This study was designed to investigate the effect of resveratrol on the proliferation and apoptosis of renal tubular epithelial cells in a high glucose environment and explore the underlying mechanism. Four experimental groups were set up. Group A was treated with normal glucose (5 mM), group B was treated with normal glucose (5 mM) and resveratrol, group C was treated with high glucose (30 mM) and resveratrol, and group D was treated with high glucose (30 mM). HK-2 cells which were cultured in vitro were selected. Cell proliferation was detected using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide assay, while cell apoptosis was measured using Terminal-deoxynucleoitidyl Transferase Mediated Nick End Labelling. Reactive oxygen species were detected by direct centrifugal flotation. It was found that resveratrol could inhibit the proliferation and apoptosis of HK-2 cells under high glucose. Cells under normal glucose had decreased proliferation and apoptosis than cells under high glucose. The cell proliferation and apoptosis rates in group C were significantly different from those in group D (20% vs. 48%; 2.2% vs. 14.7%) (p < 0.05). The inhibitory effect on reactive oxygen species was the strongest when the concentration of resveratrol was 10 µM. The addition of resveratrol under high glucose condition resulted in a remarkable decrease in the expression of α-SMA and Fibronectin i.e. Epithelial-Mesenchymal Transition, in group C and D (p < 0.05). It is concluded that resveratrol can inhibit the proliferation and apoptosis of renal tubular epithelial cells under high glucose condition and the generation of reactive oxygen species and Epithelial-Mesenchymal Transition, which may play a role in the treatment of diabetic nephropathy.
Factors that contribute to the renal damage in diabetics include, hyperglycemia, hypertension, positive family history of nephropathy and hypertension, and smoking. Therefore, key elements in the primary care of diabetes must include glycemic control, blood pressure control, and screening for microalbuminuria (Ahmad, 2015). Although the mechanism underlying tissue damage in DN is not fully understood, studies on diabetic rats displayed tissue hypoxia throughout the kidney, glomerular hyperfiltration, increased oxygen consumption, increased total mitochondrial leak respiration, and decreased tubular sodium transport efficiency (Nordquist et al., 2015). Activation of mitochondrial reactive oxygen species (ROS) and non-myeloid cell NLRP3 inflammatory body has been shown to aggravate diabetic nephropathy (Shahzad et al., 2015; Kamiyama et al., 2013; Jung et al., 2015). Based on these observations, control of inflammation has been suggested as one approach for prevention of DN.

Resveratrol is a natural plant polyphenol known to diminish oxidative damage and tumor inflammations (Szkudelska and Szkudelska 2015). Therefore, we have investigated the effect of resveratrol on the proliferation and apoptosis of renal tubular epithelial cells in a high glucose environment and explored the underlying mechanism.

**MATERIALS AND METHODS**

**Materials**

The experimental materials include: Anti-Fade solution, D-glucose (Wuhan Procell Life Science & Technology Co., Ltd., China), solution (Shanghai Kemin Biotech Co., Ltd., China), resveratrol (Shanghai Jimian Industrial Co. Ltd., China), fetal bovine serum culture solution (Shanghai Yubo Biotech Co., Ltd., China), phosphate buffered saline (PBS), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) solution, serum-free culture medium, equilibration buffer (Thermo Fisher Scientific, USA), dimethylsulfoxide (Chengbang Chemical Co., Ltd., China), paraformaldehyde, propidium iodide solution (Jianglan Pure Biological Reagent Co., Ltd., China), pancreatin digestion cell solution (Chinese Equipment Website) and 2', 7'-Dichlorodihydrofluorescin diacetate (DCFH-DA) medium (Beijing Chongqingyuan filter equipment Co., Ltd., China).

A 1M stock glucose solution was prepared in de-ionized water, sterilized ultraviolet for 30 min and stored at -20 °C under sterile conditions in 1.5 ml aliquots. A 100 mM stock resveratrol solution was prepared in DMSO and stored at -20 °C under sterile conditions in 1.5 ml aliquots.

**Cell Culture and treatment**

The human proximal tubular epithelial cells (HK-2) (ATCC Cell Bank, USA) were inoculated into low-glucose Dulbecco’s modified eagle medium (DMEM) containing 10% fetal bovine serum and transferred into an incubator (5% CO₂, 37 °C). The culture medium was exchanged every 48h until the cells reached confluency. Then, 0.25% trypsin was used for digestion and passage of cells. Cells were divided into four treatment groups.

- **Group A**: control group, cells grown in medium containing normal (low) glucose (5 mM)
- **Group B**: cells grown in medium containing normal (low) glucose (5 mM) + resveratrol (10 µM)
- **Group C**: cells grown in medium containing high glucose (30 mM) + resveratrol (10 µM)
- **Group D**: cells grown in medium containing high glucose (30 mM).

**Cell proliferation test with MTT**

A 96-well culture plate was inoculated with 5,000 cells/well and cultured in DMEM containing 10% fetal bovine serum until cells attached to the plate. Twenty-four hours later, cells were switched to DMEM containing 1% fetal bovine serum and cultured at room temperature until cells reached confluence. Then the supernatant was removed and cells were divided into 4 groups for treatment as described earlier for Group A to Group D with 6 wells per treatment group. In addition, a group of 6-wells before initiation of any treatment were set aside as zero time control. After 24h, the culture medium was removed, and the cells were washed with PBS. A 100 µl of PBS and 20 µl of MTT solutions were added to each well, and then the plate was incubated. Five hours later, 150 µl of DMSO was added to each well and vibrated for 10 minutes. Then the optical density value was measured at 490 nm.

**Cell apoptosis test with TUNEL**

Six wells were inoculated with 5,000 cells/well and cultured in a serum-free medium. Twelve hours later, the medium was removed. The cells were then washed using PBS thrice, 10 min each time. Then the cells were fixed using 4% paraformaldehyde (PH 7.4) for 30 minutes at 4°C, followed by five times of washing with PBS, 5 minutes each time. Next, the cells were incubated using 3% H₂O₂-methanol at room temperature. After 15min, the peroxidase was removed, followed by PBS washing. The glass slide was immersed with 0.2% Triton X-100 solution containing 0.1% sodium citrate solution for five minutes. Next, the cells were washed using PBS. A 100 µl of PBS and 20 µl of TdT labeling buffer were added to each well, and then the plate was incubated. Five hours later, 150 µl of DMSO was added to each well and vibrated for 10 minutes. Then the optical density value was measured at 490 nm.
photographed under a fluorescence microscope and the rate of apoptosis was calculated.

**Cell reactive oxygen species detection**

Cells were grown to confluency in six culture bottles. One ml of 0.25% trypsin was added to each bottle and the bottle was shaken gently and periodically examined under microscope. When cells turned round, 3 ml of medium was added to stop digestion followed by centrifugation at 1200 rpm for 5 minutes. Cells were gently mixed with 3 ml of culture medium to obtain cell suspension and cell count obtained. Then the cells were inoculated (1,000 cells/well) to a 96-well plate and 100 µl of culture medium was added. The cells were mixed using crossing method. Five hours later, the cells were observed under a microscope. When cell attached to the plate, the medium was removed, and the cells washed using fetal calf serum-free medium. 2', DCFH-DA medium was diluted to 10 µM with serum-free medium. To each well, 100 µl of diluted DCFH-DA culture medium was added and cells were incubated at 37 °C. After 30 min, medium was removed and cells were rinsed with serum-free medium and washed using PBS. A 200 µl aliquot of PBS was added, ROS fluorescence intensity was observed at excitation wavelength was 490 nm, and the emission wavelength was 525 nm and photographed under a laser microscope. The effects of resveratrol at the concentrations of 10, 20 and 50 µM on the cells in high glucose state were observed.

**Detection of alpha-smooth muscle actin (α-SMA) and Fibronectin in HK-2 cells**

Two sets of HK-2 cells were grown in the presence of low (5 mM) or high (30 mM) glucose for 50 hours. One of the two sets also received resveratrol (10 µM) and the other served as control. Cells were collected, washed and lysed for Western blot analysis for α-SMA and Fibronectin.

**Data analysis**

The data are expressed as mean ± standard deviation (SD) and analyzed using SPSS ver. 18.0. One-way analysis of variance was used for intergroup comparison. Difference was considered as statistically significant if p < 0.05.

**RESULTS**

**Detection of cell proliferation and apoptosis**

As shown in Table 1, the addition of resveratrol resulted in the reduction of proliferation rate of HK-2 cells under the same conditions, and the difference between group A and B (at same concentration) was statistically significant (p < 0.05). Under high glucose condition, the cell proliferation rate of group C which was added with resveratrol additionally was significantly lower than that of group D (p < 0.05). The cell proliferation rate in group A and D under high glucose condition was higher than that under normal glucose condition. As shown in Figure 1 and Table 1, the addition of resveratrol resulted in the reduction of cell apoptosis in high glucose environment. The apoptosis rate of group B was lower than that of group A, but there was no remarkable difference (p > 0.05). Group C and D had higher apoptosis rate compared to group B, and the differences were remarkable (p < 0.05), suggesting that high glucose was prone to induce cell apoptosis. Under high glucose condition, the cell apoptosis rate of

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>% Cell proliferation rate</th>
<th>% Cell apoptosis rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5 mM Glucose</td>
<td>22.18±0.32</td>
<td>2.23±0.18</td>
</tr>
<tr>
<td>B</td>
<td>5 mM Glucose + 10µM Resveratrol</td>
<td>10.36±0.21*</td>
<td>1.86±0.13</td>
</tr>
<tr>
<td>C</td>
<td>30 mM Glucose + 10 µM Resveratrol</td>
<td>23.52±0.42#</td>
<td>8.37±0.26#</td>
</tr>
<tr>
<td>D</td>
<td>30 mM Glucose</td>
<td>39.64±0.57</td>
<td>14.81±0.43#</td>
</tr>
</tbody>
</table>

**FIGURE 1. Effects of resveratrol on cell apoptosis under normal and high glucose exposure**
Resveratrol and renal tubular epithelial cells

Detection of cell reactive oxygen species

Under high glucose condition, resveratrol had certain inhibitory effects on ROS level. Moreover, the inhibitory effect was the strongest when the concentration of resveratrol was 10 µM, as shown in Figure 2.

FIGURE 2. Effects of resveratrol on ROS levels of the cells under high glucose exposure

Resveratrol lowers Epithelial-Mesenchymal Transition (EMT) in HK-2 cells

EMT describes a mechanism by which cells lose their epithelial characteristics and acquire more migratory mesenchymal properties. This process is transient and reversible. As shown in Table 2, the expression of alpha-smooth muscle actin (α-SMA) and Fibronectin was higher under high glucose conditions than under normal glucose conditions. The expression of α-SMA and Fibronectin in group B was slightly lower but statistically insignificant compared to group A (p > 0.05). The addition of resveratrol under high glucose conditions resulted in a significant decrease of α-SMA and Fibronectin expressions (p < 0.05).

DISCUSSION AND CONCLUSION

Renal tubular epithelial cell is a kind of cell on the periphery of renal tubules. ETM may occur in the late stage of DN (Zhao et al., 2016). The proliferation of renal tubular epithelial cells contributes to the increased reabsorption of the proximal tubules. Therefore, the proliferation of renal tubular epithelial cells is an important pathophysiological change of DN (Takahashi 2015). HK-2 cell is one kind of renal tubular epithelial cells. Ficarra et al. (2016) studied the effect of trans-δ-viniferin (TVN), a dimer of resveratrol, on human erythrocytes and found an antioxidant effect.

In the present study, the effects of resveratrol on the proliferation and apoptosis of renal tubular epithelial cells, HK-2, were investigated, and relevant mechanisms of ROS and EMT were explored. It was found that resveratrol had a favorable inhibitory effect on the proliferation and apoptosis of renal tubular epithelial cells, HK-2, under high glucose condition. ROS is the product of intracellular aerobic metabolism. The increase of ROS will promote the proliferation and activity of renal interstitial fibroblasts and the occurrence of EMT in renal tubular epithelial cells (Kärkönen and Kuchitsu 2015; Sun et al., 2006). The experimental results of this study revealed that resveratrol had an inhibitory effect on ROS level and the expression of α-SMA and Fibronectin. Therefore, resveratrol is worth promotion as it has a good development prospect and great practical significance.

CONFLICT OF INTEREST DISCLOSURE

The authors declare no conflict of interest.
REFERENCES


