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Original Research Article

Luteolin attenuates high glucose-induced cytotoxicity by suppressing TXNIP expression in neuronal cells

Tuerhong Tuerxun*, Xiaopeng Li, Long Ma, Yi Wang, Bo Liu, Xiangyou Yu
Department of Intensive Care Unit, The First Affiliated Hospital of Xinjiang Medical University, Xinjiang 830054, China

For correspondence: **Email: TuerhongTuerxunsdr@163.com; **Tel:** 86+0991-4319317*

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Abstract

Purpose: *To determine the potential effect of luteolin in neuroprotection using an in vitro model of diabetic neuropathy (DN) in PC12 cells by high glucose (HG)-induced neurotoxicity.*
Methods: *PC12 cells were pretreated with HG media for 3, 6, 12, and 24 h, followed by treatment with increasing concentrations of luteolin (10, 25, and 50 ug/ml) for 24 hours. Following luteolin treatment, the cells were transfected with a plasmid expressing thioredoxin-interacting protein (TXNIP). To evaluate HG-induced cytotoxicity, the expression levels of the inflammatory markers interleukin (IL)-8, IL-6, and tumor necrosis factor-α (TNF-α) were evaluated by quantitative reverse transcription PCR (qRT-PCR) and ELISA. In addition, the apoptotic cells were assessed by flow cytometry. The expression levels of TXNIP protein and mRNA were determined by western blotting and qRT-PCR, respectively.*
Results: *Luteolin decreased the expression levels of TNF-α, IL-1β, and IL-6 in a dose-dependent manner at both the protein and mRNA level. Luteolin also decreased HG-induced apoptosis in PC12 cells ($p < 0.05$). The expression of B-cell lymphoma 2 (BCL-2) was suppressed, whereas those of cleaved PARP and cleaved caspase-3 were increased following HG treatment. Luteolin treatment had the opposite effect in a dose-dependent manner ($p < 0.05$). Luteolin reduced HG-induced inflammation and apoptosis in PC12 cells by inhibiting TXNIP expression ($p < 0.05$).*
Conclusion: *These data indicate that the neuroprotective effects of luteolin is probably exerted its anti-apoptotic and anti-inflammatory activities via the TXNIP pathway.*

Keywords: *Luteolin, Diabetic neuropathy, High glucose, PC12 cells, Inflammation, Apoptosis*

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INTRODUCTION

Glucose is the main source of energy for maintaining the function of mammalian brain. Abnormal glucose metabolism, such as hyperglycemia, often causes diabetic neuropathy and leads to neurological injury by inducing cell apoptosis [1]. Inhibition of apoptosis may prevent the initiation and progression of neuropathy [2].

Diabetic nephropathy is a microvascular complication of diabetes and the leading cause of end-stage renal illness in the world [3]. Although the pathogenesis of diabetic nephropathy is still unclear, it is universally

accepted that inflammation is a key result [4]. The metabolic disorder and disruption in renal hemodynamics caused by hyperlipidemia and chronic hyperglycemia may stimulate inflammation, resulting in the infiltration of immune cells in early diabetic nephropathy [5]. This is an example of an immune response that represents the microinflammatory state related to innate immunity [6].

Luteolin is a flavonoid found in carrots, peppers, celery, olive oil, and mint. Many studies show that it has anti-tumor, anti-oxidant, and anti-inflammatory properties [7]. Luteolin has also been shown to effectively attenuate external cytotoxicity and protect nerve cells. For example, luteolin attenuates hydrogen peroxide-induced neuronal apoptosis [8]. Luteolin also protects neurons from MPP +-induced cytotoxic damage by modulating the ERK-dependent Keapl-Nrf2-ARE pathway [9]. By regulating the ERK-induced expression of heme oxygenase-1 and NF-E2–related factor 2, luteolin plays a role in protecting nerve cells [10] and regulating the growth of prominent cells by inducing microRNA-132 expression [11]. In addition, luteolin inhibits apoptosis and inflammation in human umbilical vein endothelial cells via regulation of thioredoxin-interacting protein (TXNIP) expression [12].

In this study, high glucose (HG)-induced cytotoxicity in a neuronal cell line, PC12, was measured.

EXPERIMENTAL

Cell culture and treatment

PC12 cells were obtained from the American Type Culture Collection (ATCC, USA) provided the PC12 cells. The cells were incubated in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) with penicillin-streptomycin (100 U/ml, Gibco, USA) and fetal bovine serum (FBS, 10%, Gibco, USA), and maintained in a humidified incubator (5% CO_2, 37°C). To induce hyperglycemia, the cells were pretreated with DMEM containing 50 mM D-glucose. The cells were then incubated in HG medium for 3, 6, 12, and 24 h. Luteolin (Sigma-Aldrich, USA) was dissolved in dimethyl sulfoxide (DMSO, 5%, Sigma-Aldrich). The TXNIP plasmid and the respective negative control plasmid were synthesized by GenePharma (Shanghai, China). Lipofectamine 2000 (Invitrogen, USA) was used to transfect cells with 50 nM of the TXNIP plasmid and the negative control plasmid in differentiated podocytes, according to the manufacturer's instructions. After 2 days in culture, the original medium was discarded and replaced with RPMI-1640 (Thermo Fisher Scientific, USA) culture medium.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. Then, SuperScript reverse transcriptase kit (Invitrogen, USA) was used to reverse transcribe 2 µg of total RNA. PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, USA) with Applied Biosystems PRISM 7900 Sequence Detection System (Applied Biosystems, USA) were used to perform qRT-PCR. The primer sequences are shown in Table 1. The $2^{-\Delta\Delta Ct}$ method was used to detect the expression levels of mRNA, normalized to the expression level of *GAPDH*.

Enzyme-linked immunosorbent assay (ELISA)

The expression levels of TNF-α, IL-1β, and IL-6 protein were evaluated using an ELISA kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. A spectrophotometer was used to read the absorbance of each well at 450 nm and the concentration of protein in each well was calculated using the standard curve.

Table 1: Primer sequences used for qRT-PCR

Gene	Forward (5′–3′)	Reverse (5′–3′)
IL-1β	CCCTGAACTCAACTGTGAAATAGCA	CCCAAGTCAAGGGCTTGGAA
IL-6	ATTGTATGAACAGCGATGATGCAC	CCAGGTAGAAACGGAACTCCAGA
TNF-α	CCTCTTCTCATTCCTGCTC	CTTCTCCTCCTTGTTGGG
TXNIP	CCACGCTGACTTTGAGAACA	GGAGCCAGGGACACTAACATA
GAPDH	AAGGAAATGAATGGGCAGCC	TAGGAAAAGCATCACCCGGA

Flow cytometry

PC12 cells were harvested, washed twice with ice-cold PBS, and fixed using ethanol (70 %, v/v) overnight. Then, the cells were stained with propidium iodide in PBS containing RNase in the dark (30 min, 25°C). A Coulter Epics XL flow cytometer (Beckman-Coulter Inc., USA) was used to read the sample.

Western blotting

Total protein was harvested from cells by RIPA buffer. Immunoblot analysis was performed using monoclonal antibodies against TXNIP, cleaved caspase-3, cleaved PARP, and BCL-2, (all 1:1,000, Santa Cruz, USA). β-actin (1:5,000, Santa Cruz) was used as the loading control. The secondary antibody, labeled using horseradish peroxidase (HRP) (1:1,000, Santa Cruz). was incubated with the blots for 1 h at 25°C. Band densities were quantified using the LICOR Odyssey infrared imaging system (LICOR Bio-science, USA).

Statistical analysis

GraphPad 8.0 was used to analyze the data. All experiments were repeated three times, and the data were expressed as mean ± standard deviation (SD). Analysis of variance was used to analyze the significance of differences among experimental groups. $p < 0.05$ was regarded as statistically significant.

RESULTS

HG-induced inflammation, apoptosis, and TXNIP expression

As shown in Figure 1, PC12 cells treated with HG media showed time-dependent expression of TNF-α, IL-1β, and IL-6. The expression levels of TNF-α, IL-1β, and IL-6 mRNA and protein were evaluated after 3 h of stimulation with HG media. Maintenance of this high expression was observed for up to 24 h. Culture in high-glucose media also led to an increase in apoptosis in a time-dependent manner, compared to the control group (Figure 2 A). Furthermore, PC12 cells treated with HG media showed time-dependent activation of TXNIP, after 3 h of stimulation with HG. The expression of TXNIP protein increased significantly and continued to increase to 24 h (Figure 2 B). These results indicate that HG induces inflammation, apoptosis, and TXNIP expression in PC12 cells.

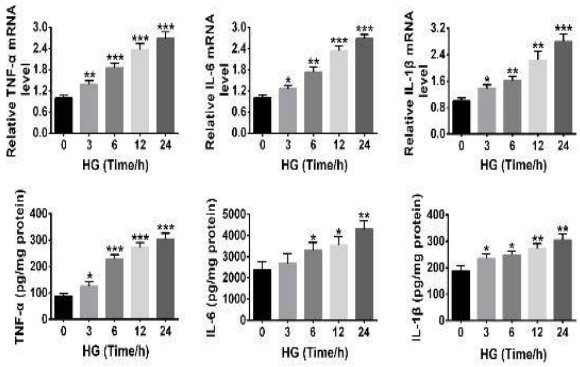

Figure 1: Effect of HG on inflammation, apoptosis, and TXNIP expression in PC12 cells incubated with high glucose (50 mM) for 0, 3, 6, 12, and 24 h. qRT-PCR and ELISA were used to evaluate the expression levels of inflammatory factors TNF-α, IL-1β, and IL-6; *$p < 0.05$ **$p < 0.01$, ***$p < 0.001$ vs. control

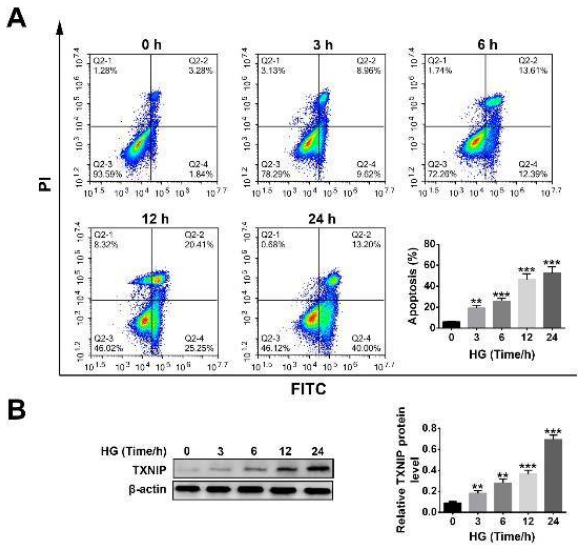

Figure 2: Effects of HG on inflammation, apoptosis, and TXNIP expression in PC12 cells incubated with high glucose (50 mM) for 0, 3, 6, 12, and 24 h. (A) Flow cytometry was used to evaluate changes in apoptosis, **$p < 0.01$, ***$p < 0.001$ vs. control group. (B) Western blot analysis was performed to evaluate TXNIP expression, **$p < 0.01$, ***$p < 0.001$ vs. control group

Luteolin attenuated HG-induced inflammation and apoptosis

To evaluate the effect of luteolin in HG-induced cellular inflammation and apoptosis, PC12 cells were stimulated with HG media and then treated with 10, 25, and 50 μg/mL luteolin for 24 h, and cellular inflammation and cell apoptosis were investigated. As shown in Figure 3, HG media significantly increased the mRNA and protein expression levels of TNF-α, IL-1β, and IL-6 in PC12 cells. Compared to HG-only controls, luteolin significantly decreased both the mRNA

and TNF-α, IL-1β, and IL-6 protein levels in a dose-dependent manner. Compared to untreated controls, HG media-treated cells had significantly higher apoptosis rate. Luteolin treatment significantly reduced the apoptosis rate in HG media-treated PC12 cells (Figure 4A). Next, the influences of luteolin on cleaved caspase-3, BCL-2, and cleaved PARP apoptotic protein were evaluated. As shown in Figure 4 B, BCL-2 expression was markedly suppressed, whereas the levels of cleaved PARP and cleaved caspase-3 were markedly increased by HG media treatment. Treatment with luteolin reversed these effects in a dose-dependent manner. These data indicate that luteolin protects PC12 cells from HG-induced inflammation and apoptosis.

Luteolin decreased HG-induced inflammation in PC12 cells by inhibiting TXNIP expression

As shown in Figure 5 A, PC12 cells pretreated with HG media showed increased expression of TXNIP. The expression levels of TXNIP protein were markedly lower after luteolin treatment. To overexpress TXNIP in PC12 cells, the cells were transiently treated with the TXNIP expression plasmid (Figure 5 B). The TXNIP protein expression was markedly increased by transfection with the TXNIP plasmid, and this effect was reversed with luteolin treatment (Figure 5 C). Furthermore, after transfection with the TXNIP plasmid, TNF-α, IL-1β, and IL-6 expression levels were significantly higher in PC12 cells after HG stimulation. Luteolin treatment significantly reversed the pro-inflammatory effect of TXNIP (Figure 6). These data suggest that luteolin reduced HG-induced inflammation by suppressing TXNIP expression PC12 cells.

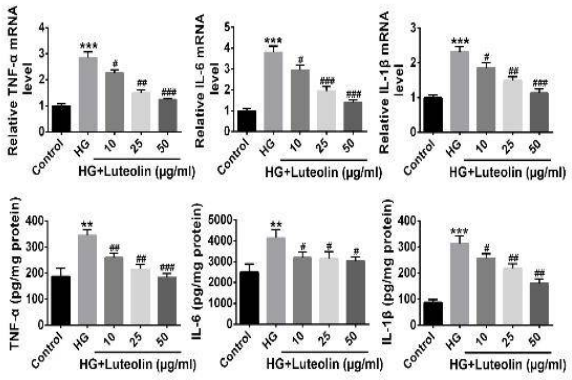

Figure 3: Effect of luteolin on HG-induced inflammation and apoptosis in PC12 cells. qRT-PCR and ELISA were used to detect the expression levels of the inflammatory factors TNF-α, IL-1β, and IL-6; ***p < 0.001 vs. control group, $^{\#}p$ < 0.05, $^{\#\#}p$ < 0.01, and $^{\#\#\#}p$ < 0.001 vs. the HG group

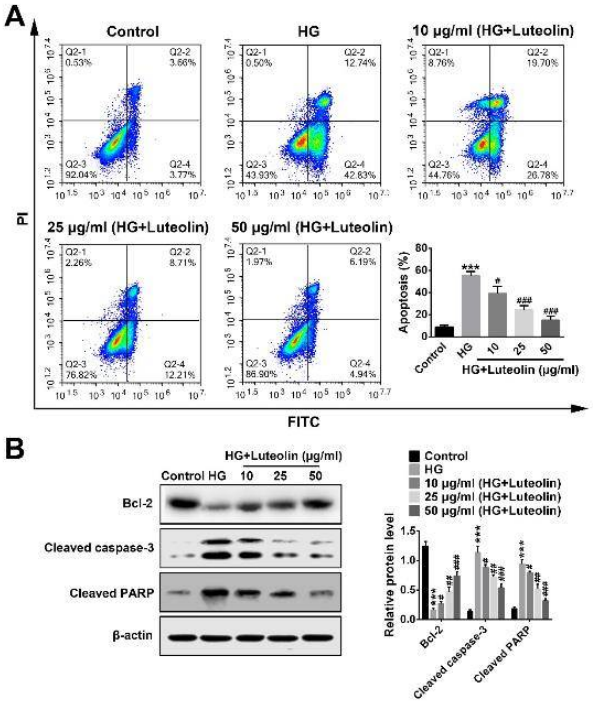

Figure 4: Effect of luteolin on HG-induced inflammation and apoptosis in PC12 cells. (A) Flow cytometry was used to evaluate changes in apoptosis, ***p < 0.001 vs. control group, $^{\#}p$ < 0.05, $^{\#\#\#}p$ < 0.001 vs. HG group. (B) Western blot analysis was performed to evaluate the levels of indicated proteins, ***p < 0.001 vs. control group, $^{\#}p$ < 0.05, $^{\#\#}p$ < 0.01, $^{\#\#\#}p$ < 0.001 vs. HG group

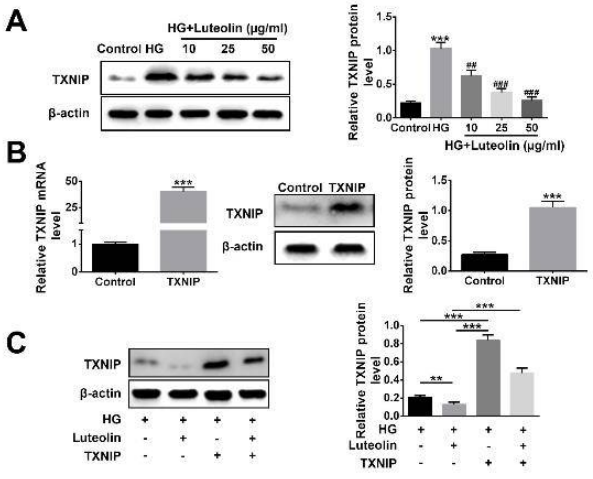

Figure 5: Luteolin reduced HG-induced inflammation by inhibiting TXNIP expression in PC12 cells. (A) Western blot analysis was performed to evaluate TXNIP protein expression. (B) Quantitative reverse transcription PCR and western blot analysis were used to evaluate TXNIP transfection efficiency, ***p < 0.001 vs. control group; ***p < 0.001 vs. control group; $^{\#}p$ < 0.05, $^{\#\#\#}p$ < 0.001 vs. HG group. (C) Western blot analysis was performed to evaluate TXNIP protein expression

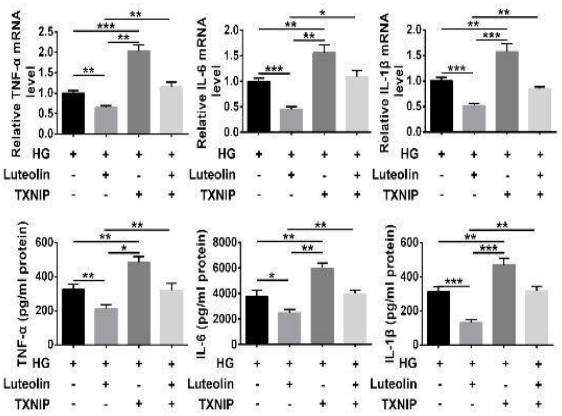

Figure 6: Luteolin reduced HG-induced inflammation by inhibiting TXNIP expression in PC12 cells. Quantitative reverse transcription PCR and ELISA were used to evaluate the levels of TNF-α, IL-1β, and IL-6.

Luteolin reduced HG-induced apoptosis by inhibiting TXNIP expression

Compared to HG alone group, luteolin treatment significantly reduced apoptosis in PC12 cells. After transfection with the TXNIP plasmid, HG-induced apoptosis increased, but the effect was markedly attenuated when cells were treated with the TXNIP plasmid and with luteolin simultaneously (Figure 7 A). As shown in Figure 7B, BCL-2 expression was markedly suppressed, whereas the expression levels of cleaved PARP and cleaved caspase-3 were markedly increased upon HG media treatment. These effects were reversed upon luteolin treatment. However, transfection with the TXNIP plasmid inhibited HG-induced BCL-2 upregulation, and decreased the expression levels of cleaved PARP and cleaved caspase-3. Moreover, treatment with luteolin significantly decreased BCL-2 expression, which is a downstream protein of cleaved caspase-3 and cleaved PARP in PC12 cells, when treated with HG media and transfected with the TXNIP plasmid. These data indicate that luteolin reduced HG-induced apoptosis by inhibiting TXNIP expression.

DISCUSSION

Chronic low-grade inflammation is caused by the infiltration of cytokines and immune cells into kidney tissue [13]. Immune-mediated inflammation is a key component of diabetic nephropathy and hyperglycemia [14]. The expression levels of inflammatory factors such as TNF-α, IL-1β, and IL-6 are markedly increased in renal tissues during diabetic nephropathy, and suppressing these cytokines expression may confer protection against diabetic renal injury [15].

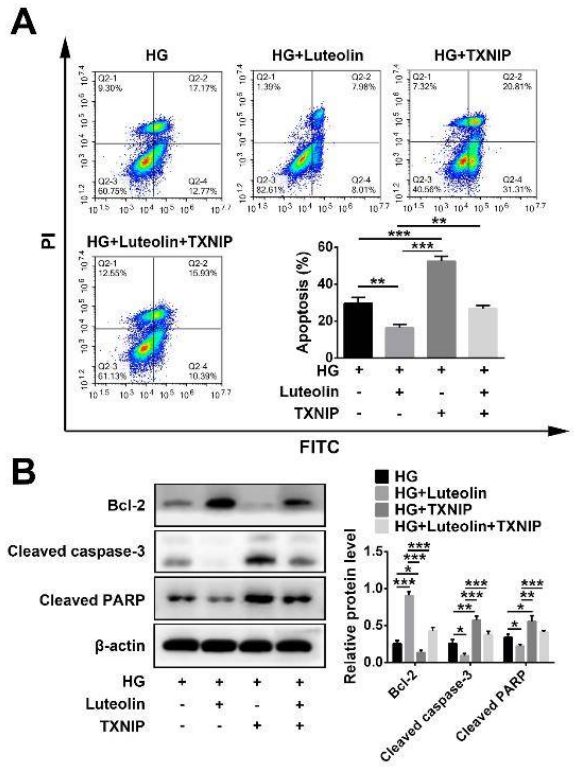

Figure 7: Luteolin reduced HG-induced apoptosis by inhibiting TXNIP expression. (A) Flow cytometry was used to detect changes in apoptosis, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. the HG group or HG + luteolin group, respectively. (B) Western blot analysis was performed to evaluate BCL-2 expression, cleaved caspase-3, and cleaved PARP protein expression; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. the HG group or HG + luteolin group, respectively

HG-induced apoptosis is a critical mechanism in the pathogenesis of diabetic nephropathy and a possible mechanism of HG-induced neuronal dysfunction and death [16,17]. Consistent with this, HG has been reported to induce apoptosis both *in vivo* [18,19] and *in vitro* in neurons such as primary dorsal root ganglion neurons [16], SH-SY5Y human neuroblastoma cells, and PC12 cells [20,21].

Apoptosis is an intrinsic cell-suicide mechanism with a highly regulated mechanism. Two important protein families, including the BCL-2 and caspase families, have a crucial effect in regulating apoptosis [22,23]. The TXNIP pathway plays a key role in the initiation of apoptosis of mitochondrial in cells [24,25]. The present study showed that 50 mM of HG media is able to induce neurotoxicity in PC12 cells by modulating the inflammation, apoptosis, and TXNIP pathways.

Luteolin is a natural plant extract found in various vegetables and fruits in the form of glycosides [17]. It has strong anti-tumor, anti-oxidative, anti-

infection, immune conditioning, and cardio protective properties. [18]. Recent studies show that luteolin can markedly reduce the inflammatory response of activated macrophages and downregulate the expression levels of pro-inflammatory factors such as TNF-α, IL-1β, and IL-6 [19]. The present findings demonstrate that luteolin attenuated the HG-induced release of TNF-α, IL-1β, and IL-6 in HG-sensitized PC12 cells. Moreover, luteolin markedly decreased apoptosis, as measured by cleaved PARP and cleaved caspase-3 levels, in HG-induced PC12 cells. In contrast, the level of BCL-2 increased with luteolin treatment.

Recently, it has been demonstrated that polyphenolic compounds, such as quercetin and epigallocatechin gallate, induce cell inflammation and apoptosis mediated by NLRP3 and TXNIP in endothelial cells [20]. TXNIP is a thioredoxin endogenous inhibitor protein that reduces the ability of cells to mediate oxidative stress. The interaction of thioredoxin with proteins is significantly increased in humans and rats with diabetic nephropathy, and is closely related to renal fibrosis, urinary albumin, and reactive oxygen species [29,30].

High glucose treatment significantly upregulated TXNIP expression, even at 3h, in this *in vitro* study. These data indicate that TXNIP is an early response gene that is readily induced by diabetic nephropathy and hyperglycemia. Consistently, the levels of TNF-α, IL-1β, and IL-6 markedly increased upon treatment with HG media in a time-dependent manner.

These data indicate a subtle relationship between the TXNIP and the inflammatory factors. Surprisingly, the induction of TXNIP, TNF-α, IL-1β, and IL-6 by HG media treatment was markedly suppressed by luteolin treatment. This reveals a close relationship between inflammatory factor expression and TXNIP in the activation of an inflammatory response through the TXNIP pathway. Inflammation promotes the activation of the TXNIP pathway, which is suppressed by reduced inflammation. Furthermore, TXNIP and inflammation signaling exert a key effect in the initiation of mitochondria-dependent cell apoptosis [24,25]. Mitochondrial dysfunction activates scorpion venom cysteine proteases, leading to the release of cytochrome C and cell apoptosis [21]. Following treatment of PC12 cells with luteolin, there was a marked decrease in the protein levels of BCL-2, which is downstream of cleaved caspase-3 and cleaved PARP in PC12 cells. Luteolin protected cells from death and inflammation, indicating its beneficial effect on cytotoxicity.

CONCLUSION

The findings of this study show that luteolin suppresses HG-induced inflammation and apoptosis in PC12 cells by inhibiting TXNIP expression. The beneficial effects of luteolin observed in this study support the previously reported neuroprotective and anti-inflammatory properties of luteolin. Although luteolin should be considered a promising treatment for diabetic nephropathy, further studies using *in vivo* models will be necessary to confirm this conclusion.

DECLARATIONS

Acknowledgement

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was carried out by the researchers listed in this manuscript. All liabilities regarding the content of this manuscript will be borne by the authors. Tuerhong Tuerxun and Xiaopeng Li designed all the experiments and revised the paper. Bo Lou, Long Ma and Yi Wang performed the experiments. Tuerhong Tuerxun and Xiangyou Yu wrote the manuscript.

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