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Simvastatin inhibites high glucose-induced renal tubular epithelial cells apoptosis by down-regulating miR-92a

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Abstract: This work aims to investigate the role of simvastatin (SIM) in renal tubular epithelial cells (HK-2) proliferation. The apoptosis model of HK-2 cells induced by high glucose was established; HK-2 cells were cultured in vitro and randomly divided into control group, model group, SIM low-dose group, SIM medium-dose group and SIM high-dose group. After 24 h culture, the inhibitory effect of SIM on high glucose- induced proliferation of HK-2 cells was evaluated by MTT method. The expression of cysteinyl aspartate specific proteinase (Caspase-3) in apoptosis-related protein was evaluated by Western blotting; miR-92a expression in HK-2 cells was measured by RT-qPCR. High glucose group had significantly lower HK-2 cell survival rate than the control group (p<0.05); SIM middle-dose and high-dose groups had higher HK-2 cell survival rate than the model group, (p<0.05); SIM low, medium and high-dose groups had lower HK-2 cell apoptosis rate, Caspase-3 protein and miR-92a expression levels than the model group (p<0.05), all showing dose-dependence.

Keywords: Simvastatin; miR-92a; renal tubular epithelial cells; apoptosis

INTRODUCTION

Diabetie nephropathy is a harmful diabetic microvascular complication (El-Horany *et al*., 2017). With the increasing incidence of diabetes, DN-induced chronic renal failure has become an important cause of death for diabetes patients (Bao *et al*., 2019). Under the stimulation of DN metabolic disorders, inflammatory factors and other factors, renal tubular epithelial cells can secrete cytokines, which participate in the occurrence and development of DN (Hu *et al*., 2019). The pathogenesis of DN is complex. Clinically, it is mainly treated by lowering blood glucose and blood pressure to delay the progression of the disease, but some patients' condition still cannot be effectively controlled. Statins is a recognized lipid-lowering drug (Zhang *et al*., 2019). Simvastatin (SIM) can inhibit recombinant human normal T cell expression and over expression of secretory factors, thereby protecting the kidney (Shen *et al*., 2013). However, the effect of simvastatin on apoptosis of renal tubular epithelial cells still remains unclear. Therefore, this study investigates such issue in order to provide theoretical reference for the clinical use of SIM in DN treatment.

MATERIALS AND METHODS

Reagents and Instruments

HK-2 was purchased from the Cell Center of CAS; SIM was purchased from Sigma, USA; penicillin and streptomycin were purchased from North China Pharmaceutical Co., Ltd., MTT were purchased from Beijing Yami Biotechnology Co., Ltd.; Cysteinyl aspartate specific proteinase and β-actin antibody were purchased from Wuhan AmyJet Scientific Inc.; optical microscope was purchased from Leica, Germany; microplate reader, carbon dioxide cell incubator, real-time fluorescence quantitative detection PCR instrument, flow cytometer were all purchased from Thermo Fisher, USA (Cooper *et al*., 2018).

HK-2 Cell Culture Experiment

The purchased HK-2 cells were placed in a -80℃ refrigerator for 24h, thawed in a 37℃ water bath, centrifuged (1200r/min, 5min) in a sterile environment, followed by removal of the supernatant was discarded. Subsequently, low-glucose/high-glucose DMEM medium was added, and the mixed solution was transferred into a culture bottle for incubation.

MTT Experiment

High glucose- induced cell activity experiment

Inoculate the cells in logarithmic growth phase in a 96-well plate. After the cells were fully adhered, HK-2 cells were treated with DMEM containing glucose (0, 5.5, 20, 35mmol/L) for 0 h and 24h and 6 replicate wells were set in each group. MTT solution was added for incubation, followed by discarding the supernatant. Then dimethyl sulfoxide was added to measure the OD value and calculate the cell survival rate.

Detection of cell activity after simvastatin treatment

According to the method of 1.3.1, the role of simvastatin on the activity of HK-2 cells was studied. A total of 6 groups including control group, model group, SIM low-dose group, SIM medium-dose group, SIM high-dose group were arranged and 6 parallels were set for each concentration. Cells were collected for subsequent research after 24 h culture.

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Apoptosis Experiment

The HK-2 cells collected in 1.3.2 were wash twice with pre-chilled PBS, followed by addition of Binding Buffer (500μL) to resuspend the cells. After mixing, the mixed solution was kept in the dark for 15min before conducting flow cytometry to calculate the apoptosis rate.

Western Blotting Experiment

The total protein of each group of cells collected in 1.3.2 was extracted using ristocetin-induced platelet aggregation lysate. After that, we performed protein quantification (BCA method), boiled for 5 min, loaded the sample, and transferred it to polyvinylidene fluoride membrane after separation by 10% SDS-PAGE, followed by blocking with 5% skimmed milk powder. Subsequently, Caspase-3 and β-actin antibody were added to incubate at 4℃ overnight, followed by membrane washing, and addition of secondary antibody for incubation.

RT-Qpcr Experiment

The total RNA of each groups of HK-2 cells collected in 1.3.2 was extracted using RNA extraction kit according to the steps of reverse transcription kit and RT-qPCR reaction instructions. Relative change of miRNA-92a was calculated using $2^{-\Delta\Delta CT}$ method (table 1).

STATISTICAL ANALYSIS

The test used for statistical methods, all data are represented by Mean±SD. The experimental data were processed with SPSS 22.0. The measurement data was described as $\overline{x} \pm s$. The different was considered significant when $p<0.05$.

RESULTS

High Glucose Inhibits the Proliferation of HK-2 Cells

In comparison to the control group, the survival rate of HK-2 cells in the low-glucose group was not statistically significant ($p>0.05$) and that in the medium-glucose group and high-glucose group was reduced ($p<0.05$). In comparison to the low-glucose group and medium-glucose group, high-glucose group had significantly lower survival rate of HK-2 cells ($p<0.05$) (table 2).

SIM on Survival Rate of HK-2 Cells

The model group had significantly higher HK-2 cell survival rate than the control group ($p<0.05$); SIM middle-dose and high-dose groups had higher HK-2 cell survival rate than the model group, ($p<0.05$) (table 3).

SIM on Apoptosis of HK-2 Cells

The model group had significantly higher HK-2 cell apoptosis rate than control group ($p<0.05$); SIM low, medium, and high-dose groups had lower HK-2 cell apoptosis rate than the model group ($p<0.05$) (fig. 1 and table 4).

SIM on the Expression of Caspase-3 in HK-2 Cells

The model group had significantly higher Caspase-3 protein expression than the control group, ($p<0.05$); SIM low, medium and high-dose groups had lower Caspase-3 protein expression than the model group, ($p<0.05$), showing dose-dependence (fig. 2 and table 5).

SIM on the Expression Level of miR-92a in HK-2 Cells

The model group had significantly higher miR-92a expression level than the control group ($p<0.05$). The SIM low, medium and high-dose groups had lower miR-92a expression level than the model group, ($p<0.05$) (table 6).

DISCUSSION

DN is a result of changes in pathological structure and function of the kidney of diabetic patients under the influence of physiological, genetic and environmental factors. In recent years, the prevalence of DN has been increased quickly in our country. It is not easy to diagnose early DN. Once it enters mass albuminuria period, it develops into terminal end-stage renal disease at a rate 14 times faster than other kidney diseases (Cheng *et al.*, 2015). Renal tubular interstitial fibrosis is an important factor that directly leads to the pathogenesis of DN (Xu *et al*., 2018). In a high glucose environment, under influence of metabolic disorders, inflammation, changes in hemodynamics and changes in urine composition, oxidative stress reactions will occur in renal tubular epithelial cells, thereby mediating DN occurrence and development (Li and Liu, 2018). The pathogenesis of DN is complex, and the existing research results are far from enough to solve DN. At present, DN treatment is to control blood glucose and pressure, reduce urinary protein and perform anticoagulation. However, specific treatment therapy is lacked. Therefore, investigation into DN pathogenesis and clinical exploration of ideal DN treatment modes mean important significance for early prevention of DN incidence and for improvement of survival rate in DN patients.

Statins can regulate metabolism in the body and reduce the mortality of cardiovascular diseases (Huang, 2016). Simvastatin belongs to a kind of statin. Liang Wenjian et al. (Liang and Wang, 2018) found that simvastatin combined with compound Qima capsules can significantly reduce the degree of vascular injury in patients with carotid atherosclerosis. Lin Jie *et al.* (Lin *et al*., 2019) showed that simvastatin can effectively improve inflammation and hemorheology dysfunction in patients with hypertension and dyslipidemia. Yu Dongdong et al. (Yu *et al*., 2019) applied simvastatin to osteoclasts and found that it can promote osteoclast apoptosis through the NFATc1 pathway. Li Huimei (Li, 2018) found that simvastatin application in the early treatment of DN can produce significant therapeutic effects and strengthen renal protection, but the action mechanism of simvastatin on DN remains unclear.

Table 1: MiR-92a, U6 primer sequences

Gene	Upstream Primer (5′--3′)	Downstream Primer (5′--3′)
miR-92a	CGCGGATCCTGGATTTAGTAAGATTTGGGC	CCGGAATTCACATGCAATTCAGGTCAGTG
U6	GTGCTCGCTTCGGCACATATAC	AAAAATATGGAACGCTCACGAATTTG

Table 2: The effect of glucose on the survival rate of HK-2(χ±s, n=6)

Group	Glucose concentration (mmol/L)	HK-2 survival rate (%)	
		0 h	24 h
Control group	0	101.87±3.15	106.84±5.72
Low-glucose group	5.5	102.98±9.31	102.58±7.67
Medium-glucose group	20	102.32±5.99	70.96±2.37^{abd}
High-glucose group	35	100.96±9.44	41.36±7.96^{abcd}

Note: Compared with control group, ap<0.05; Compared with Low-glucose group, bp<0.05; Compared with Medium-glucose group, cp<0.05; Compared with 0h, dp<0.05.

Table 3: The effect of SIM on HK-2 survival rate (χ±s, n=6)

Group	Glucose concentration (mmol/L)	SIM concentration (μmol/L)	HK-2 survival rate (%)	
			0 h	24 h
Control group	0	0	100.15±5.84	107.11±6.2
Model group	35	0	99.77±6.14	54.94±4.69^{a}
SIM low-dose group		2	100.22±6.31	60.84±5.17^{a}
SIM medium-dose group		4	101.23±8.02	82.98±5.70^{abc}
SIM high-dose group		6	102.37±7.41	89.36±6.67^{abcd}

Note: Compared with Control group, ap<0.05; Compared with Model group, bp<0.05; Compared with SIM low-dose group, cp<0.05; Compared with SIM medium-dose group, dp<0.05.

Table 4: Comparison of apoptosis rate of HK-2 cells (χ±s, n=6)

Group	Glucose concentration (mmol/L)	SIM concentration (μmol/L)	HK-2 cell apoptosis rate (%)
Control group	0	0	2.13±1.02
Model group	35	0	45.91±6.11^{a}
SIM low-dose group		2	37.44±5.13^{ab}
SIM medium-dose group		4	26.98±6.01^{abc}
SIM high-dose group		6	10.34±3.79^{bcd}

Table 5: Caspase-3 protein expression in HK-2 cells (χ±s, n=6)

Group	Glucose concentration (mmol/L)	SIM concentration (μmol/L)	Caspase-3/β- actin
Control group	0	0	0.31±0.04
Model group	35	0	1.59±0.06^{a}
SIM low-dose group		2	1.21±0.10^{ab}
SIM medium-dose group		4	0.73±0.07^{abc}
SIM high-dose group		6	0.47±0.04^{abcd}

Table 6: MiR-92a expression in HK-2 cells ($\bar{\chi}$±s, n=6)

Group	Glucose concentration (mmol/L)	SIM concentration (μmol/L)	miR-92a/U6
Control group	0	0	1.03±0.19
Model group	35	0	2.51±0.23^{a}
SIM low-dose group		2	1.97 ±0.17^{ab}
SIM medium-dose group		4	1.46±0.11^{abc}
SIM high-dose group		6	1.09±0.21^{bcd}

Note: Compared with Control group, ap<0.05; Compared with Model group, bp<0.05; Compared with SIM low-dose group, cp<0.05; Compared with SIM medium-dose group, dp<0.05.

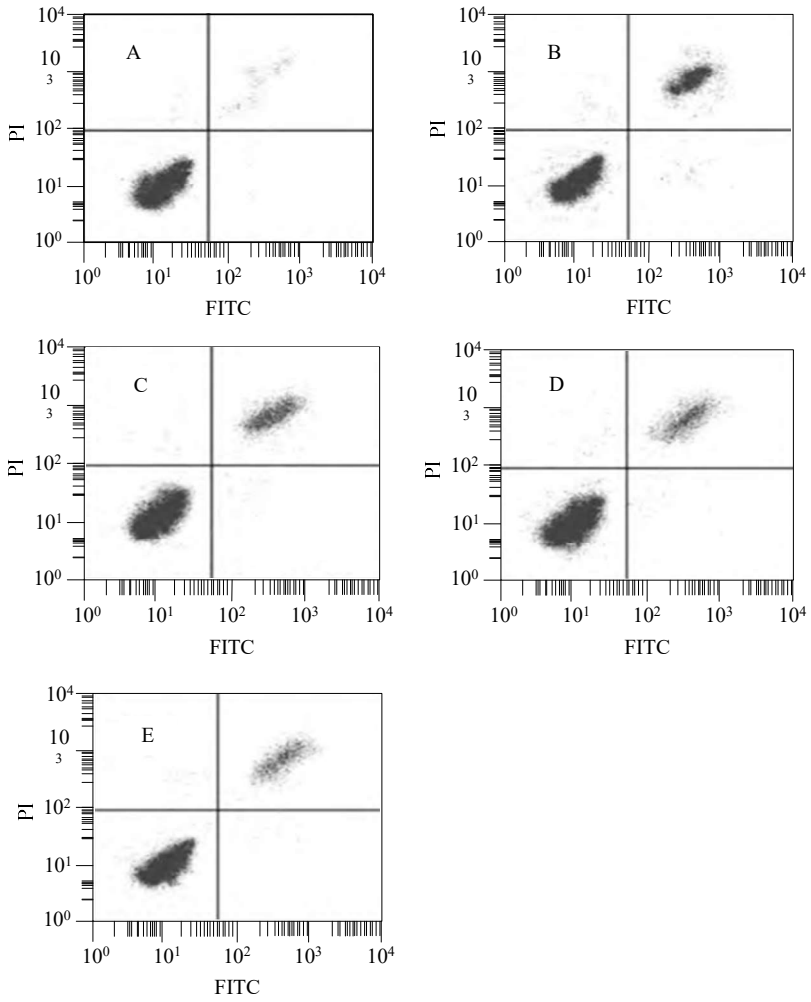

Fig. 1: SIM on high glucose-induced apoptosis of HK-2 cells

It was found that at 24h, the model group had significantly higher HK-2 cell survival rate than the control group, suggesting that glucose can effectively inhibit the survival of HK-2 cells; the SIM medium-dose and high-dose groups had lower HK-2 cell survival rate than model group, suggesting that medium and high concentrations of simvastatin can promote high glucose-induced growth of HK-2 cells, so it may act as one of the effective drugs for clinical treatment of DN.

The Caspase protein family is of great importance to the apoptosis of mammalian cells. Caspase-3 can promote apoptosis after activation. Detection of Caspase-3 expression in cells can determine cell apoptosis status (Yang *et al*., 2018). In this work, the model group had significantly higher HK-2 cell apoptosis rate and Caspase-3 protein expression levels than the control group, suggesting that high glucose can induce HK-2 cell apoptosis. SIM low, medium and high-dose groups had lower HK-2 cell apoptosis rate and Caspase-3 protein expression level than the model group.

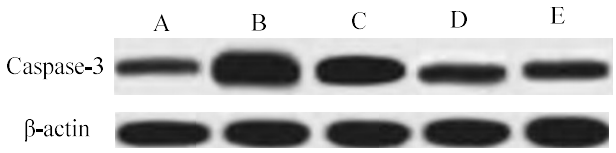

Fig. 2: Electrophoretogram of Caspase-3 protein expression in HK-2 cells

MiR-92a as a type of microRNA (miRNA) is involved in physiological activities such as cell inflammatory response. Blocking miR-92a expression can reduce endothelial inflammation damage and inhibit cervical cancer cell growth (Hu et al., 2018; Wang et al., 2019). Sun et al. (Sun et al., 2019) found that alkanes can promote the apoptosis of hepatoma carcinoma cells and exert anti-tumor activity by down-regulating miR-92a. However, it has not been reported whether simvastatin inhibits HK-2 cell apoptosis pathway in a way associated with miR-92a. Through RT-qPCR, it was found that the model group had significantly increased miR-92a

expression level in the HK-2 cells than the control group (Meng and Yang, 2018). SIM low, medium and high-dose groups had lower miR-92a expression level in HK-2 cells than the model group, indicating that simvastatin can dose-dependently down-regulate miR-92a expression level.

CONCLUSION

Simvastatin can inhibit HK-2 cells apoptosis by down-regulating miR-92a expression. This provides theoretical reference for clinical application of simvastatin in DN treatment. However, this paper only made a preliminary exploration into action mechanism of simvastatin through *in vitro* studies, so further in-depth researches are needed.

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