

Induction of proliferation and activation of rat hepatic stellate cells via high glucose and high insulin

Q. LI¹, X. LI², C.-L. DENG¹

¹Department of Infectious Diseases, The Affiliated Hospital of Southwest Medical University, Luzhou, China

²School of Pharmacy, The Southwest Medical University, Luzhou, China

Abstract. – **OBJECTIVE:** To further investigate the occurrence mechanism of diabetic hepatic fibrosis through observing the effects of insulin and glucose in different concentrations on hepatic stellate cell (HSC) proliferation, and mRNA expressions of transforming growth factor- β 1 (TGF- β 1) and tissue inhibitor of metalloproteinase-1 (TIMP-1) in rats, so as to provide a theoretical and experimental basis for the occurrence, prevention and treatment of diabetic hepatic fibrosis (HF).

MATERIALS AND METHODS: The HSCs in rats were cultured *in vitro* with high glucose alone and high glucose + high insulin as the stimulating factors and mannitol as the high osmotic pressure control. After the above 10 groups of HSC were cultured for some time, the absorbance value of each group was determined using the Cell Counting Kit-8 (CCK-8) to clarify the number of proliferative HSC. Moreover, the count per minute (Cpm) of DNA in HSC was detected via the 3H-thymidine incorporation (3H-TDR incorporation) to clear the proliferation status of HSC. Finally, the mRNA expressions of TGF- β 1 and TIMP-1 in HSC in each group were detected via Real-time fluorescence quantitative polymerase chain reaction (RT-FQ-PCR).

RESULTS: Both HSC proliferation and DNA synthesis were increased in a glucose concentration-dependent manner, while the HSC proliferation and DNA synthesis in glucose groups with insulin were significantly higher than those in glucose groups without insulin ($p < 0.05$). The DNA synthesis in insulin + mannitol group was higher than that in insulin + normal glucose group. The mRNA level in TGF- β 1 in glucose groups with insulin was decreased, but that in TIMP-1 was increased.

CONCLUSIONS: Both high glucose and high insulin can induce the HSC proliferation, and high insulin can further activate HSC and promote the progression of hepatic fibrosis course.

Key Words:

HSC, Hepatic fibrosis, High glucose, High insulin, TGF- β 1, TIMP-1.

Introduction

Hepatic fibrosis (HF) is a common pathological change of a variety of chronic liver diseases, and it is characterized by excessive deposition of collagen-dominated extracellular matrix (ECM) in the liver. The hepatic stellate cell (HSC) is the central link of HF and the most important collagen-synthesizing cell in the liver, which is closely related to the occurrence and development of HF. Diabetes mellitus (DM) is a group of metabolic diseases, and high glucose can cause a variety of acute and chronic complications, which can significantly promote the renal, nerve and vascular fibrosis. Recent studies have shown that hyperglycemia can promote the formation of fibrosis, but the research data are mostly from the research results of diabetic nephropathy model¹. In addition, although hyperglycemia and hyperinsulinemia are important features of type 2 DM and hepatogenous DM², there are few reports on the correlation of high glucose and high insulin with HF.

Transforming growth factor- β 1 (TGF- β 1) is the most potent HF-promoting factor at present, and its expression level has a significantly positive correlation with the severity of HF^{3,4}. In normal liver, the vast majority of TGF- β 1 mRNAs are distributed in Kupffer cells, followed by HSC. In fibrous liver, the mRNA expression of TGF- β 1 in HSC is increased by 12 times compared with that in normal liver, which exceeds that in Kupffer cells^{4,5}. TGF- β 1 widely influences the HF-related cells, growth factors and a variety of enzymes, leading to HF, which can not only promote the matrix synthesis, but also inhibit its degradation. At the same time, TGF- β 1 can also inhibit the regeneration and promote the apoptosis of liver cells, thus resulting in the ratio imbalance between parenchymal hepatic cells and mesenchymal

components in the liver, so it plays a core role in the occurrence and progression of HF and even the formation of liver cirrhosis.

The tissue inhibitor of metalloproteinase-1 (TIMP-1) is a kind of glycoprotein produced by macrophages and connective tissue cells, which can inhibit the activity of matrix metalloproteinases (MMPs), especially MMP-1⁶, and it plays an important role in degrading the major components of ECM, Type I and III collagen, in fibrous liver⁷. In the HF process, TIMP-1 inhibits the degradation of Type I and III collagen through inhibiting the activity of MMPs, leading to the excessive deposition of ECM in the liver, and promoting the formation and development of HF⁸. Studies have shown that TIMP-1 and TIMP-2 are expressed in normal liver tissues⁸, and the activated HSC can secrete a large number of TIMP-1 and promote the fibrosis in the liver⁹. The expression level of TIMP-1 is decreased when HF is reversed, so it can be seen that TIMP-1 plays an important role in the maintenance of HF¹⁰.

Hyperglycemia can promote the occurrence and development of HF mainly through stimulating the activation and proliferation of HSC¹¹. However, there has been no report on whether the high glucose alone *in vitro* can directly promote the activation and proliferation of HSC, thus leading to the increased secretion of TGF- β_1 and TIMP-1. In addition, insulin, as a kind of multi-functional hormone protein, can not only regulate the body's metabolism, but also promote the division and proliferation of cells cultured *in vitro*, such as glomerular mesangial cells and liver cells; however, the role of insulin in HSC has not been elucidated yet. Some studies found that insulin plays an inhibiting effect on cell growth, and such an effect is more significant with the increase in insulin concentration¹², while some showed that insulin can stimulate the HSC mitosis and collagen synthesis¹³. Whether high insulin inhibits the HSC growth or promotes its activation needs further experiment, so it is worthwhile to investigate the correlation of high glucose and high insulin with HSC.

Materials and Methods

Cell Culture and Grouping

Rat HSC lines were purchased from Shanghai University of Traditional Chinese Medicine (Shanghai, China). This study was approved by the Animal Ethics Committee of South-West Medical University Animal Center. The supernatant

in HSC culture flask was discarded and 4-5 mL of normal glucose Dulbecco's modified Eagle medium (DMEM) was added for incubation in an incubator containing 5% CO₂ at 37°C. According to the cell growth situation, the fluid was replaced once every 1-2 days, and the experiments were performed after cells covered the bottom of bottle and fused.

The glucose groups without insulin included normal glucose group (5.6 mmol/L), high glucose A group (15 mmol/L), high glucose B group (25 mmol/L), high glucose C group (35 mmol/L), mannitol group (29.4 mmol/L mannitol + 5.6 mmol/L glucose); the glucose groups with insulin included insulin + normal glucose group (5.6 mmol/L glucose + 150 nmol/L insulin), insulin + high glucose A group (15 mmol/L glucose + 150 nmol/L insulin), insulin + high glucose B group (25 mmol/L glucose + 150 nmol/L insulin), insulin + high glucose C group (35 mmol/L glucose + 150 nmol/L insulin), insulin + mannitol group (29.4 mmol/L mannitol + 5.6 mmol/L glucose + 150 nmol/L insulin).

Detection of HSC Proliferation

The cell proliferation level was detected via Cell Counting Kit-8 (CCK-8). After 80% HSCs were fused, the supernatant was discarded and 0.25% trypsin was added for digestion, and then discarded. 4 mL normal glucose DMEM was added to terminate the digestion, and the cells were gently blown to form the cell suspension. After centrifugation at 1000 rpm for 5 min at room temperature, the supernatant was discarded and 3 mL low-glucose full DMEM was added to prepare the cell suspension. Then, the cell suspension mixed was inoculated into 40 wells (100 μ L/well) of the 96-well plate (2×10^4 cells/mL) for incubation in an incubator containing 5% CO₂ at 37°C. After 24 h, the supernatant was discarded and replaced with the serum-free normal-glucose DMEM culture solution, followed by incubation in an incubator for 24 h to synchronize the cells in the G0 phase. The CCK-8 solution was added after 72 h, and the absorbance value at 450 nm of each group was determined using the microplate reader.

Determination of HSC Incorporation Amount (Cpm)

The 96-well plate incubated for 7 h was taken out, ³H-thymidine (³H-TDR) 2 μ Ci was added into each of the 40 wells containing cells, and incubated in an incubator containing 5% CO₂ at 37°C for 12 h. After that, the supernatant was discarded and 60 μ L cell digestion solution was added

into each well for digestion for 1.5 min and then discarded. The mixture was washed twice with 200 μ L cold phosphate-buffered saline (PBS) and added with 150 μ L PBS to blow and beat cells to prepare them into the cell suspension. Next, the cell suspension was transferred into the centrifuge tube, followed by centrifugation at 2000 rpm for 10 min, and the supernatant was gently absorbed and discarded. The remaining cell suspension in the centrifuge tube was transferred onto the prepared glass fiber paper according to grouping, and dried at 60°C for 40 min. The glass fiber paper, with the face adhered by cells upward, was placed into the bottle with toluene scintillation solution, and the incorporation amount of HSC DNA was measured using a liquid scintillation counter.

Cell RNA Extraction

The supernatant in the 6-well plate was discarded and the cells were washed twice with phosphate-buffered saline (PBS). 200 μ L RNA lysis buffer (RLA) reagent was added into each well for splitting. The sample was transferred to a 1.5 mL Eppendorf (EP) (Hamburg, Germany) tube and placed at room temperature for 5 min, followed by centrifugation at 12000 rpm at 4°C for 5 min for separation: aqueous phase in upper layer, intermediate phase, and organic phase in lower layer. The aqueous phase in upper layer was removed into another clean centrifuge tube, added with 200 μ L W3. Then, the mixture was transferred into the adsorption column, followed by centrifugation at 12000 rpm at 4°C for 30 s; the liquid in collection tube was discarded, and the adsorption column was placed into the collection tube. Next, 500 μ L working solution from the RNA extraction kit was added into the adsorption column, followed by centrifugation at 12000 rpm for 30 s, and the adsorption column was transferred into another clean collection tube.

500 μ L extraction solution was added into the adsorption column and placed at room temperature for 1 min, followed by centrifugation at 12000 rpm for 30 s. The liquid in collection tube was discarded and the adsorption column was placed into the collection tube. The empty column was centrifuged at 12000 rpm for 1 min. The adsorption column was transferred into a clean 1.5 mL EP tube, and 50 μ L RNase-free pure water was added to the center of adsorption membrane; the tube was placed at room temperature for 5 min and centrifuged at 12000 rpm for 1 min; finally, the RNA extracted was in the EP tube.

CDNA Synthesis and Amplification

According to the instructions of reverse transcription-polymerase chain reaction (RT-PCR) kit, the reaction system was added successively, and after transient centrifugation, the reverse transcription was performed: 30°C for 10 min, 50°C for 25 min, 99°C for 5 min and 5°C for 5 min. The housekeeping gene, β -actin, was used as the internal control. Primers were designed according to the sequences of TGF- β_1 , TIMP-1 and β -actin in National Center of Biotechnology Information (NCBI) Genbank.

The cDNA obtained via reverse transcription received the degeneration at 94°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 2 min for a total of 35 times. After PCR amplification, a certain number of PCR products were taken for agarose gel electrophoresis to confirm the presence of target gene and the quality of primer design. After electrophoresis under 120 V for 50 min, the product was observed under ultraviolet lamp, and photographed on the DNA imager. The remaining PCR products were stored at -20°C.

Real-time Fluorescence Quantitative PCR (RT-FQ-PCR)

The standard sample was made using the agarose gel DNA, and the concentration of DNA and $A_{260\text{nm}}/A_{280\text{nm}}$ were measured on an ultraviolet spectrophotometer using 1 μ L sample. The ratio of absorbance of the extracted samples was between 1.8 and 2.2, suggesting that the purity of DNA was high and could be used as the standard sample. RT-FQ-PCR was performed using RealMasterMix SYBR Green reagent to obtain the amplification curves, standard curves and melting curves of the housekeeping gene and target gene, respectively, clarify the amplification efficiencies of the housekeeping gene and target gene and the linear relationship shown by the standard curve. 1 μ L cDNA obtained via reverse transcription was taken from each group of samples and added into the 20 μ L reaction system, and 6 repeated wells were set. The housekeeping gene was used for correction. The initial amount of target gene of each group was divided by that of the housekeeping gene to obtain the original relative amount of the target gene, by which the differences in mRNA among groups were obtained.

Statistical Analysis

The data in each group were presented as mean $\pm$ standard deviation ($\bar{x} \pm s$), and Statistical Product and Service Solutions (SPSS) 13.0 software

Table I. Analysis of effects of glucose and insulin on the absorbance value of HSC.

Group	Average absorbance ($\bar{x} \pm s$)		Total	F	p
	Without insulin	With insulin			
Normal glucose group	1.5720 $\pm$ 0.01418	1.5480 $\pm$ 0.1670	1.56 $\pm$ 0.019	3.6	0.131
High glucose A group	1.6680 $\pm$ 0.00529	1.6603 $\pm$ 0.01779	1.66 $\pm$ 0.012	0.51	0.541
High glucose B group	1.7657 $\pm$ 0.01604	1.7527 $\pm$ 0.06486	1.76 $\pm$ 0.043	0.11	0.753
High glucose C group	1.8497 $\pm$ 0.01607	1.8140 $\pm$ 0.02066	1.83 $\pm$ 0.026	5.57	0.078
Mannitol group	1.5837 $\pm$ 0.02899	1.5667 $\pm$ 0.03259	1.58 $\pm$ 0.029	0.46	0.537
Total	1.6878 $\pm$ 0.1114	1.6683 $\pm$ 0.1109	1.68 $\pm$ 0.197	3.61*	0.072*
F	134.64	31.72	104.64*	F= 0.22 [#]	
p	0.000	0.000	0.000*	p= 0.92 [#]	

*F and p values of main effect; # F and p values of interaction effect; there is no interaction between glucose and insulin. There are statistically significant differences between the other groups and the normal glucose group, except that between mannitol group and normal glucose group.

(Version X; IBM, Armonk, NY, USA) was used for statistical analysis. In this study, two-factor factorial design was adopted, and the analysis of variance based on factorial design was used for the analysis of main effects of glucose and insulin and the interaction effect between them. Student Newman Keuls-q (SNK-q) test was used for the pairwise comparison among several sample means, and the Dunnett-t test was used for the comparisons of means among the several experimental groups and the normal glucose group. $p < 0.05$ suggested that the difference was statistically significant.

Results

Normal Glucose Cell Culture

Under the phase contrast microscope, it could be seen that the cells were in a star shape with multiple angles, the cell body was larger with rich cytoplasm and many cytoplasmic processes. After the treatment factors were added, the cell morphology had no significant change.

High Glucose Promoted the Proliferation of HSC

The cell proliferation in each group was detected via Cell Counting Kit-8 (CCK-8). The absorbance values of HSC in high glucose A, B and C groups in glucose groups with and without insulin had statistically significant differences compared with those in normal glucose group ($p < 0.05$), and the value in each group was increased in a glucose concentration-dependent manner. The absorbance value had no statistically significant difference in the pairwise comparison between

glucose groups with and without insulin under the same glucose concentration ($p > 0.05$). The absorbance value of HSC had no statistically significant difference between mannitol group and normal glucose group, between insulin + mannitol group and insulin + normal glucose group ($p > 0.05$), suggesting that the high glucose can promote the proliferation of HSC, and the proliferation is increased in a glucose concentration-dependent manner. However, the glucose and insulin have no combined effect on the proliferation of HSC, and the stimulation of cells via high insulin and high osmotic pressure for 72 h has no significant effect on the proliferation of HSC (Table I and Figure 1).

High Glucose and High Insulin Promoted the DNA Synthesis in HSC

It could be seen from the statistical table and statistical chart that the HSC DNA incorporation amount (Cpm) in high glucose A, B and C

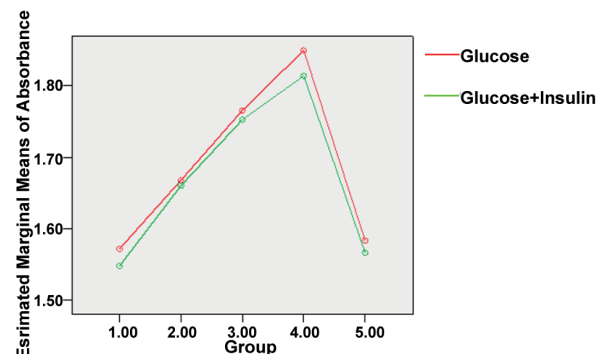


Figure 1. Skeleton map of interaction effect between glucose and insulin in detection of HSC absorbance values via CCK-8.

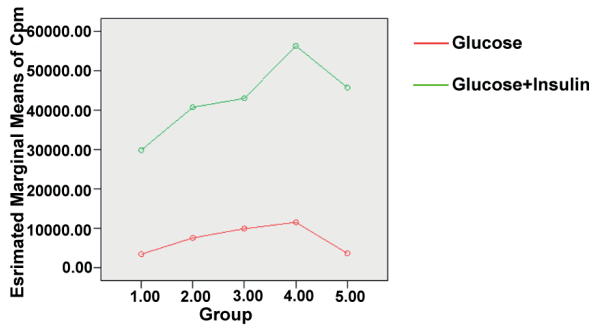


Figure 2. Skeleton map of interaction effect between glucose and insulin in HSC DNA incorporation (Cpm).

groups in glucose groups with and without insulin had statistically significant differences compared with that in normal glucose group ($p < 0.05$), and it was increased in each group in a glucose concentration-dependent manner. Cpm had statistically significant differences in the pairwise comparison between glucose groups with and without insulin under the same glucose concentration, and it was significantly higher in glucose groups with insulin than that in glucose groups without insulin ($p < 0.05$). The change in mannitol group was not compared with that in normal glucose group ($p > 0.05$), and it was higher in insulin + mannitol group than that in insulin + normal glucose group ($p < 0.05$), suggesting that the high glucose and high insulin can promote the activation of HSC, the high glucose combined with high insulin has a more significant activating effect on HSC than high glucose alone, and there is an interaction between glucose and insulin. Simple high osmotic pressure has

no significant effect on the HSC DNA content, but high osmotic pressure combined with high insulin can promote the synthesis of HSC DNA (Table II and Figure 2).

PCR Detection of Standard Sample

The electrophoretic bands in the three genes, PCR amplification product segment 205 bps of housekeeping gene β -actin, amplification product segment 233 bps of target gene TIMP-1 and amplification product segment 209 bps of TGF- β_1 , and the DNA Marker I control, were within the 200+ bp, which were consistent with the designed primer product, and there was no non-specific band, indicating that the primers were ideal meeting the experimental requirements (Figure 3A).

After the PCR of standard sample made by DNA obtained via cDNA amplification, the better standard curve and melting curve could be obtained. The standard curve showed a good linear relationship (housekeeping gene β -actin: $R^2 = 0.996$; target gene TIMP-1: $R^2 = 0.997$; target gene TGF- β_1 : $R^2 = 0.99$, $R^2 > 0.98$), and the accurate quantification could be realized in a wide range (β -actin: $E = 99\%$; TIMP-1: $E = 90\%$; TGF- β_1 : $E = 104\%$, $0.8 < E < 1.2$). The melting curve had single peak of wave without impure peak, suggesting that the housekeeping gene and target gene have high specificities, and no other non-specific products affect the quantification of target gene. The fluorescence signal was collected once in each cycle of the amplification curve, which reflected the fluorescence intensity and the number of amplification cycles of samples (Figure 3B).

Table II. Analysis of effects of glucose and insulin on the HSC DNA incorporation (Cpm).

Group	Average absorbance ($\bar{x} \pm s$)		Total	F	p
	Without insulin	With insulin			
Normal glucose group	3419 $\pm$ 180.37	29832 $\pm$ 3229.95	16625.5 $\pm$ 14276.30	266.66	0.000
High glucose A group	7562 $\pm$ 1239.61	40707.5 $\pm$ 6331.83	24134.75 $\pm$ 18213.55	105.56	0.000
High glucose B group	9886 $\pm$ 307.17	42960 $\pm$ 7029.69	26423 $\pm$ 18269.07	88.38	0.000
High glucose C group	11516.5 $\pm$ 3066.66	56275 $\pm$ 7159.59	33895.75 $\pm$ 24461.75	132.09	0.000
Mannitol group	3619 $\pm$ 699.31	45716 $\pm$ 6218.03	24667.5 $\pm$ 22871.61	181.05	0.000
Total	7200.50 $\pm$ 1003.55	43098.1 $\pm$ 1003.55	25149.30 $\pm$ 19710.64	15.10	0.000
F	22.98	9.54	639.77*	F = 5.52#	
p	0.000	0.000	0.000*	p = 0.002#	

* F and p values of main effect; # F and p values of interaction effect; there is no interaction between glucose and insulin. There are statistically significant differences between the other groups and the normal glucose group, except that between mannitol group without insulin and normal glucose group without insulin.

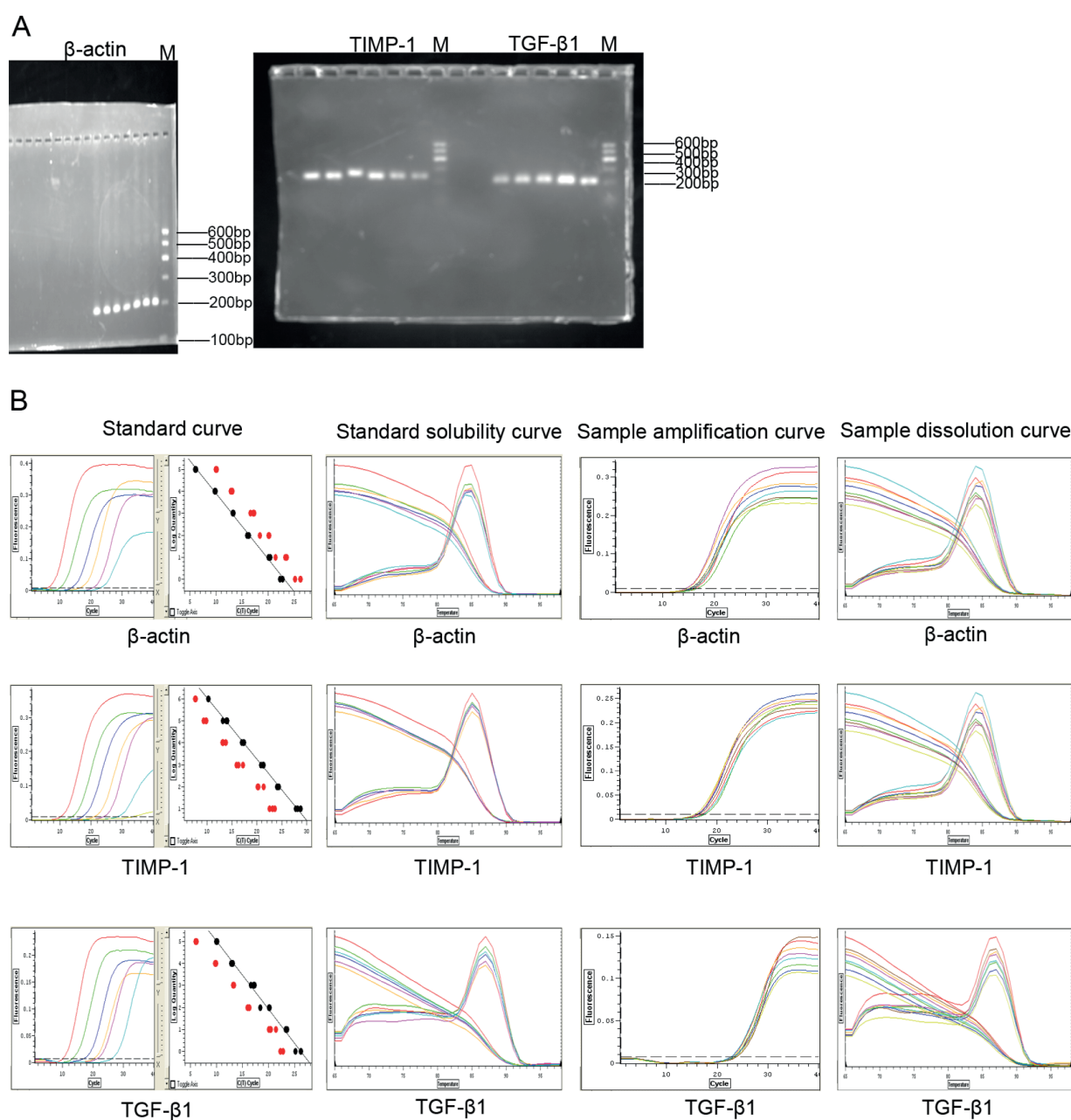


Figure 3. *A*, Agarose gel electrophoresis for PCR amplification products after the addition of corresponding primers into HSC cDNA; *B*, RT-FQ-PCR curves of β-actin, TIMP-1 and TGF-β1.

High Insulin Promoted the Increase of TIMP-1 mRNA in HSC

From the statistical data, it could be seen that although the TIMP-1 mRNA expression of HSC had no statistically significant differences in the pairwise comparison between glucose groups with and without insulin under the same glucose concentration, comparisons within glucose groups with insulin ($p > 0.05$), the TIMP-1 mRNA expres-

sion of HSC was not highly correlated with the glucose concentration. However, it could be seen from the skeleton map of interaction effect between glucose and insulin that the TIMP-1 mRNA expression in glucose groups with insulin was higher than that in glucose groups without insulin, indicating that high insulin may promote the increased TIMP-1 mRNA expression in activated HSC to a certain extent (Tables III-IV and Figure 4A).

Table III. Relative amount of target gene TIMP-1.

Group	No.	β -actin amount	TIMP-1 amount	Relative TIMP-1 amount
Normal glucose group	6	3226.67 $\pm$ 348.47	12468.67 $\pm$ 1479.26	3.88 $\pm$ 0.52
High glucose A group	6	5680.00 $\pm$ 628.65	18400.00 $\pm$ 5879.63	3.33 $\pm$ 1.31
High glucose B group	6	3495.00 $\pm$ 360.65	14360.00 $\pm$ 1978.71	4.15 $\pm$ 0.83
High glucose C group	6	1523.00 $\pm$ 486.31	7497.00 $\pm$ 870.41	5.33 $\pm$ 1.93
Mannitol group	6	3967.67 $\pm$ 697.41	18086.67 $\pm$ 1958.17	4.66 $\pm$ 0.97
Insulin + normal glucose group	6	6266.33 $\pm$ 1174.05	29156.67 $\pm$ 7019.99	4.63 $\pm$ 0.43
Insulin + high glucose A group	6	6087.67 $\pm$ 2161.70	26103.33 $\pm$ 3154.37	4.54 $\pm$ 1.11
Insulin + high glucose B group	6	2935.33 $\pm$ 699.54	12190.00 $\pm$ 2221.24	4.31 $\pm$ 1.15
Insulin + high glucose C group	6	50866 $\pm$ 1279.42	27830 $\pm$ 3265.10	5.65 $\pm$ 1.11
Insulin + mannitol group	6	4375.33 $\pm$ 1957.49	23136.67 $\pm$ 5541.20	5.91 $\pm$ 2.70

Table IV. Analysis of effects of glucose and insulin on the relative amount of target gene TIMP-1 in HSC.

Glucose group	TIMP-1 ($\bar{x}\pm s$)		Total	F	p
	Without insulin	With insulin			
Normal glucose group	3.88 $\pm$ 0.52	4.63 $\pm$ 0.43	4.26 $\pm$ 0.59	3.69	0.13
High glucose A group	3.33 $\pm$ 1.31	4.54 $\pm$ 1.11	3.94 $\pm$ 1.27	1.49	0.29
High glucose B group	4.15 $\pm$ 0.83	4.31 $\pm$ 1.15	4.23 $\pm$ 0.90	0.04	0.86
High glucose C group	5.33 $\pm$ 1.93	5.65 $\pm$ 1.11	5.49 $\pm$ 1.42	0.06	0.82
Mannitol group	4.66 $\pm$ 0.97	5.91 $\pm$ 2.70	5.29 $\pm$ 1.94	0.57	0.49
Total	4.27 $\pm$ 1.24	5.01 $\pm$ 1.43	4.64 $\pm$ 1.37	2.18*	0.16*
F	1.19	9.54	1.57*	F=0.203 [#]	
p	0.69	0.61	0.22*	p= 0.93 [#]	

*F and p values of main effect; # F and p values of interaction effect; there is no interaction between glucose and insulin. There are no statistically significant differences among other groups.

Table V. Relative amount of target gene TGF- β 1.

Group	n	β -actin amount	TGF- β ₁ amount	Relative TGF- β 1 amount
Normal glucose group	6	673.90 $\pm$ 9.47	430.50 $\pm$ 17.85	0.64 $\pm$ 0.03
High glucose A group	6	674.40 $\pm$ 15.12	419.63 $\pm$ 11.41	0.62 $\pm$ 0.01
High glucose B group	6	641.33 $\pm$ 18.75	407.33 $\pm$ 19.13	0.64 $\pm$ 0.04
High glucose C group	6	732.47 $\pm$ 37.58	439.30 $\pm$ 13.47	0.60 $\pm$ 0.01
Mannitol group	6	706.97 $\pm$ 7.86	450.63 $\pm$ 22.44	0.64 $\pm$ 0.33
Insulin + normal glucose group	6	768.17 $\pm$ 52.56	423.23 $\pm$ 14.45	0.55 $\pm$ 0.04
Insulin + high glucose A group	6	746.63 $\pm$ 53.12	425.77 $\pm$ 15.38	0.57 $\pm$ 0.03
Insulin + high glucose B group	6	655.07 $\pm$ 19.31	407.57 $\pm$ 21.69	0.62 $\pm$ 0.02
Insulin + high glucose C group	6	809.67 $\pm$ 42.51	477.00 $\pm$ 32.44	0.59 $\pm$ 0.08
Insulin + mannitol group	6	824.00 $\pm$ 44.55	451.70 $\pm$ 14.27	0.55 $\pm$ 0.02

High Insulin Inhibited the Increased TGF- β 1 mRNA in HSC

From the statistical data, it could be seen that although the TGF- β ₁ mRNA expression of HSC had no statistically significant differences in the pairwise comparison between glucose groups with and without insulin under the same glucose concentration, comparisons within glucose groups with insulin and comparisons within glucose groups without insulin ($p>0.05$), the

TGF- β ₁ mRNA expression of HSC was not highly correlated with the glucose concentration. However, it could be seen from the skeleton map of interaction effect between glucose and insulin that the TGF- β ₁ mRNA expression in glucose groups with insulin was lower than that in glucose groups without insulin, indicating that insulin may inhibit the increased TGF- β ₁ mRNA expression in activated HSC to a certain extent (Tables V-VI and Figure 4B).

Table VI. Analysis of effects of glucose and insulin on the relative amount of target gene TGF- β 1 in HSC.

Glucose group	TIMP-1 ($\bar{x} \pm s$)		Total	F	P
	Without insulin	With insulin			
Normal glucose group	0.64 $\pm$ 0.03	0.55 $\pm$ 0.04	0.60 $\pm$ 0.06	9.40	0.077
High glucose A group	0.62 $\pm$ 0.01	0.57 $\pm$ 0.03	0.60 $\pm$ 0.03	10.12	0.073
High glucose B group	0.64 $\pm$ 0.04	0.62 $\pm$ 0.02	0.63 $\pm$ 0.03	0.28	0.62
High glucose C group	0.60 $\pm$ 0.01	0.59 $\pm$ 0.08	0.58 $\pm$ 0.03	4.63	0.098
Mannitol group	0.64 $\pm$ 0.33	0.55 $\pm$ 0.02	0.59 $\pm$ 0.05	17.78	0.054
Total	0.63 $\pm$ 0.03	0.57 $\pm$ 0.03	0.60 $\pm$ 0.04	2.49*	0.096*
F	0.95	4.13	31.01*	F=2.14 [#]	
p	0.47	0.061	0.051	p=0.11 [#]	

*F and p values of main effect; [#]F and p values of interaction effect; there is no interaction between glucose and insulin. There are no statistically significant differences among other groups.

Discussion

In this investigation, HSC cultured *in vitro* was stimulated using different concentrations of glucose with mannitol as the high osmotic pressure control, and it was found that different concentrations of glucose could activate HSC, resulting in HSC proliferation and increased DNA synthesis, and the HSC proliferation and DNA synthe-

sis were increased with the increase in glucose concentration in a dose-dependent manner. Moreover, high insulin could induce the HSC activation and increase the DNA content, while high insulin combined with high glucose might induce the proliferative HSC to secrete more TIMP-1 but less TGF- β 1.

With the improvement of living conditions, the prevalence rate of DM has been increasing, and there is a close relationship between DM and liver disease^{14,15}. At present, scholars generally believe that the activation of HSC is the main source and target of important effector and cytokines of HF, and the central link in the formation and development of HF, as well as the ultimate common pathway of HF¹¹. Insulin resistance often exists in type 2 diabetes mellitus and hepatogenous DM, and hyperinsulinemia has even become an important feature of hepatogenous DM¹⁶. At present, the study on relationship between high glucose and HF showed that the concentrations of IL-6, TNF- α and serum IL-1 are increased under high glucose¹⁷, thus activating HSC and leading to HF.

Insulin, as a multi-functional hormone protein, not only regulates the sugar, fat and protein metabolism in the body, but also binds to most specific receptors on the cell surface. In type 2 DM and hepatogenous DM, insulin resistance often exists and the insulin level increases, thus increasing the production of oxygen free radicals and inducing the release of inflammatory mediators, such as tumor necrosis factor- α (TNF- α), activating HSC and leading to HF.

The results of this experiment showed that in terms of inducing HSC DNA synthesis, HSC DNA in the five groups with insulin was significantly higher than that in the five groups without

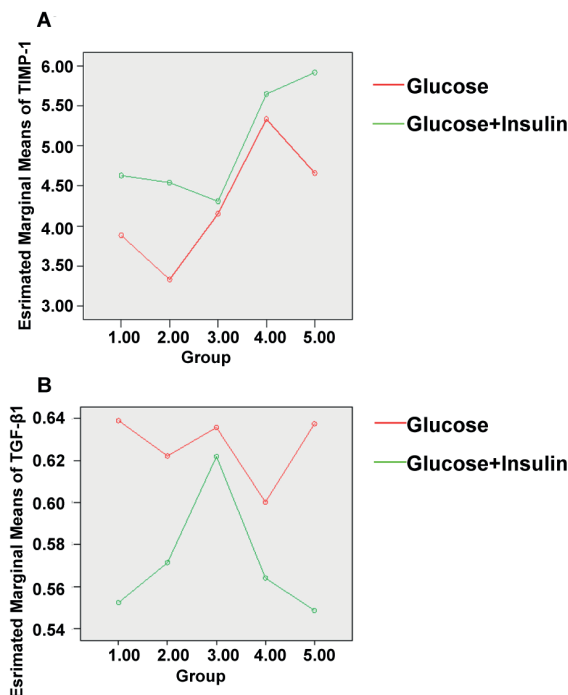


Figure 4. A, Trend chart of TIMP-1 expressions in glucose and insulin groups; B, Trend chart of TGF- β 1 expressions in glucose and insulin groups.

insulin, and high insulin could promote the HSC DNA synthesis under high osmotic pressure, indicating that there is a synergistic effect between high insulin and high glucose, and insulin alone can promote the HSC activation. However, in terms of inducing the HSC proliferation, the effect of high insulin combined with high glucose on HSC proliferation was consistent with that of high glucose alone. In addition, insulin could lead to the increased HSC DNA synthesis, but there was no increase in the number of cells; it is speculated that its mechanism may be that the high insulin stimulates the HSC activation and inhibits the TGF- β_1 secreted by HSC, thereby inhibiting the HSC proliferation.

TGF- β_1 can activate the static HSC and convert its phenotype to myofibroblasts (MFB), thus synthesizing a large number of ECM and forming the autocrine loop of TGF- β_1 ⁴. TIMPs are the major endogenous inhibitory factors in MMPs. In the process of HF, MMP-1 plays an important role in degrading the main components of ECM, type I and III collagen, in fibrous liver⁷, and TIMP-1 inhibits the degradation of ECM, type I and III collagen, through inhibiting the activity of MMPs, mainly MMP-1, thereby leading to the excessive deposition of ECM in the liver and promoting the formation and development of HF.

In this study, the effects of high glucose alone and high glucose + insulin on the mRNA expression of TGF- β_1 and TIMP-1 in HSC, were studied via RT-FQ-PCR. The results showed that the high glucose alone did not lead to the increased secretion of TGF- β_1 and TIMP-1 in HSC, but the mRNA expression of TGF- β_1 was decreased but the mRNA expression of TIMP-1 was increased after the insulin was added under the stimulation of high-concentration glucose. According to the above-mentioned speculation, it can be seen that the main mechanism of hepatogenous DM- and type 2 DM-induced HF is the non-TGF- β_1 pathway, but has a great relationship with the TIMP-1 pathway.

Conclusions

High glucose can induce the proliferation of rat HSC cultured *in vitro* and increase the DNA content. High insulin can increase the DNA content in rat HSC and has a synergistic effect with high glucose, but it does not increase the number of HSC. In addition, high insulin combined with high glucose can induce the proliferative HSC to secrete more TIMP-1 but less TGF- β_1 .

Conflict of interest

The authors declare no conflicts of interest.

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