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Original Research Article

Effects of icariin and quercetin on high glucose-induced neuronal cell apoptosis

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Abstract

Purpose: *To study the effects of icariin and quercetin on cell apoptotic changes in neurons induced by elevated glucose condition, and the mechanism involved.*
Methods: *Neonatal male Sprague Dawley rats (n = 48) weighing 5 – 7 g were used. Neuronal cells were isolated from rat hippocampus and cultured after purification. The cells were randomly assigned to six groups: control, high glucose, icariin, quercetin, serine/threonine-specific protein kinase (Akt) inhibitor, and Akt agonist groups. The Akt inhibitor and agonist used in this study were MK-22062hci and SC79, respectively. The influence of icariin and quercetin on neuronal apoptotic changes were determined flow cytometrically, while their effects on levels of expression of Akt, p-Akt, bax and bcl-2 were determined with Western blotting.*
Results: *Treatment with icariin or quercetin significantly inhibited apoptosis induced by high glucose. The concentrations of Akt, p-Akt, and bcl-2 proteins were markedly upregulated in high glucose group, relative to control ($p < 0.05$). The corresponding expression of bax was significantly down-regulated in high glucose group, relative to control ($p < 0.05$). Treatment with icariin or quercetin, or their agonists reversed high glucose-mediated alterations in these protein levels ($p < 0.05$).*
Conclusion: *Icariin and quercetin inhibit neuronal cell apoptosis induced by high glucose through upregulation of bcl-2 expression and down-regulations of bax expression and Akt-induced protein phosphorylation. Thus, Icariin and quercetin possess potential benefits for the treatment of neurological diseases.*

Keywords: *Apoptosis, High glucose condition, Hippocampal neurons, Icariin, Quercetin*

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INTRODUCTION

Diabetes mellitus (DM) is a mixed group of syndromes marked by elevated fasting blood glucose (FBG) due to relative or complete absence of insulin. In 2018, the global prevalence of type-2 diabetes mellitus (T2DM) was about half a billion, thereby creating financial and psychological hardship for sufferers [1]. In 2015, the International Diabetes Union reported that more than 400 million people worldwide suffered from T2DM. The incidence of the disease increases on yearly basis, especially in developing countries [2]. Complications of DM are either microvascular or macrovascular, and some patients may suffer cognitive decline [3].

There is a direct relationship between the hippocampus and memory: neuronal information coding has been linked to memory loss, to some extent [4].

Although high glucose condition enhances the proliferation of adult neural progenitor cells, it poses serious threat to their survival [5]. Hippocampal neurons are involved in learning and memory. Persistent hyperglycemia, an important clinical manifestation of DM, inhibits proliferation of hippocampal neurons [6]. Chronic hyperglycemia causes neuronal damage and morphological alteration of hippocampal structure, thereby leading to cognitive impairment [7]. This is believed to be responsible for cognitive impairment and brain atrophy in patients with T2DM.

Icariin has been shown to possess pharmacological properties such as antitumor, immunomodulating and anti-apoptotic effects. Quercetin exerts anti-inflammatory, antihyperglycemic and anti-hypercholesterolemic properties, and effectively inhibits H_2O_2-induced neuronal and glial cell death [8].

The serine/threonine-specific protein kinase (Akt) exists at the intersection of many signal pathways, and is involved in cell viability, while inhibiting cell apoptosis [9]. Studies have reported the involvement of bcl-2 and bax in apoptosis [10]. This investigation was focused on the influence of icariin and quercetin on neuronal cell apoptosis induced by high glucose condition, and the possible mechanisms involved.

EXPERIMENTAL

Materials

Icariin was obtained from Hubei Jiangmin Taihua Chemical Co. Ltd. (China), while quercetin was purchased from Hubei Yuancheng saichuang Technology Co. Ltd. (China). Serine/threonine-specific kinase (Akt) inhibitor (MK-22062hci) and SC79 were products of Selleck (China). This research received approval from the Animal Ethical Committee of The First People's Hospital of WenLing (with approval number of 20198731), and was performed according to guidelines of "Principles of Laboratory Animal Care" (NIH article no. 85-23, 1985) [11].

Rats

Newborn male Sprague Dawley rats (n = 48) weighing 5 – 7 g (mean weight = 5 ± 2 g) were bought from Beijing Weitong Lihua Experimental Animal Technology Co. Ltd (no scxk 2018-0010). The animals were housed in an aseptic environment with equal light/dark photoperiod at 25 °C and 50 % relative humidity. They were acclimatized to laboratory condition for seven days before commencement of study, and had free access to standard feed and clean drinking water.

Isolation of rat hippocampus and grouping of cells

The hippocampi of the rats were isolated and cultured after purification in Dulbecco's modified Eagle medium (DMEM) for 3 days at 37 °C in an atmosphere of 5 % CO_2. The medium was refreshed every 2 days. Cells at logarithmic growth phase were used in the study.

The cultured hippocampal neurons were then randomly assigned to six groups: control, high glucose, icariin, quercetin, Akt inhibitor, and Akt agonist groups. The Akt inhibitor and agonist used in this study were MK-22062hci and SC79, respectively. With the exception of control group, the cells were treated with either glucose, icariin, quercetin, MK-22062hci or SC79. The effects of the different treatments on neuronal cell apoptosis, and levels of expression of various proteins were determined using flow cytometry and Western blotting, respectively.

Determination of apoptosis

The cultured hippocampal cells were in 6-well plates at (2.5×10^6 cells/well) and cultured for 24 h. They were treated with either glucose, icariin, quercetin, MK-22062hci or SC79, and the medium was thereafter incubated for another 72 h, washed with phosphate-buffered saline (PBS), and properly blended with 300 µL binding buffer. This was followed with staining in the dark using Annexin V-fluorescein isothiocyanate and PI (5 µL each) within 25 min, and flow cytometric analysis for apoptosis at 485 nm.

Western blotting

The cells were rinsed with PBS, and lysed using ice-cold RIPA buffer laced with protease inhibitor. The resultant lysate was subjected to centrifugation, and the protein content of the supernatant was measured using bicinchoninic acid assay kits. Then, protein (30 µg) was resolved with SDS-PAGE, and transferred to PVDF membrane for 120 min. The membrane was incubated with non-fat milk solution to block non-specific interaction of the blot. Thereafter, the membrane was subjected to incubation for 12 h at 4 °C with 1° antibodies for Akt, p-Akt, bcl-2, bax and β-actin, each diluted 1 to 1000. Then,

the membrane was washed thrice with TBS-T, followed by incubation with HRP-conjugated secondary antibody for 90 min. x-ray films were applied in development of the blots, while Bio-rad gel imaging technique was used for Grayscale analysis of bands. β-Actin served as housekeeping gene.

Statistics

Data are shown as mean ± SEM. The results were statistically analyzed with SPSS ver. 19.0. Group comparison was done with Student *t*-test. Statistical significance of differences was fixed at $p < 0.05$.

RESULTS

Effect of icariin and quercetin on neuronal cell apoptosis

The population of apoptotic cells was markedly higher in high glucose group than in control, but it was markedly decreased following exposure to icariin, quercetin, MK-22062hci or SC79 ($p < 0.05$). Apoptosis was comparable amongst the treatment groups ($p > 0.05$; Table 1).

Table 1: Neuronal cell apoptosis in the various treatment groups

Group	Level of neuronal apoptosis (%)
Control	11.68
High glucose	24.57
Icariin	15.83
Quercetin	15.73
Icariin + MK-22062hci	19.58
Quercetin + MK-22062hci	18.77
SC79	15.68

Effect of icariin on expressions of p-Akt and Akt

As presented in Table 2 and Figure 1, the expressions of Akt and p-Akt in high glucose group were significantly down-regulated, relative to control group, but were significantly upregulated after treatment with icariin or SC79 ($p < 0.05$). Protein expressions of Akt and p-Akt were significantly higher in icariin group than in icariin + MK-22062hci cells. Similarly, Akt and p-Akt expressions were markedly higher in SC79 group than in icariin-treated cells.

Effect of icariin on expressions of bcl-2 and bax

The expressions of bcl-2 and bcl-2/bax were markedly decreased in high glucose group, while bax protein expression was markedly upregulated, relative to control. The bcl-2 level, and bcl-2/bax ratio were markedly elevated in icariin and SC79-treated cells, relative to high glucose-treated cells ($p < 0.05$). However, corresponding bax protein levels were markedly lower in icariin and SC79 groups than in high glucose group. Moreover, in icariin-treated cells, the proportion of bcl-2:bax was markedly higher than that in icariin + MK-22062hci group, while the corresponding bax expression was significantly lower than that in icariin + MK-22062hci-treated cells. However, no marked differences were seen in bcl-2 level, and bcl-2/bax ratio between icariin and SC79 groups (Table 3 and Figure 1).

Table 2: Levels of expression of Akt and p-Akt in the various treatment groups

Group	p-AKT	AKT
Control	118.69 ± 0.34	92.03 ± 0.14
High glucose	100.73 ± 0.58	81.38 ± 0.02
Icariin	107.00 ± 1.61[ac]	113.82 ± 1.61[ac]
Icariin + MK-22062hci	88.05 ± 0.55[b]	84.47 ± 0.55[b]
SC79	128.21 ± 0.98[a]	127.60 ± 0.18[a]

[a, b, c]$P < 0.05$ ([a]vs high glucose; [b]vs icariin; [c]vs SC79)

Table 3: Effects of icariin and its agonist and inhibitor on the expressions of bax and bcl-2

Group	Bcl-2	Bax	Bcl-2/Bax
Control	93.08 ± 0.08	73.82 ± 0.03	121.93 ± 0.11
High glucose	89.04 ± 0.09	92.39 ± 0.11	96.48 ± 0.04
Icariin	99.86 ± 0.12[ac]	82.85 ± 0.03[ac]	120.67 ± 0.16[ac]
Icariin + MK-22062hci	79.29 ± 0.13[b]	102.00 ± 0.09[b]	77.82 ± 0.08[b]
SC79	102.60 ± 0.58[b]	82.64 ± 0.69[a]	124.29 ± 1.00[a]

[a, b, c]$P < 0.05$ ([a]vs high glucose; [b]vs icariin; [c]vs MK-22062hci)

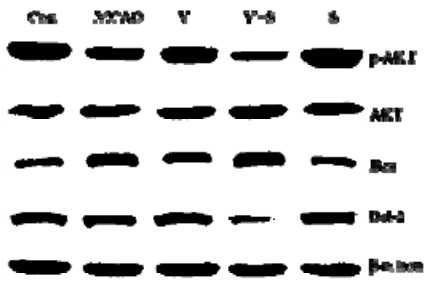

Figure 1: Akt, p-Akt, bcl-2 and bax levels in the various groups. Con: control; MCAO: high glucose; Y: icariin group; Y + S: icariin + MK-22062hci; S: MK-22062hci

Effect of quercetin on expressions of Akt and p-Akt proteins

The expression levels of Akt and p-Akt proteins were markedly decreased in high glucose group, relative to control group, but they were markedly upregulated in quercetin group, relative to high glucose group, and were significantly downregulated in quercetin + MK-22062hci group, when compared to control. The expressions of these genes were significantly upregulated by quercetin, when compared with SC79 ($p < 0.05$; Table 4).

Table 4: Levels of expression of Akt and p-Akt in the various treatment groups

Group	p-AKT	AKT
Control	68.35 ± 0.39	90.45 ± 0.24
High glucose	36.33 ± 0.03	78.31 ± 0.27
Quercetin	59.26 ± 0.42[a]	84.46 ± 0.47[a]
Quercetin + MK-22062hci	55.72 ± 0.24	75.37 ± 0.28
SC79	71.55 ± 0.71[b]	101.49 ± 0.80[b]

[a, b, c]$P < 0.05$ ([a]vs high glucose; [b]vs quercetin)

Levels of expression of bax and bcl-2

The bcl-2 level, and bcl-2/bax were markedly decreased in high glucose group, relative to control ($p < 0.05$). However, their corresponding levels were markedly increased in quercetin group, relative to high glucose-treated cells. The bcl-2 protein, and bcl-2/bax were also markedly lower in quercetin + MK-22062hci group, relative to high glucose group, and their concentrations were markedly smaller in quercetin group than in SC79-exposed cells. These results are shown below.

Table 5: Effect of quercetin and its agonist and inhibitor on concentrations of bax and bcl-2

Group	Bcl-2	Bax	Bcl-2/Bax
Control	117.26 ± 1.67	70.67 ± 0.54	166.12 ± 1.62
High glucose	83.15 ± 0.45	89.00 ± 0.09	94.48 ± 0.44
Quercetin	89.25 ± 0.93[ac]	73.25 ± 0.34[ac]	121.99 ± 1.26[ac]
Quercetin + MK-22062hci	80.14 ± 0.56[b]	86.99 ± 0.35[b]	92.23 ± 0.42[b]
SC79	104.19 ± 1.50[a]	81.48 ± 0.77[a]	128.01 ± 1.02[a]

[a, b, c]$P < 0.05$ ([a]vs high glucose; [b]vs quercetin; [c]vs SC79)

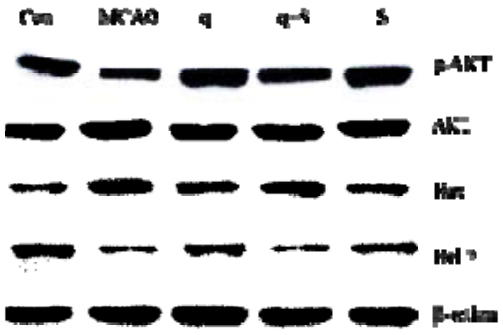

Figure 2: Levels of Akt, p-Akt, bcl-2 and bax in various groups. Con: Control; MCAO: High glucose; q: Quercetin; q + S: Quercetin + MK-22062hci; S: MK-22062hci

DISCUSSION

Apoptosis, which is important in the growth of multicellular organisms via maintenance of their internal and external environment, has been implicated in the pathophysiology of T2DM. Chronic hyperglycemia promotes apoptosis, thereby leading to cognitive dysfunction [12].

It is known that PKB or Akt is involved in regulation of cellular events such as transcription, carbohydrate metabolism, apoptosis, as well as cell proliferation and migration. It has been shown to mediate the survival of neurons. Dysregulation of this protein produces stress-induced pathological and degenerative disorders which are related to the pathogenesis of a variety of neurological diseases. Studies have shown that Akt is involved in bcl-2/bax-dependent apoptotic pathway, and plays a crucial role in neuronal cell apoptosis [13].

In Traditional Chinese Medicine (TCM), “Bushen Huoxue Recipe” functions in *tonifying the kidney* and *benefiting essence*. It has shown potential in improving learning and memory function of mice with cerebral ischemia [14]. Epimedium *tonifies the kidney,* strengthens *Yang*, *eliminates wind* and *dehumidifies*, and is used in TCM to treat infertility and polyuria [15]. Icariin, a flavonoid glycoside compound, is the active principle in epimedium. Cuscuta is a genus of about 100 – 170 species of yellow, orange, or red parasitic plants. Extracts and isolates from the plants have been shown to *nourish* the liver and kidney. They possess antioxidant and antitumor properties. Quercetin, a plant flavonol from the flavonoid group of polyphenols, is a bioactive compound present in Cuscuta [16].

The present study involved investigation of the effects of icariin and quercetin on neuronal cell apoptotic changes induced by high glucose, and the likely associated mechanism. The findings revealed that icariin and quercetin significantly inhibited neuronal cell apoptosis promoted by high glucose state. Treatment in the presence of Akt inhibitor (MK-22062hci) produced a similar effect. These results suggest that long-term high blood glucose level may induce apoptosis in neurons, thereby adversely affecting hippocampal function and memory.

It is likely that icariin and quercetin are potential inhibitors of high glucose-mediated apoptotic lesions in hippocampal neuronal cells. The results obtained from Western blotting indicate that the expressions of PKB, p-PKB, and bcl-2, and bcl-2/bax were markedly lower in high glucose group than in control, but the corresponding expression of bax was markedly higher in high glucose group than in control. The expressions of these genes were significantly higher in icariin group than in high glucose group. However, bax expression was significantly down-regulated in high glucose group, relative to control. The expressions of Akt, p-Akt and bcl-2, and bcl-2/bax were higher in icariin group than in icariin + MK-22062hci group, but the corresponding bax protein expression was significantly reduced, relative to icariin + MK-22062hci group.

These results show that icariin and quercetin may be effective in reversing high glucose-induced Akt protein phosphorylation, and are in agreement with results of previous studies [9,10]. Inhibition of Akt pathway may have significantly reduced the protective effect of these compounds on neuronal cell apoptosis, an indication that they exerted their effects via the Akt pathway.

CONCLUSION

Icariin and quercetin inhibit neuronal cell apoptosis induced by conditions of high glucose through upregulation of bcl-2 expression, and downregulations of bax and Akt-induced protein phosphorylation. These findings have potential for use in the development of new drugs for the management of neurological diseases.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

This study was done by the authors named in this article, and the authors accept all liabilities resulting from claims which relate to this article and its contents. The study was conceived and designed by Mengqian Dong; Mengqian Dong, Ying Jin, Peifen Huang, Zhiyang Chen collected and analyzed the data; Zhiyang Chen wrote the text. All authors read and approved the manuscript for publication.

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