Original Article
Effects of curcumin pretreatment on cell proliferation, oxidative stress, and Nrf2 pathways in HK-2 cells cultured in high glucose medium

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Abstract: Objective: The aim of this study was to examine the protective effects of curcumin on proliferation of human renal tubular epithelial HK-2 cells treated with glucose and to preliminarily explore its mechanisms. Methods: HK-2 cells were cultured in vitro and divided into three groups: negative control, glucose (40 mmol/L), and curcumin pretreatment (50 μmol/L) plus glucose. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was used to determine effects of glucose, with and without curcumin, on proliferation of HK-2 cells. Intracellular reactive oxygen species (ROS), malondialdehyde (MDA), glutathione (GSH) levels, and total superoxide dismutase (SOD) activity were examined. The mRNA expression levels of Nrf2 were determined by real-time quantitative polymerase chain reaction. Results: Glucose treatment inhibited growth of HK-2 cells, increased intracellular ROS and MDA levels, and decreased GSH levels and SOD activity. Relative expression levels of Nrf2 mRNA in cells pretreated with curcumin were significantly increased compared to cells treated with glucose alone. This suggests that curcumin pretreatment could significantly enhance expression of Nrf2. Conclusion: Curcumin alleviates cytotoxicity, improves cell viability, reduces intracellular ROS levels, decreases intracellular lipid peroxidation, and enhances the content and activity of intracellular antioxidants in HK-2 cells cultured in a high glucose medium.

Keywords: HK-2, curcumin, Nrf2, oxidative stress

Introduction
Diabetic nephropathy (DN), one of the most commonly occurring chronic microvascular renal complications in patients with type 1 and type 2 diabetes mellitus, is frequently seen in clinical practice [1]. There are over 350 million diabetic patients in the world. This number is expected to reach 600 million by 2035 [2]. In developed countries, type 2 diabetes mellitus accounts for 80-90% of all diabetes cases, whereas the proportion in developing countries may be higher. In addition, 20% of type 2 diabetes mellitus patients face the risk of DN [3]. The pathogenesis of DN is complex. However, an increasing number of studies have shown that oxidative stress injury may be an important contributor to the pathogenesis of DN. Increased lipid peroxide levels and decreased antioxidant enzyme activities have been observed in DN patients in clinical practice [5]. Therefore, inhibiting or reducing levels of reactive oxygen species (ROS) in patients is important for DN treatment.

Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is one of the most important oxidative stress pathways found, to date [4]. Nrf2 activates phase II reaction enzymes and antioxidant genes to protect cell and tissue function, playing a protective role in a variety of diseases related to oxidative stress [5]. Curcumin is a plant polyphenol extracted from Curcuma species [6]. Contemporary studies have suggested that curcumin has antioxidant, anti-inflammatory, anti-fibrotic, and anti-cancer properties [7]. Some studies have shown that curcumin can reduce levels of triglycerides and phospholipids, accelerate the dissolution of fibrin, and inhibit lipid peroxidation [8]. However, its spe-
Effect of curcumin on cell proliferation and its mechanisms

cific mechanism of action remains unknown. Therefore, this study explored its inhibitory and protective effects by treating human renal epithelial HK-2 cells with curcumin prior to culturing in high glucose medium.

Materials and methods

Cells

HK-2 cells were purchased from Biological Research Institute, Chinese Academy of Sciences (Beijing, China).

Main reagents and instruments

Glucose was purchased from Yihui Biological Technology (Shanghai, China). Dimethyl sulfoxide, trypsin, and Dulbecco’s modified Eagle’s medium (low carbohydrate) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation, RNA extract TRIzol, and quantitative real-time polymerase chain reaction (qRT-PCR) kits were purchased from Invitrogen (Carlsbad, CA, USA). Reverse-transcription and TaqMan miRNA kits were purchased from Applied Biosystems (Foster City, CA, USA). ROS and malondialdehyde (MDA) kits were purchased from Shanghai Beinuo Biological Technology (Shanghai, China). Glutathione (GSH) and superoxide dismutase (SOD) kits were purchased from Beyotime Biotechnology Research Institute (Haimen, China). ABI 7900 PCR instrument was purchased from Applied Biosystems.

Cell culture

HK-2 cells were cultured in Dulbecco’s Modified Eagle’s medium, containing 10% fetal bovine serum and 100 g/L penicillin-streptomycin, and incubated at 37°C under 5% CO₂. Cells were passaged when they reached 90% confluence. Cells were divided into three groups including a negative control group where no treatment was given, glucose group in which cells were cultured with 40 mmol/L glucose, and curcumin pretreatment group in which cells were treated with curcumin (50 μmol/L) 2 hours prior to addition of 40 mmol/L glucose to the culture medium.

Detection of cell proliferation by MTT assay

Cells were collected in the exponential growth phase, re-suspended, adjusted to 5 × 10⁵ cells, transferred to 96-well plates, and cultivated as previously described. After 24 hours, the medium was replaced with serum-free medium and cultivation was continued for an additional 24 hours. Cell proliferation was then determined by MTT assay, with absorbance measuring at 570 nm using a microplate reader. The experiment was repeated three times.

ROS assay

When adherent growth of cells was observed, cells in the logarithmic growth phase were collected, re-suspended, adjusted to 2 × 10⁵, and inoculated in 6-well plates. Subsequently, the cells were cultured as described above. ROS assay was carried out, in accordance with manufacturer instructions, adjusting the probe concentration to 1:500. Cells were then incubated under 5% CO₂ at 37°C for 40 minutes. Trypsin was applied for digestion and medium was added to stop the digestion. Cells were collected by centrifugation and supernatant was discarded. Finally, the cells were resuspended in phosphate-buffered saline and analyzed. The experiment was performed in triplicate.

MDA assay

Cells in the logarithmic growth phase were collected, resuspended, adjusted to 2 × 10⁵ cells, and inoculated onto 6-well plates. Subsequently, the cells were cultured as described above. After 24 hours of cultivation, the medium was replaced with serum-free medium and cells were cultured for 24 hours, and then lysed. The lysate was centrifuged, supernatant collected, and protein concentration determined using a bicinchoninic acid assay kit. Finally, manufacturer instructions for the MDA kit were followed in performing the assay. The experiment was performed in triplicate.

GSH and SOD assays

Cells were collected during the logarithmic growth period, resuspended, adjusted to 2 × 10⁵ cells, and plated in 6-well plates. Cells were cultured as described above. After 24 hours of cultivation, the medium was replaced with serum-free medium and cells were incubated for an additional 24 hours. Trypsin was used for digestion. Following centrifugation, supernatant was discarded. Cells were resuspended in saline, disrupted by sonication, and
Effect of curcumin on cell proliferation and its mechanisms

Table 1. Primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Upstream primers</th>
<th>Downstream primers</th>
</tr>
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<tbody>
<tr>
<td>Nrf2</td>
<td>5'-CACATTCCCAACAAGATGC-3'</td>
<td>5'-TCTTTTTCCAGCAGGAGAT-3'</td>
</tr>
<tr>
<td>β-actin</td>
<td>5'-CTCCATCTGCGCTGCTG-3'</td>
<td>5'-GCTGTCACCTTCACCGTTCC-3'</td>
</tr>
</tbody>
</table>

Primer sequences are shown in Table 1.

Statistical analysis

Data collected in this study were analyzed using SPSS 20.0 software package (IBM, Chicago, IL, USA). Data are expressed as mean ± standard deviation. Comparisons among multiple groups were carried out using one-way analysis of variance and least significant difference methods. P < 0.05 indicated a statistically significant difference.

Results

Effects of curcumin on the proliferative capacity of HK-2 cells

As shown in Figure 1, proliferative capacity of cells cultured, in the presence of 40 mmol/L glucose, was significantly inhibited compared to the negative control group (P < 0.05). Proliferative capacity of cells treated with curcumin, before adding glucose, increased significantly compared to cells in the glucose alone treatment group (P < 0.05). Cell proliferation in the curcumin pretreatment group was not significantly different from the negative control group (P > 0.05).

Effects of curcumin on ROS levels

ROS levels of cells in the glucose alone group were significantly increased compared to cells in the negative control group (P < 0.05). ROS levels of cells in the curcumin pretreatment group were significantly decreased compared to those in the glucose alone group (P < 0.05). ROS levels in the curcumin pretreatment group were not different from the negative control group (P > 0.05) (Figure 2).

Effects of curcumin on MDA levels

MDA levels in cells of the glucose alone group were significantly increased compared to cells in the negative control group (P < 0.05). MDA levels in the curcumin pretreatment group were significantly decreased compared to cells in the group treated with glucose alone (P < 0.05). MDA levels in the curcumin pre-treatment group were not different from the negative control group (P > 0.05) (Figure 3).

Figure 1. Proliferative capacity of HK-2 cells. The proliferative capacity of HK-2 cells was detected by using the MTT assay. Data are presented as mean ± SD from three independent experiments. *P < 0.05, **P < 0.01.

Detection of Nrf2 mRNA expression in cells by qRT-PCR

Cells were dissociated using TRizol Reagent for 30 minutes, then shaken. Purity and concentration of total extracted RNA were detected using ultraviolet spectrophotometry. The A$_{260/280}$ ratio was between 1.8 and 2.1. DNA synthesis was carried out in strict accordance with instructions for use of the cDNA kit. The reaction consisted of 2 μL of DNA template, 0.5 mol/L primer, 2.0 μL 2 × dNTP, 2.5 μL buffer solution, 1.5 mol/L MgCl$_2$, and 1.0 international units of TaqDNA polymerase. The volume was adjusted to 20 μL with nuclease-free water. PCR reaction conditions were as follows: 95°C pre-denaturation for 3 minutes, followed by 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. β-Actin was used as the reference gene.
Effects of curcumin on GSH levels and SOD activity

GSH levels in cells of the glucose alone group were significantly decreased compared to cells in the negative control group (P < 0.05), whereas GSH levels in cells of the curcumin pretreatment group were significantly increased compared to the glucose alone group (P < 0.05). GSH levels in the curcumin pretreatment group showed no significant differences compared to the negative control group (P > 0.05).

SOD activity in cells of the glucose alone group were significantly decreased compared to cells in the negative control group (P < 0.05), whereas SOD activity in cells of the curcumin pretreatment group were significantly increased compared with the glucose alone group (P < 0.05). SOD activity in the curcumin pretreatment group was not significantly different from the negative control group (P > 0.05) (Figure 4A, 4B).

Effects of curcumin on expression of Nrf2 mRNA

Using qRT-PCR, relative expression of Nrf2 mRNA in the glucose alone group was significantly increased compared to the negative control group (P < 0.05), whereas relative expression in cells of the curcumin pretreatment group was significantly increased compared to the glucose alone treatment group (P < 0.01). Relative expression of Nrf2 mRNA in the curcumin pretreatment group was significantly increased compared to the negative control group (P < 0.05) (Table 2).

Discussion

DN is a common clinical disorder in China, affecting a large number of patients [9]. At present, the occurrence and development of DN, in its initial stage, are relatively unclear and hard to identify due to lack of obvious symptoms. As a result, many patients miss the optimal treatment time because the disease has progressed to an advanced stage by the time it is diagnosed clinically [10, 11]. Advanced DN is extremely complicated. Treatment becomes much more difficult as the disease progresses because kidney damage is irreversible. Therefore, timely discovery and earlier treatment, with a suitable therapy, is particularly important for DN patients. Currently, Western medicine does not have a specific treatment scheme for DN. However, traditional Chinese medicine (TCM) has made advancements in DN treatment based on principles of TCM differentiation of symptoms and signs [12].
Curcumin, a plant polyphenol extracted from turmeric, is nontoxic and applicable as a natural pigment [13]. Turmeric has been used in the clinical practice of TCM for a very long time. Importantly, studies [14] have shown that curcumin is able to inhibit proliferation of tumor and T-cells, as well as vascular smooth muscle cells, and decrease secretion of various inflammatory factors produced by fibroblasts and macrophages. Other studies have indicated that curcumin is effective in inhibiting oxygen free radicals, reducing fibronectin, and enhancing activity of GSH and related enzymes in kidneys, resulting in the protection of this organ [15].

In this current study, it was found that glucose was toxic to HK-2 cells at a concentration of 40 mmol/L. When HK-2 cells grown in the presence of glucose were pretreated with curcumin, cell proliferation improved significantly compared to cells exposed to glucose alone.

This indicates that curcumin can suppress glucose toxicity in HK-2 cells.

A natural byproduct of oxygen metabolism, ROS plays important roles for transferring cell signals and maintaining balance in the human body. ROS levels can increase significantly under environment stress, possibly damaging cell structure [16]. MDA, a product of lipid peroxidation originating from polyunsaturated fatty acids, is used as a marker for oxidative stress in the organism [17]. GSH is an important factor in maintaining normal immune function of the human body and plays a role as an antioxidant and free radical scavenger to maintain stability of the internal environment [18]. SOD catalyzes the formation of oxygen and hydrogen peroxide from superoxide and is an important antioxidant enzyme [19].

It was found that ROS and MDA levels were significantly increased in HK-2 cells, cultured in the presence of high glucose. In addition, GSH levels and SOD activity in cells, cultured in the presence of glucose, were reduced, thereby impairing endogenous antioxidant activity. Therefore, this study concluded that HK-2 cells cultured in the presence of glucose...
have undergone oxidative stress. Furthermore, it was speculated that SOD and GSH were reduced because of increased consumption caused by increased levels of ROS. Together, these effects will induce an imbalance in regulation of the cellular antioxidant capacity, an increase in cell lipid peroxidation, and decrease in cell proliferation.

Nrf2 is an important signaling pathway involved in cellular antioxidant and inflammatory reactions, playing vital roles in combatting oxidant stress [20]. Recent studies [21, 22] have shown that there are more than 200 endogenous genes affected by Nrf2 signaling pathways and these genes play significant protective roles due to their anti-tumor, anti-inflammatory, and anti-apoptotic properties. This pathway is beneficial for catalyzing the elimination of free radicals and in maintaining redox balance of the organism [23, 24].

In this current study, expression of Nrf2 mRNA in HK-2 cells was evaluated. It was found that pretreatment with curcumin maintained mRNA levels significantly higher than cells in both the negative control and glucose alone groups. Accordingly, it was speculated that Nrf2 pathways of HK-2 cells were activated by curcumin treatment. In addition, this study determined that GSH level in cells of the curcumin pretreatment group were higher than the glucose alone group and that SOD activity was increased similarly. These results suggest that, after activation of Nrf2 pathways, GSH, SOD, and other endogenous antioxidants are gradually enhanced to maintain intracellular redox balance and effectively inhibit glucose-induced oxidative stress.

There are some limitations to this study. First, results from a cell line may not reflect what happens in clinical practice. Thus, whether the observed effects of curcumin occur in diabetic patients still requires assessment. Second, indicators of glucose and curcumin effects were measured at only a single time point and only the key gene in Nrf2 pathways was examined. Thus, future studies are necessary in which samples are collected from patients and several time points are examined, along with determination of additional indicators. This information will help to verify the validity and feasibility of treating DN patients with curcumin.

In conclusion, in HK-2 cells exposed to high glucose, curcumin can reduce cytotoxicity, intracellular ROS, and intracellular lipid peroxidation. Curcumin can also increase cell viability and the content and activities of intracellular enzymatic and non-enzymatic antioxidants. This suggests that curcumin may be a useful agent in the treatment of DN.

Disclosure of conflict of interest

None.

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Effect of curcumin on cell proliferation and its mechanisms


