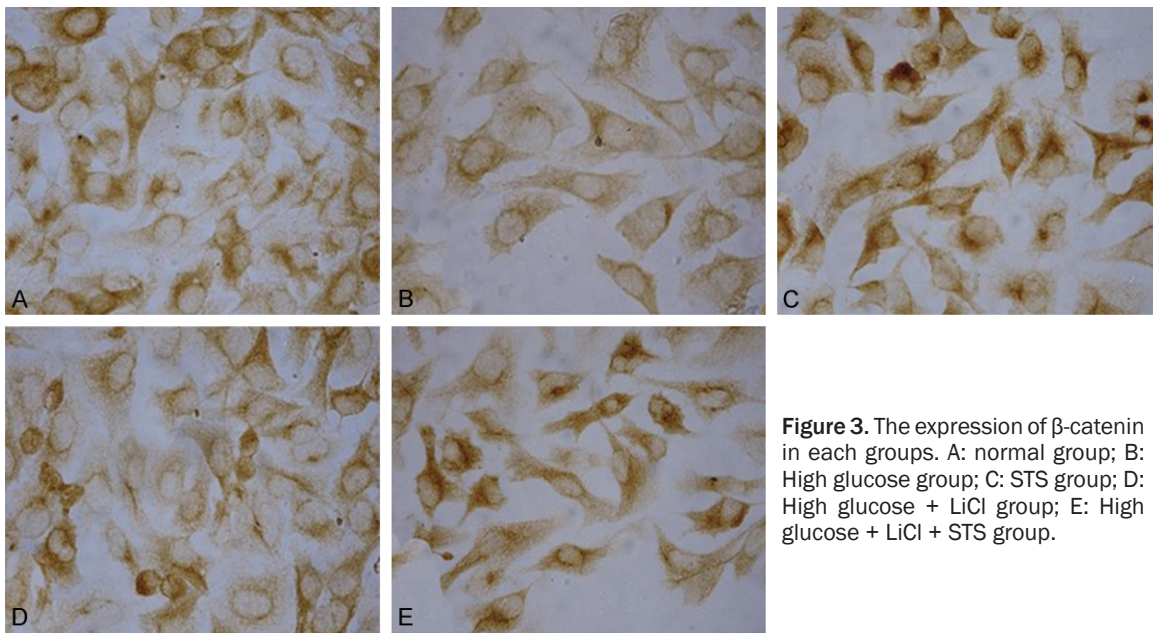


Figure 3. The expression of β -catenin in each groups. A: normal group; B: High glucose group; C: STS group; D: High glucose + LiCl group; E: High glucose + LiCl + STS group.

effective dose of intervention, we chose 5 mg/L as the pre-concentration of STS (**Figure 1**).

Detection of apoptosis in HUVECs

After treatment with 33.3 mmol/L high glucose, the apoptosis rate of HUVECs was significantly increased compared with normal group (**Table 2; Figure 2**, $P < 0.05$). STS could reduce the apoptosis rate of HUVECs when it cultured in low glucose, but there is no obviously difference with normal group. However, the apoptosis could be significant decreased by STS pre-

conditioning when HUVECs cultured in high glucose environment (**Table 2; Figure 2**, $P < 0.05$), and the apoptosis rate will further declined when it was co-pretreated by STS and LiCl. Moreover, these two group is different with the normal group (**Table 2; Figure 2**, $P < 0.05$).

Sodium tanshinone IIA sulfonate improves β -catenin and p-GSK-3 β (Ser9) expression, and inhibites FKN levels induced by high glucose

The immunohistochemistry assay results indicated that, β -catenin and p-GSK-3 β positive

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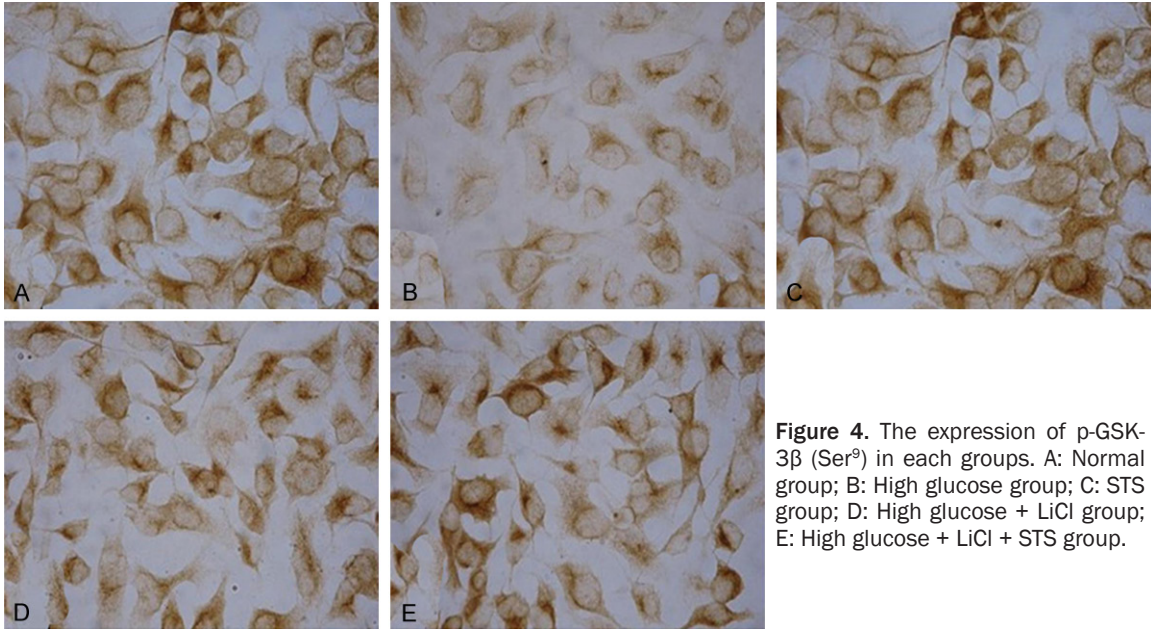


Figure 4. The expression of p-GSK-3 β (Ser⁹) in each groups. A: Normal group; B: High glucose group; C: STS group; D: High glucose + LiCl group; E: High glucose + LiCl + STS group.

Table 3. The protein levels of β -catenin, p-GSK-3 β (Ser9) and FKN in HUVECs ($x \pm s$)

Groups	β -catenin η	p-GSK-3 β (Ser9) η	FKN ψ
Normal	0.153 \pm 0.021	0.162 \pm 0.014	0.038 \pm 0.004
High glucose	0.102 \pm 0.007*	0.097 \pm 0.008*	0.095 \pm 0.017*
Tanshinone IIa	0.157 \pm 0.017	0.165 \pm 0.010	0.042 \pm 0.011
HG + STS	0.129 \pm 0.019* \diamond	0.128 \pm 0.009* \diamond	0.075 \pm 0.007* \diamond
HG + STS + LiCl	0.139 \pm 0.009* \diamond	0.151 \pm 0.008* \diamond \triangle	0.062 \pm 0.005* \diamond \triangle

HG: high glucose; STS: sodium tanshinone IIa sulfonate; LiCl: thium chloride; HUVECs: human umbilical vein endothelial cells. The data were analyzed by the gray scan according to the western blot images. * $P < 0.05$ VS N; $\diamond P < 0.05$ VS HG; $\triangle P < 0.05$ VS HG + STS.

expression cells in high glucose group were significantly more compared to the normal group (Figures 3, 4). The western blot assay results also showed that the expression of HUVECs β -catenin and p-GSK-3 β (Ser9) protein was significant decreased in high glucose group compared to the normal group (Table 3, $P < 0.05$). For the above changes, which will also be inhibited by STS preconditioning. Furthermore, compared with the high glucose group, the expression of β -catenin and p-GSK-3 β (Ser9) protein was increased in the sts+HG group (Table 3, $P < 0.05$), but it still lower than the normal group (Table 3, $P < 0.05$). Meanwhile, the western blot assay showed that the expression of HUVECs FKN protein was obviously increased in high glucose group compared with the normal group (Table 3, $P < 0.05$), and this effect will partially suppressed by STS precondition-

ing. The immunohistochemistry assay results also indicated that compared with the high glucose group, the expression of FKN was obviously declined in STS + HG group (Figure 5), and it still higher than the normal group. Moreover, after co-pretreatment by STS and LiCl, these effects were further strengthened. Compared with the high glucose group, the expression of β -catenin

and p-GSK-3 β (Ser9) was increased, and FKN was declined in the STS + LiCl group. Compared with STS + HG group, the expression of p-GSK-3 β (Ser9) was further increased in co-pretreatment group and the expression of FKN protein was decreased (Table 3, $P < 0.05$). β -catenin protein expression is slightly elevated, but no statistical significance. However, the β -catenin and p-GSK-3 β (Ser9) protein expression in co-pretreatment group were still lower than normal group, and the FKN expression was higher than normal group (Table 3, $P < 0.05$).

Sodium tanshinone IIa sulfonate inhibited GSK-3 β and FKN mRNA induced by high glucose

The mRNA expression level of HUVECs GSK-3 β , FKN increased apparently (Table 4, $P < 0.05$)

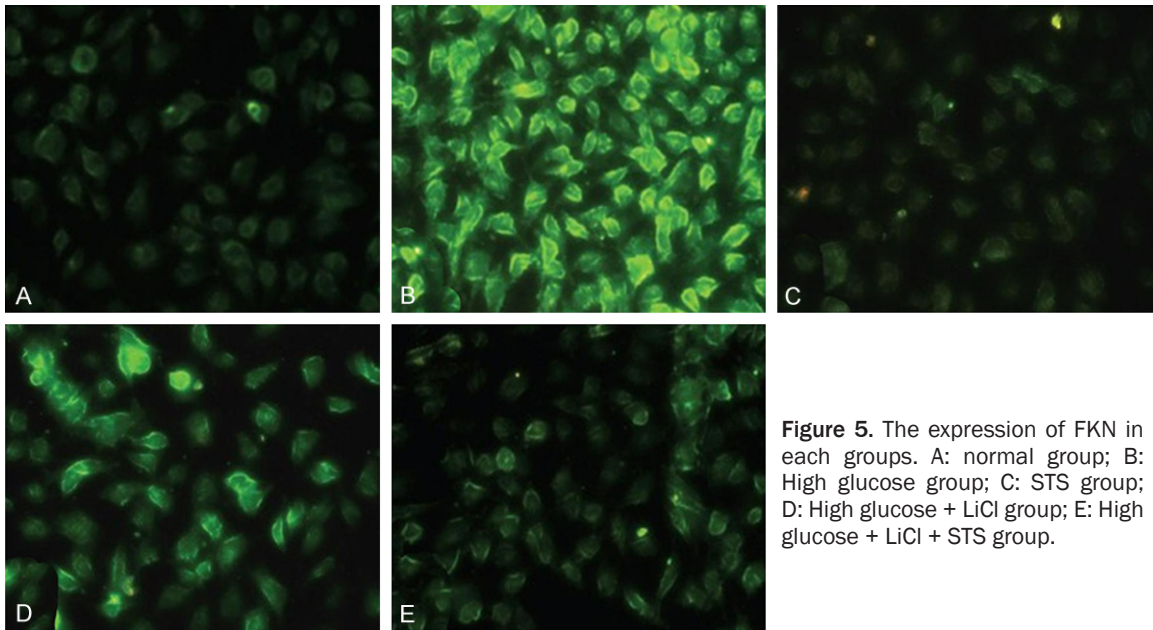


Figure 5. The expression of FKN in each groups. A: normal group; B: High glucose group; C: STS group; D: High glucose + LiCl group; E: High glucose + LiCl + STS group.

Table 4. The mRNA levels of GSK-3 β and FKN in each groups ($x \pm s$)

Groups	GSK-3 β	FKN
Normal (N)	0.047 \pm 0.001	0.046 \pm 0.001
High glucose (HG)	0.237 \pm 0.005*	0.503 \pm 0.007*
Tanshinonell A	0.059 \pm 0.002	0.060 \pm 0.002
HG + STS	0.149 \pm 0.007* \blacklozenge	0.394 \pm 0.009* \blacklozenge
HG + STS + LiCl	0.126 \pm 0.004* \blacklozenge \blacktriangle	0.217 \pm 0.009* \blacklozenge \blacktriangle

HG: high glucose; STS: sodium tanshinone IIa sulfonate; LiCl: thium chloride; HUVECs: human umbilical vein endothelial cells. * $P < 0.05$ VS N; $\blacklozenge P < 0.05$ VS HG; $\blacktriangle P < 0.05$ VS HG + STS (n = 7).

than the normal group, and this effect will be partially inhibited preconditioning by STS. The expression level of GSK-3 β , FKN mRNA reduced significantly in STS+HG group than HG group (Table 4, $P < 0.05$). Meanwhile, the expression level of GSK-3 β , FKN mRNA in co-pretreatment group even lower than in HG group, but it still higher than normal group (Table 4, $P < 0.05$).

Discussion

Fractalkine (FKN) as CX3CL1, it belong to the type I transmembrane protein which discovered by Bazan et al [8], and this molecule can exist in two forms: either membrane-anchored or as a shed 95K glycoprotein. The high expression of FKN in HUVEC and Vascular smooth muscle cells (VSMC) can be induced by high glucose exposure, advanced glycation end products (AGEs) [9], TNF- α , INF- γ and other pro-

inflammatory cytokines [9]. Moreover, diabetic stimuli and AGEs increased the expression of the adhesive chemokine fractalkine (FKN) in HVSMCs, and Human aortic VSMCs (HVSMCs) treated with high glucose and AGEs, exhibited significantly increased binding of THP-1 monocytic cells. All these promoted the development of diabetes and its complications [10]. STS is the derivatives of tanshinone IIa. Current, it is used for the treatment of diabetic nephropathy and realized its effects by anti-inflammatory, anti-fiber. The c-reactive protein of T2DM patients could decreased with STS therapy [3]. These research demonstrate that high glucose significant induced HUVEC to express FKN, and pretreatment by STS could markedly decreased the expression of FKN protein and mRNA which is induced by high glucose. It further suggest that STS has anti-inflammatory effects.

High glucose leads to the down-regulation of HUVECs p-GSK-3 β (Ser9) protein, up-regulation of GSK-3 β mRNA expressed and decreased the β -catenin level in cytoplasm; After pretreatment by STS, HUVEC β -catenin and p-GSK-3 β (Ser9) protein expression level increased, and the expression of GSK-3 β mRNA decreased. This effects is similar to the role of LiCl. Association STS with LiCl to pretreatment HUVEC, the expression of β -catenin and p-GSK-3 β (Ser9) elevated significantly than pretreatment by STS only, and the expression of FKN

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further reduced. It suggests that STS and the activation of Wnt signaling pathway as associated. Because of the expression of FKN may involve in the Wnt signaling pathway, we deduce that STS might be reduced the expression of FKN via Wnt signaling pathway.

TCF7L2 (Transcription factor 7-like 2) as a considerable factor of Wnt signaling pathway, its genetic variation can increase the incidence risk of T2DM [4, 11]. Wnt signaling pathway can regulate the survival and proliferation of HUVEC [12] and kidney mesangial cells [13] with the stimulation of high glucose. The previous study demonstrates that the reduction of HUVEC, myocardial β -catenin and p-GSK-3 β (Ser9) can be induced by high glucose exposure. Pretreatment by LiCl would antagonistic the effect of high glucose via active Wnt signaling pathway and increased the expression and apoptosis [7, 14], it also suggests that Wnt signaling pathway has a protective effect to HUVEC in high glucose. Compound danshen dripping pills could reduce the myocardial injury of cardiac ischemia zone in rat model of myocardial infarction by adjusting Wnt signaling pathways and some other genes and signaling pathways [15, 16], it illustrates that salvia class preparation could realize the effect of anti-inflammatory by regulated Wnt signaling pathway.

In these study, we found that high glucose inhibited Wnt signaling pathway in HUVECs, and induced the expression of Chemokine fractalkine; STS can activate the canonical Wnt pathway in partly, inhibit FKN expressed, and the expression of FKN significant declined when added LiCl which is the agonist of canonical Wnt pathway, it shows that STS may play the role of anti-inflammatory by regulate canonical Wnt pathway to inhibit the expression of FKN induced by high glucose. Thereby, it will provide an effective therapeutic target for DM and its complication, and it also further reveals the mechanism of STS.

Acknowledgements

This work was supported by National Key Clinical Specialties Construction Program of China (No. [2013] 544).

Disclosure of conflict of interest

None.

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References

- [1] Song Y, Li Y, Wang PJ and Gao Y. Contrast-enhanced ultrasonography of skeletal muscles for type 2 diabetes mellitus patients with microvascular complications. *Int J Clin Exp Med* 2014; 7: 573-579.
- [2] Bazan JF, Bacon KB, Hardiman G, Wang W, Soo K, Rossi D, Greaves DR, Zlotnik A and Schall TJ. A new class of membrane-bound chemokine with a CX3C motif. *Nature* 1997; 385: 640-644.
- [3] Bergmann K and Sypniewska G. Secreted frizzled-related protein 4 (SFRP4) and fractalkine (CX3CL1)-potential new biomarkers for beta-cell dysfunction and diabetes. *Clin Biochem* 2014; 47: 529-532.
- [4] Hisa I, Kaji H, Inoue Y, Sugimoto T and Chihara K. Fasting plasma glucose levels are related to bone mineral density in postmenopausal women with primary hyperparathyroidism. *Int J Clin Exp Med* 2008; 1: 319-326.
- [5] Chong ZZ, Shang YC and Maiese K. Vascular injury during elevated glucose can be mitigated by erythropoietin and Wnt signaling. *Curr Neurovasc Res* 2007; 4: 194-204.
- [6] Manduteanu I, Pirvulescu M, Gan AM, Stan D, Simion V, Dragomir E, Calin M, Manea A and Simionescu M. Similar effects of resistin and high glucose on P-selectin and fractalkine expression and monocyte adhesion in human endothelial cells. *Biochem Biophys Res Commun* 2010; 391: 1443-1448.
- [7] High glucose induces fractalkine expression and apoptosis in human umbilical vein endothelial cells. *Acta Acad Med Militaris Tertiae* 2011; 33: 699-702.
- [8] Bazan JF, Bacon KB, Hardiman G, Wang W, Soo K, Rossi D, Greaves DR, Zlotnik A and Schall TJ. A new class of membrane-bound chemokine with a CX3C motif. *Nature* 1997; 385: 640-644.
- [9] Meng L, Park J, Cai Q, Lanting L, Reddy MA and Natarajan R. Diabetic conditions promote binding of monocytes to vascular smooth muscle cells and their subsequent differentiation. *Am J Physiol Heart Circ Physiol* 2010; 298: H736-H745.
- [10] Bordonaro M. Role of Wnt signaling in the development of type 2 diabetes. *Vitam Horm* 2009; 80: 563-581.
- [11] Liu Z, Huang Q, Liu G, Dang L, Chu D, Tao K and Wang W. Presence of FOXP3 (+) Treg cells is

STS affects FKN expression and apoptosis

- correlated with colorectal cancer progression. *Int J Clin Exp Med* 2014; 7: 1781-1785.
- [12] Masckauchan TN, Shawber CJ and Funahashi Y. Wnt/beta-catenin signaling induces proliferation, survival and interleukin-8 in human endothelial cells. *Angiogenesis* 2005; 8: 43-51.
- [13] Lin CL, Wang JY and Huang YT. Wnt/beta-catenin signaling modulates survival of high glucose-stressed mesangial cells. *J Am Soc Nephrol* 2006; 17: 2812-2810.
- [14] Zhu D, Yu H, He H, Ding J, Tang J, Cao D and Hao L. Spironolactone inhibits in rat mesangial cells under hyperglycaemic conditions via the Wnt signaling pathway. *Mol Cell Biochem* 2013; 380: 185-193.
- [15] Yuan X, Jing S, Wu L, Chen L and Fang J. Pharmacological postconditioning with tanshinone IIA attenuates myocardial ischemia-reperfusion injury in rats in activating phosphatidylinositol 3-kinase pathway. *Exp Ther Med* 2014; 8: 973-977.
- [16] Ay Y, Kara I, Aydin C, Ay NK, Teker ME, Senol S, Inan B, Basel H, Uysal O and Zeybek R. Effects of ischemic preconditioning and iloprost myocardial ischemia-reperfusion damage in rats. *Int J Clin Exp Med* 2013; 6: 516-523.