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Effect of *Polygonatum odoratum* ethanol extract on high glucose-induced tubular epithelial cell apoptosis and oxidative stress

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Abstract: This work aims to analyze the effect of the ethanol extract from *Polygonatum odoratum* on high glucose-induced tubular epithelial cell apoptosis and oxidative stress. HK-2 injury of tubular epithelial cells was induced by high glucose, and the ethanol extract from *Polygonatum odoratum* was given. HK-2 cell activity and apoptosis were detected by MTT method and flow cytometry, respectively. Western blot was performed to analyze Cleaved-caspase3, Pro-caspase3, Nrf2, HO-1 protein expression. The levels of MDA, GSH, SOD were evaluated using commercial Kit. si-Nrf2 was transfected into HK-2 cells and high-glucose induction and ethanol extract from *Polygonatum odoratum* were given to observe the changes of cell apoptosis and oxidative stress. Ethanol extract from *Polygonatum odoratum* increased the high glucose-induced HK-2 cell activity, Pro-caspase3, Nrf2, HO-1 protein, GSH, SOD levels and decreased its apoptosis rate, Cleaved-caspase3 protein and MDA levels, showing statistically significant difference ($p<0.05$). After Nrf2 interference, high glucose-induced HK-2 cell activity, Pro-caspase3 protein, GSH, and SOD levels were decreased under the action of ethanol extract from *Polygonatum odoratum*, while the apoptosis rate, Cleaved-caspase3 protein, and MDA levels were increased significantly ($p<0.05$). The ethanol extract from *Polygonatum odoratum* can inhibit high glucose-induced tubular epithelial cell apoptosis and reduce oxidative stress by activating the Nrf2-ARE signaling pathway.

Keywords: Ethanol extract from *Polygonatum odoratum,* tubular epithelial cells, Nrf2-ARE signaling pathway, apoptosis, oxidative stress.

INTRODUCTION

Tubular epithelial cell apoptosis can be used to indicate the progression of kidney disease (Wei and Szeto, 2019). High glucose-induced oxidative stress significantly affects the pathogenesis of diabetic nephropathy, so the treatment of high glucose-induced proximal tubule injury has attracted an increasingly attention (Liu *et al*., 2020; Tong *et al*., 2018). The dried rhizome of *Polygonatum odoratum*, a Liliaceous plant, is a common clinical drug for the treatment of diabetes, which nourishes yin to moisturize dryness, engenders liquid to allay thirst. Its ethanol extract and chloroform separation parts have significant kidney protection function in diabetic rats (Shi *et al*., 2007). In the diabetic model rats induced by streptozotocin, the extract from *Polygonatum odoratum* can alleviate rat symptoms and treat diabetic nephropathy. Nrf2-ARE signaling pathway is a key pathway involved in diabetic nephropathy. Resveratrol can improve oxidative stress by enhancing Nrf2-ARE pathway activity, thereby protecting the kidneys of diabetic nephropathy mice (Gao *et al*., 2019). However, the effect of ethanol extract from *Polygonatum odoratum* on the high glucose-induced tubular epithelial injury and whether it plays a role through Nrf2-ARE signaling pathway remain unknown. In this study, the effect of ethanol extract from *Polygonatum odoratum* on the function is studied to provide new enlightenment and clues for the application of *Polygonatum odoratum* as clinical drug.

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MATERIALS AND METHODS

Drugs, Cells and Reagents

Polygonatum odoratum was derived from a hospital pharmacy and identified. After crushing the medicinal materials, add 5 times amount of 80% absolute ethanol, reflux for 2h, repeat 3 times, combine the filtrate of 3 refluxes, and concentrate on a rotary evaporator under reduced pressure to obtain a brown extract (yield 25%), which is the ethanol extract from *Polygonatum odoratum*. The ethanol extract from *Polygonatum odoratum* was stored in a refrigerator at 4°C and diluted with culture medium.

DMEM medium was purchased from Gibco, USA, MTT was purchased from Beijing Sunshine Co., Ltd, radioimmuno precipitation assay buffer, malondialdehyde (MDA) kit, bicinchoninic acid protein assay kit, horseradish peroxidase conjugate goat anti-rabbit IgG were purchased from Jiangsu Beyotime Institute of Biotechnology, Nrf2 small interfering RNA si-Nrf2, negative control si-NC were purchased from Guangzhou RiboBio, SOD and GSH-Px kits were purchased from Nanjing Jiancheng Bioengineering Company, RT-PCR kit was purchased from Guangzhou Dingguo Biotechnology Co., Ltd., polyvinylidene fluoride membrane and chemiluminescence reagents were purchased from EMD

Millipore, USA, anti-activation (Cleaved)-aspartic acid specific cysteine protease 3 (caspase3), anti-Pro-caspase3, anti-Nrf2, anti-heme oxygenase (HO-1) primary antibodies were purchased from Abcam, Shanghai.

Cell Culture and Experiment Grouping

HK-2 cells were routinely cultured in DMEM medium containing 10% fetal bovine serum under humid condition at temperature of 37°C with 5% CO2 in air. At the logarithmic growth phase of HK-2 cells, the designated treatments were performed for control group (HK-2 cells), model group (30 mmol/L glucose treatment of HK-2 cells for 8h), low-dose group (after HK-2 cell treatment for 24 h by 12.5μg/mL ethanol extract from *Polygonatum odoratum*, 30mmol/L glucose treatment for 8 h), medium-dose group (after HK-2 cell treatment for 24h by 25μg/mL ethanol extract from *Polygonatum odoratum*, 30 mmol/L glucose treatment for 8 h), high-dose group (after HK-2 cell treatment for 24h by 50μg/mL ethanol extract from *Polygonatum odoratum*, 30mmol/L glucose treatment for 8h, si-NC group (HK-2 cell transfection with si-NC for 24h), si-Nrf2 group (HK-2 cell transfection with si-Nrf2 for 24 h), high-dose drug + si-NC group (HK-2 cell transfection with si-NC, treatment by 50 μg/mL ethanol extract from *Polygonatum odoratum* for 24 h and 30 mmol/L glucose treatment for 8 h), high-dose drug + si-Nrf2 (HK-2 cell transfection with si-Nrf2, treatment by 50 μg/mL ethanol extract from *Polygonatum odoratum* for 24 h and 30 mmol/L glucose treatment for 8 h). The transfection of HK-2 cells into si-Nrf2 and si-NC was performed using Lipofectamine 2000 transfection reagent (Yang and Zhou, 2019; Wang *et al*., 2019).

Detection of HK-2 Cell Activity

3×10^5 HK-2 cells were incubated for 48h in a 96-well plate, followed by addition of 20 μL MTT dye solution for another 3-4 h of incubation. Then, the supernatant was removed, added with dimethyl sulfoxide (200μL) and then shaked for 20 min before measuring cell viability (Chen *et al*., 2019).

Detection of HK-2 Cell Apoptosis

HK-2 cells were resuspended in binding buffer and incubated with Annexin V-FITC and propidium iodide (PI) at room temperature. Finally, BD FASCCalibur analyzed the cells using flow cytometry. The total apoptosis rate (%) is defined as the sum of the upper and lower right quadrants of the flow cytometry dot chart, namely the sum of early apoptosisand late apoptosis.

Western Blot Analysis of Cleaved-caspase3, Pro-caspase3, Nrf2, HO-1 Protein Expression

HK-2 cells were lysed in radioimmuno precipitation assay buffer containing a mixture of protease and phosphatase inhibitors, placed on ice for 40 min and centrifuged at 15000×g at 4°C for 20 min before quantifying the protein concentration. Afterwards, 40μg protein sample was separated by SDS-PAGE and then transferred to a polyvinylidene fluoride membrane.

Kit detection of the levels of oxidative stress indicators MDA, GSH and SOD

Collect HK-2 cells to be tested in each group, refer to the operation manual of MDA, GSH, SOD kits, and detect the levels of oxidative stress indicators MDA, GSH, SOD in the cell supernatant (Lin *et al*., 2020).

RT-PCR Detection of Nrf2 mRNA Expression

Trizol reagent (1 mL) was added to HK-2 cells to extract total RNA, and RNA concentration was measured in a nucleic acid detector (Yang *et al*., 2019). Afterwards, Nrf2 mRNA expression was detected according to the instructions of RT-PCR kit. Nrf2 mRNA expression was calculated according to the threshold cycle $2^{-\Delta\Delta Ct}$ method. See attached table 1 for primer information (Meng and Young, 2018).

Table 1: RT-PCR primer sequence

Name	Sequence (5'-3')	Product length
Nrf2	forward CACATTCCCAAACAAGATGC	374 bp
	ReverseTCTTTTTCCAGCGAGG AGAT	
β-actin	forward CTCCATCCTGGCCTCGCTGT	268 bp
	Reverse GCTGTCACCTTCACCGTTCC	

Table 2: Effect of ethanol extract from *Polygonatum odoratum* on high glucose-induced HK-2 cell activity

Group	OD value
Control group	1.16±0.04
Model group	0.44±0.01[a]
Low-dose group	0.45±0.01[a]
Medium-dose group	0.72±0.02[abc]
High-dose group	0.98±0.03[abcd]
F	493.548
p	0.000

Note: In comparison to the control group, [a]p<0.05; In comparison to the model group, [b]p<0.05; In comparison to the low-dose group, [c]p<0.05; In comparison to the medium-dose group, [d]p<0.05.

STATISTICAL ANALYSIS

All experimental data was statistically analyzed in SPSS 22.0 software and expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to analyze the differences between multiple groups, t test was used to analyze the differences between the two groups and SNK-q test was used for pair-wise

Table 3: Effect of ethanol extract from *Polygonatum odoratum* on high glucose-induced HK-2 apoptosis

Group	Apoptosis rate (%)	Cleaved-caspase3	Pro-caspase3
Control group	7.29±0.26	0.22±0.01	0.62±0.03
Model group	23.72±0.44[a]	0.68±0.03[a]	0.15±0.01[a]
Low-dose group	23.76±0.46[a]	0.69±0.03[a]	0.15±0.01[a]
Medium-dose group	19.01±0.30[abc]	0.51±0.02[abc]	0.30±0.02[abc]
High-dose group	12.19±0.26[abcd]	0.32±0.02[abcd]	0.52±0.03[abcd]
F	1261.866	247.389	287.938
p	0.000	0.000	0.000

Table 4: Effect of ethanol extract from *Polygonatum odoratum* on high glucose-induced HK-2 oxidative stress

Group	MDA(nmol/mg)	GSH(mg/L)	SOD(U/mg)
Control group	2.31±0.14	25.97±0.27	4.49±0.14
Model group	6.66±0.15[a]	8.12±0.17[a]	1.66±0.09[a]
Low-dose group	6.63±0.16[a]	8.17±0.15[a]	1.68±0.09[a]
Medium-dose group	4.55±0.12[abc]	15.50±0.17[abc]	2.12±0.08[abc]
High-dose group	3.47±0.11[abcd]	21.05±0.23[abcd]	3.82±0.12[abcd]
F	589.615	4517.349	457.309
p	0.000	0.000	0.000

Table 5: Expression of Nrf2-ARE signal pathway in HK-2

Group	Nrf2	HO-1
Control group	0.10±0.01	0.05±0.01
Model group	0.23±0.02[a]	0.16±0.01[a]
Low-dose group	0.24±0.01[a]	0.17±0.01[a]
Medium-dose group	0.39±0.02[abc]	0.28±0.02[abc]
High-dose group	0.61±0.03[abcd]	0.48±0.02[abcd]
F	299.448	360.955
p	0.000	0.000

Note: In comparison to the control group, [a]p<0.05; In comparison to the model group, [b]p<0.05; In comparison to the low-dose group, [c]p<0.05; In comparison to the medium-dose group, [d]p<0.05.

Table 6: Interference with Nrf2 expression

Group	Nrf2 mRNA
si-NC	0.98±0.04
si-Nrf2	0.22±0.01a
t	31.926
p	0.000

Note: In comparison to si-NC group, [a]p<0.05.

comparisons. The p<0.05 indicates statistically significant difference.

RESULTS

Effect of Ethanol Extract from Polygonatum odoratum on High Glucose-induced HK-2 Cell Activity

As shown in table 2, the high-dose group had significantly lower high glucose-induced HK-2 cell activity than the other four groups (p<0.05). The medium and high-dose groups had significantly increased high glucose-induced HK-2 cell activity than control group (p<0.05), while low-dose group had no different with control group. The medium and high-dose groups had significantly increased high glucose-induced HK-2 cell activity than low-dose group (p<0.05). The high-dose group has significantly increased high glucose-induced HK-2 cell activity than the medium-dose group (p<0.05).

Effect of Ethanol Extract from Polygonatum odoratum on High Glucose-induced HK-2 Apoptosis

According to table 3 and fig. 1, high-dose groups had increased high glucose- induced HK-2 cell apoptosis rate, increased Cleaved-caspase3 protein level and decreased Pro-caspase3 protein level than the other four groups (p<0.05). In comparison to the model group, low-dose group had no statistically significant difference in high glucose-induced HK-2 cell apoptosis rate, Cleaved-

Table 7: Effect of Nrf2 interference on high glucose-induced activity and apoptosis of HK-2 cells with ethanol extract from *Polygonatum odoratum*

Group	OD value	Apoptosis rate (%)	Cleaved-caspase3	Pro-caspase3
High-dose drug +si-NC	0.98±0.03	12.21±0.26	0.33±0.02	0.52±0.03
High-dose drug +si-Nrf2	0.54±0.02[b]	21.86±0.36[b]	0.59±0.02[b]	0.22±0.02[b]
t	21.137	37.639	15.922	14.412
p	0.000	0.000	0.000	0.000

Table 8: Effect of Nrf2 interference on high glucose-induced HK-2 oxidative stress with ethanol extract from *Polygonatum odoratum*

Group	MDA(nmol/mg)	GSH(mg/L)	SOD(U/mg)
High-dose drug+si-NC	3.50±0.12	21.11±0.27	3.81±0.09
High-dose drug+si-Nrf2	5.26±0.11b	11.88±0.20b	1.75±0.05b
t	18.726	47.579	34.656
p	0.000	0.000	0.000

Note: In comparison to high-dose drug + si-NC group, [b]p<0.05.

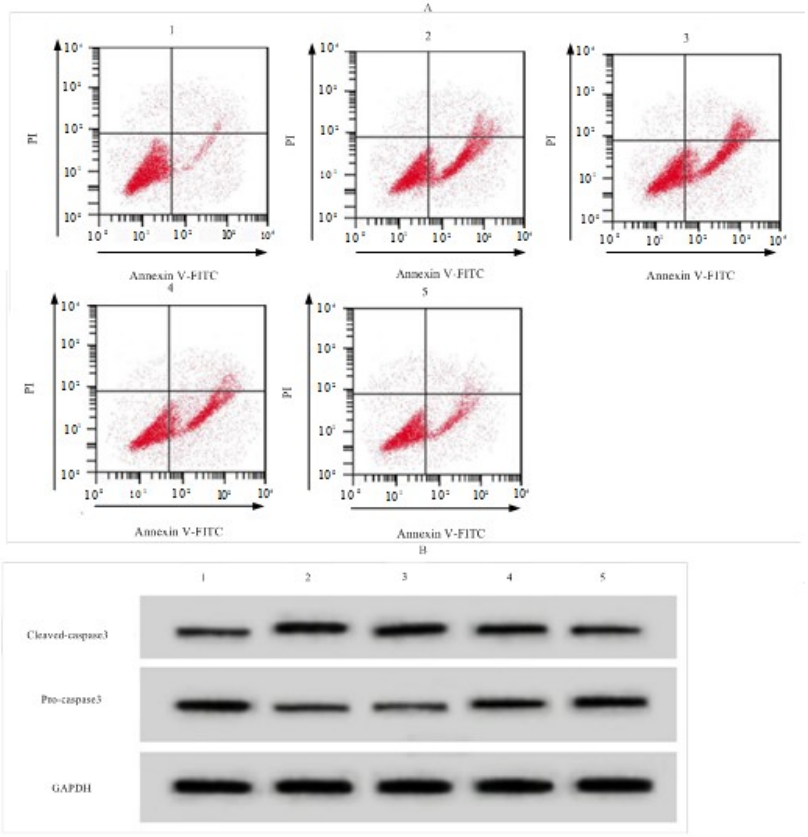

Fig. 1: Effect of ethanol extract from *Polygonatum odoratum* on high glucose-induced HK-2 apoptosis and caspase3 protein expression (1: Control group; 2: Model group; 3: Low-dose drug group; 4: Medium-dose drug group; 5: High-dose drug group).

caspase3 and Pro-caspase3 protein expression; the medium and high-dose groups had significantly reduced high glucose-induced HK-2 cell apoptosis, decreased Cleaved-caspase3 protein level, and increased Pro-caspase3 protein level (p<0.05). In comparison to the low-dose group, the medium and high-dose groups had significantly reduced high glucose- induced HK-2 cell apoptosis rate, reduced Cleaved-caspase3 protein level and increased Pro-caspase3 protein level (p<0.05). In comparison to the medium-dose group, the high-dose group had significantly reduced high glucose-induced HK-2 cell apoptosis rate, decreased Cleaved-caspase3 protein level, and increased in the Pro-caspase3 protein level, showing statistically significant difference (p<0.05).

Effect of ethanol extract from Polygonatum odorate on high glucose-induced hk-2 oxidative stress

According to table 4, high-dose groups had increased MDA level in HK-2 cells, decreased GSH and SOD levels than the other four groups ($p<0.05$). In comparison to model group, low-dose group had no significant differences in these indexes, while the medium and high-dose groups had significantly reduced MDA level in HK-2 cells and increased GSH, SOD levels ($p<0.05$). In comparison to low-dose group, the medium and high-dose groups had significantly decreased MDA level in HK-2 cells, and increased GSH, SOD levels ($p<0.05$).

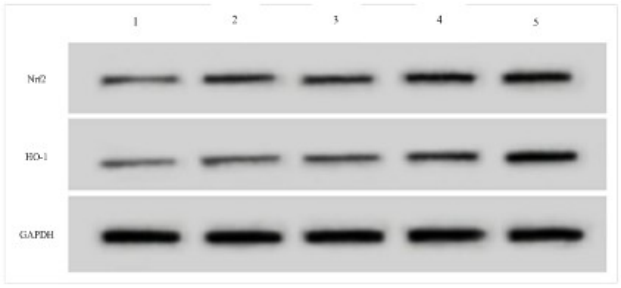

Fig. 2: Expression of Nrf2 and HO-1 protein (1: Control group; 2: Model group; 3: Low-dose drug group; 4: Medium-dose drug group; 5: High-dose drug group)

Effect of Ethanol Extract from Polygonatum odoratum on High Glucose-Induced Nrf2-ARE Signaling Pathway in HK-2 Cells

According to table 5 and fig. 2, high-dose groups had significantly increased high glucose-induced Nrf2 and HO-1 protein levels in HK-2 cells in comparison to the other four groups ($p<0.05$). In comparison to the model group, the low-dose group had no statistically significant differences in these indexes ($p<0.05$). In terms of the glucose-induced Nrf2 and HO-1 protein levels in HK-2 cells among the low, medium and high dose groups, the low-dose group ranked the lowest, the medium and high-dose groups ranked in the middle ($p<0.05$), the high-dose group ranked the highest ($p<0.05$).

Effect of Nrf2 Interference on High Glucose-induced Activity and Apoptosis of HK-2 Cells with Ethanol Extract from Polygonatum odoratum

As shown in table 6, table 7 and fig. 3, si-Nrf2 group had significantly lower Nrf2 mRNA level than si-NC group ($p<0.05$), suggesting that it successfully interfered with Nrf2 expression. In comparison to the high-dose drug +si-NC group, the high-dose drug+si-Nrf2 group had significantly decreased high glucose-induced HK-2 cell activity, increased apoptosis rate, Cleaved-caspase3 protein level and decreased Pro-caspase3 protein level ($p<0.05$).

Effect of Nrf2 interference on high glucose-induced hk-2 oxidative stress with ethanol extract from Polygonatum odoratum

As shown in table 8, in comparison to the high-dose drug + si-NC group, the high-dose drug + si-Nrf2 group had significantly increased high glucose-induced MDA levels and decreased GSH and SOD levels in HK-2 cells ($p<0.05$).

DISCUSSION

Oxidative stress and apoptosis are important mechanisms of high glucose injury in diabetic nephropathy (He *et al*., 2019). Persistent high glucose will increase the production of reactive oxygen species so that it exceeds endogenous antioxidants in quantity, thereby leading to MDA production and subsequent cell injury (Tang *et al*., 2018). SOD and GSH are important antioxidant defense systems. MDA, SOD and GSH are three biomarkers used to detect oxidative stress in diabetic nephropathy (Huang and Han, 2020). In this study, exposure of HK-2 cells to glucose led to decreased cell viability, apoptosis,

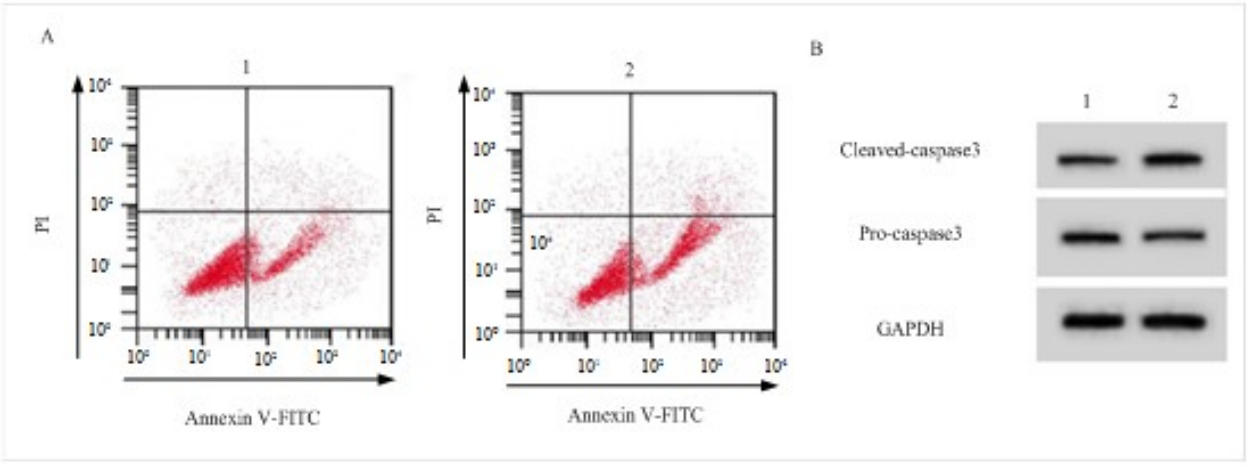

Fig. 3: Effect of Nrf2 interference on high glucose-induced HK-2 apoptosis and caspase3 protein expression with ethanol extract from *Polygonatum odoratum* (1: High-dose drug + si-NC; 2: High-dose drug + si-Nrf2)

increased oxidative stress, which was verified by increased MDA levels, Cleaved-caspase3 expression levels, SOD and GSH levels in the cell supernatant and decreased Pro-caspase3 expression level. This confirms that this model can effectively simulate oxidative stress-induced kidney injury by incubating HK-2 cells with glucose, and supports the concept that high glucose promotes oxidative stress and aggravates diabetic nephropathy. Therefore, drugs for oxidative stress provide a promising treatment strategy for tubular epithelial injury in diabetic nephropathy.

Polygonatum odoratum contains chemical substances such as flavonoids, saponins, polysaccharides. Studies have shown that in the rat diabetic model stimulated by streptozotocin, flavonoids from *Polygonatum odoratum* can improve oxidative stress of diabetic rats by inhibiting MDA production and promoting SOD activity, which has a certain preventive and therapeutic effect on diabetic nephropathy. Polysaccharides in *Polygonatum odoratum* can alleviate streptozotocin-induced diabetes in mice by inhibiting cytokines (Liu and Hu, 2009), and can also show antioxidant and hypoglycemic effects on glucose and lipid metabolism of diabetic rats (Zhu *et al*., 2008). Another study indicates that *Polygonatum odoratum* extract reverses streptozotocin-induced blood glucose increase in diabetic mice by regulating immunity (Zhang *et al*., 2012). However, the effect of ethanol extract from *Polygonatum odoratum* on high-glucose injury of tubular epithelial cells remains unknown. Therefore, this study investigated such issue. The results reveal that the ethanol extract from *Polygonatum odoratum* significantly increased high glucose-induced HK-2 cell activity, Pro-caspase3 protein, GSH and SOD levels, and reduced high glucose-induced HK-2 cell apoptosis rate, Cleaved-caspase3 Protein and MDA levels, indicating that *Polygonatum odoratum* extract protects tubular epithelial cells against high glucose injury by improving cell activity, inhibiting apoptosis and reducing oxidative stress.

Ethanol extract from *Polygonatum odoratum* can activate Nrf2 and its downstream antioxidant gene HO-1 in high glucose-induced HK-2 cells. As a transcription factor of endogenous antioxidant defense system, Nrf2 protein can reduce kidney cell injury in diabetic patients, thereby slowing down the process of diabetic nephropathy. One potential mechanism in Nrf2's protective effect is to inhibit oxidative stress (Jiang and Liu, 2020). Nrf2 is considered as a therapeutic target for diabetic nephropathy, which mediates various drugs to alleviate the process of diabetic nephropathy, such as baicalin (Yin *et al*., 2020), eucommia flavonoids (Xu *et al*., 2020), resveratrol. In this experiment, the ethanol extract from *Polygonatum odoratum* became a new Nrf2 activator in high glucose-induced tubular epithelial cells by activating the Nrf2-ARE pathway. It is because Nrf2 interference reverses the effect of ethanol extract from *Polygonatum odoratum* in promoting high glucose-induced HK-2 cell activity, Pro-caspase3 protein, GSH, SOD levels, and reverses its effect in inhibiting apoptosis, Cleaved-caspase3 protein, and MDA levels (Cooper *et al*., 2018).

CONCLUSION

To sum up, the ethanol extract from *Polygonatum odoratum* can increase high glucose-induced activity of tubular epithelial cells, inhibit apoptosis and reduce oxidative stress. In addition, the ethanol extract from *Polygonatum odoratum* can avoid high glucose-induced injury of tubular epithelial cells by regulating the Nrf2-ARE pathway.

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