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Luteolin attenuates neuronal apoptosis in the hippocampi of diabetic encephalopathy rats☆

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Abstract

Luteolin (3',4',5,7-tetrahydroxyflavone) has powerful anti-apoptotic and antioxidant properties. This study aimed to investigate the effects of luteolin on hyperglycemia-mediated apoptosis in the hippocampi of rats with streptozotocin-induced diabetic encephalopathy after injection into the tail veins, and the molecular mechanisms involved. Biochemistry and terminal deoxynucleotidyl transferase mediated dUTP nick end labelling detection results showed that luteolin treatment (given twice daily for 15 days) significantly inhibited hyperglycemia-mediated apoptosis, decreased malondialdehyde levels and increased glutathione levels in the hippocampi of streptozotocin-induced diabetic rats. Western blot analysis revealed that luteolin also inhibited the expression of apoptosis-related factors and cytochrome c release from mitochondria. Luteolin also improved the learning and memory abilities of rats with diabetic encephalopathy in a water maze test. Further western blot analysis revealed that luteolin treatment facilitated neuronal cell survival through activation of the phosphatidylinositol 3-kinase/Akt signaling pathway, an extracellular signal pathway involved in the suppression of cell apoptosis and promotion of cell survival. These experimental findings indicate that luteolin can inhibit apoptosis of hippocampal nerve cells in rats with diabetic encephalopathy, and that this effect is mediated by an indirect antioxidative effect, the inhibition of activation of apoptosis-related factors and the activation of phosphatidylinositol 3-kinase/Akt signal pathway.

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Key Words

neural regeneration; brain injury; diabetic mellitus; luteolin; apoptosis; learning and memory; spatial recognition memory; nerve cells; hippocampus; anti-oxidation; neuroregeneration

Research Highlights

(1) Luteolin is a kind of natural flavonoid existing in several plants. It has many kinds of pharmacological activities, with anti-oxidative, anti-inflammatory and anti-apoptosis effects, and can be regarded as a potential natural anti-aging protective agent, even an ideal treatment for neurological impairment.
(2) Luteolin injection *via* tail veins reduced the amount of streptozotocin-induced apoptosis in hippocampal cells in rats with diabetic encephalopathy, and the molecular mechanism was investigated.
(3) Luteolin could inhibit apoptosis of hippocampal nerve cells in rats with diabetic encephalopathy, and the mechanism of action included an indirect anti-oxidative effect and inhibition of apoptosis-related factors. In addition, luteolin activates the phosphatidylinositol 3-kinase/Akt signaling pathway, and accordingly suppresses apoptosis of hippocampal nerve cells.

INTRODUCTION

Recent reports strongly suggest that apoptosis plays a central role in the development of complications in diabetes-associated neuronal disorders. Apoptosis contributes to normal tissue homeostasis, but changes in the amount of apoptosis may lead to disease[1]. Several studies have confirmed that apoptosis of neuronal cells in diabetic patients is mediated by hyperglycemia, which leads to the opening of mitochondrial permeability transition pores, and by up-regulated expression of caspase 3 and caspase 8[2-3]. The expression levels of apoptosis-inducing factor, cytochrome c and Bax have a close relationship with apoptosis. Antioxidant strategies have shown a promise for the treatment of both acute and chronic neurodegenerative diseases by effectively reversing apoptosis[4-7]. Overproduction of reactive oxygen species by mitochondria, driven by high glucose metabolism, can trigger cell death by modulating a series of intracellular signaling pathways[8]. Understanding the regulation of apoptosis may have therapeutic relevance for diabetes-related brain disorders.

Luteolin (3',4',5,7-tetrahydroxyflavone), an important member of the flavonoid family, is present in various fruits, vegetables and medicinal herbs. It exhibits a wide spectrum of pharmacological properties including antioxidant, anti-apoptotic and anti-inflammatory properties[9]. Recently, post-ischemic administration of luteolin was shown to be effective against ischemia-reperfusion injury by exerting antioxidant and anti-apoptotic effects[9], suggesting that luteolin could offer neuro-protection against brain damage. Although the influence of luteolin on apoptosis in several cell systems has been reviewed[10-11], little is known about its effects on apoptotic events in hyperglycemia-mediated brain damage. The phosphatidylinositol 3-kinase/Akt signaling pathway is implicated in cell proliferation and apoptosis. To address the lack of knowledge about the effects of luteolin on apoptotic events in hyperglycemia-mediated brain damage, we investigated the effects of luteolin treatment on hyperglycemia-mediated apoptosis in the hippocampi of rats with streptozotocin-induced diabetic encephalopathy. To assess the mechanism(s) underlying the anti-apoptotic activity of luteolin, we measured the influence of luteolin on production of glutathione and malondialdehyde, expression of apoptosis-inducing factor and Bax, activation of caspase-3 and -8, and release of cytochrome c from mitochondria, as well as on the phosphatidylinositol 3-kinase/Akt signaling pathway.

RESULTS

Quantitative analysis of experimental animals

A total of 90 rats were equally and randomly assigned to six groups: a control group (normal feeding), a luteolin group (continuous injection of luteolin into tail veins), a dimethyl sulfoxide group (continuous injection of dimethyl sulfoxide into tail veins), a diabetes mellitus group (continuous injection of streptozotocin for 15 days), a diabetes mellitus + luteolin group (diabetes mellitus + continuous injection of luteolin for 15 days), and a diabetes mellitus + luteolin + wortmannin group (diabetes mellitus + continuous injection of luteolin for 15 days + phosphatidylinositol 3-kinase pathway inhibitor wortmannin for 15 days). Hippocampal tissues of rats in each group were collected for further study. Ninety rats were included in the final analysis.

Luteolin decreased blood glucose levels, increased body weights and improved learning and memory capacity in diabetic encephalopathy rats

After 15-day treatment with luteolin (day 80 after streptozotocin induction), fasting blood glucose levels were significantly decreased compared with the diabetes mellitus and diabetes mellitus + luteolin + wortmannin groups ($P < 0.01$; Figure 1A). The body weights of the experimental rats were also measured. Compared with age-matched non-diabetes mellitus rats, the body weights of diabetes mellitus group rats declined after streptozotocin injection ($P < 0.01$). The body weight at day 80 was greater in the luteolin group than that in the diabetes mellitus and diabetes mellitus + luteolin + wortmannin groups (Figure 1B).

Cognitive function was assessed in the Morris water maze test during days 81–84 (Figure 2). From the second day, the escape latency in the diabetes mellitus group was significantly higher than that in the control, luteolin and dimethyl sulfoxide groups ($P < 0.01$). Diabetes mellitus rats showed less ability to find the platform during the 4-day training period than did control, luteolin and dimethyl sulfoxide rats. Luteolin treatment significantly improved the escape latency to find the platform ($P < 0.01$); the escape latency in diabetes mellitus + luteolin + wortmannin group was similar to that in diabetes mellitus group.

In the probe trial of the Morris water maze, which assesses how well rats have learned and consolidated the platform location during the 4 days of training, there was a significant difference among rats in different

groups. The number of times crossing the platform and the percentage of time spent in the target quadrant are the relevant indices. Compared with the control, luteolin and dimethyl sulfoxide groups, rats in the diabetes mellitus group showed significantly fewer platform crossings and a lower percentage of time spent in the target quadrant ($P < 0.05$). Rats treated with luteolin performed similarly to the rats in the diabetes mellitus group in terms of the number of times crossing the platform (Table 1). Nevertheless, luteolin treatment significantly increased the percentage of time spent in the target quadrant ($P < 0.05$) compared with the diabetes mellitus group. The diabetes mellitus + luteolin + wortmannin group showed similar performance to the diabetes mellitus group (Table 1).

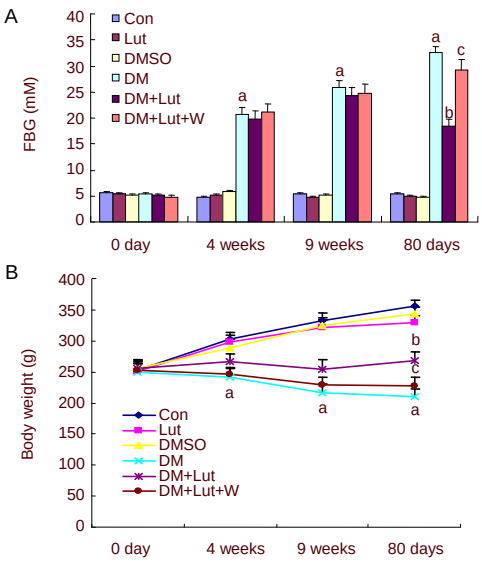

Figure 1 Effects of Lut treatment on fasting blood glucose (FBG) levels and body weights of diabetic encephalopathy rats.

(A) Fasting blood glucose levels; (B) body weight. [a]$P < 0.01$, *vs*. Con, Lut, and DMSO groups; [b]$P < 0.01$, *vs*. DM group; [c]$P < 0.01$, *vs*. DM + Lut group. Data are expressed as mean ± SD from 15 rats in each group. Differences between groups were compared using one-way analysis of variance and least significant difference *t*-test.

Con: Control; Lut: luteolin; DMSO: dimethyl sulfoxide; DM: diabetes mellitus; W: the phosphatidylinositol 3-kinase inhibitor wortmannin.

Influence of luteolin on the hippocampal malondialdehyde level in diabetic encephalopathy rats

Compared with control, luteolin and dimethyl sulfoxide groups, the malondialdehyde level in the hippocampus was significantly increased in the diabetes mellitus group ($P < 0.01$), and this increase in malondialdehyde level was attenuated by luteolin treatment. The malondialdehyde levels in the diabetes mellitus + luteolin group were significantly lower than that in diabetes mellitus group (Figure 3).

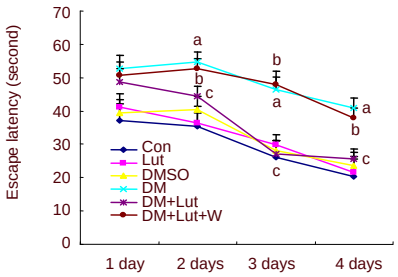

Figure 2 Effect of Lut treatment on the learning abilities of diabetic encephalopathy rats in a Morris water maze test.

[a]$P < 0.01$, *vs*. Con, Lut and DMSO groups; [b]$P < 0.01$, *vs*. DM group; [c]$P < 0.01$, *vs*. DM + Lut group. Data are expressed as mean ± SD from 15 rats in each group. Differences between groups were compared using one-way analysis of variance and least significant difference *t*-test.

Con: Control; Lut: luteolin; DMSO: dimethyl sulfoxide; DM: diabetes mellitus group; Lut: luteolin treatment; W: the phosphatidylinositol 3-kinase inhibitor wortmannin.

Table 1 Effects of Lut treatment on the memory of diabetic encephalopathy rats in a Morris water maze test

Group	Number of times crossing the platform	Percentage of time spent in the target quadrant (%)
Control	4±0	35.6±3.5
Lut	4±0	33.8±2.9
DMSO	4±0	36.1±3.8
DM	2±0[a]	25.8±3.6[b]
DM+Lut	2±0	32.5±2.9[c]
DM+Lut+W	2±0	26.4±3.0[d]

[a]$P < 0.05$, [b]$P < 0.01$, *vs*. control group; [c]$P < 0.05$, *vs*. DM group; [d]$P < 0.05$, *vs*. DM + Lut group. Data are expressed as mean ± SD from 15 rats in each group. Differences between groups were compared using one-way analysis of variance and least significant difference *t*-test. Percentage of time spent in the target quadrant was calculated from the total time spent in the target quadrant in a time period of 90 seconds.

Lut: Luteolin; DMSO: dimethyl sulfoxide; DM: diabetes mellitus; W: the phosphatidylinositol 3-kinase inhibitor wortmannin.

Luteolin increased the hippocampal glutathione level in diabetic encephalopathy rats

A significant reduction in the level of glutathione was observed in rats in the diabetes mellitus group (21.28 ± 5.20 μg/mg; $P < 0.01$) compared with the control group (116.4 ± 9.43 μg/mg), luteolin group (110.8 ± 5.31 μg/mg) and dimethyl sulfoxide group (120.1 ± 4.3 μg/mg). There was a significant rise in the level of glutathione in the diabetes mellitus + luteolin group (107.23 ± 4.7 μg/mg) compared with the diabetes mellitus group ($P < 0.01$;

Figure 4).

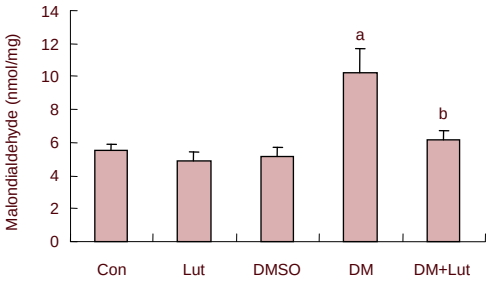

Figure 3 Effect of Lut treatment on malondialdehyde level in the hippocampi of rats with diabetic encephalopathy.

[a]*P* < 0.01, *vs*. Con, Lut and DMSO groups; [b]*P* < 0.01, *vs*. DM group. Data are expressed as means ± SD from five rats in each group. Differences between groups were compared using one-way analysis of variance and least significant difference *t*-test.

Con: Control; Lut: luteolin; DMSO: dimethyl sulfoxide; DM: diabetes mellitus.

Influence of luteolin on streptozotocin-induced neuronal morphology in diabetic encephalopathy rats

Hematoxylin-eosin staining revealed a significant decrease in the number of hippocampal neurons in diabetes mellitus rats compared with the control, luteolin and dimethyl sulfoxide groups (*P* < 0.01). After treatment with luteolin, hippocampal neurons appeared to be arranged in an orderly manner, and the number of neurons remained at normal levels. The number of hippocampal neurons in the diabetes mellitus + luteolin + wortmannin group was also decreased (*P* < 0.01; Figures 5, 6).

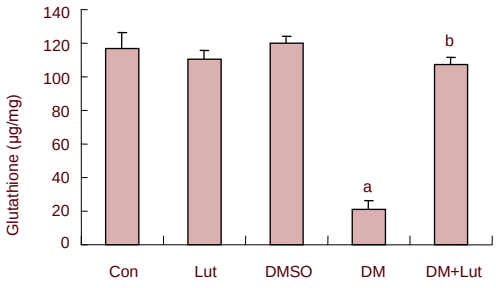

Figure 4 Effect of Lut treatment on glutathione level in the hippocampi of rats with diabetic encephalopathy rats.

[a]*P* < 0.01, *vs*. Con, Lut and DMSO groups; [b]*P* < 0.01, *vs*. DM group. Data are expressed as mean ± SD from five rats in each group. Differences between groups were compared using one-way analysis of variance and least significant difference *t*-test.

Con: Control; Lut: luteolin; DMSO: dimethyl sulfoxide; DM: diabetes mellitus.

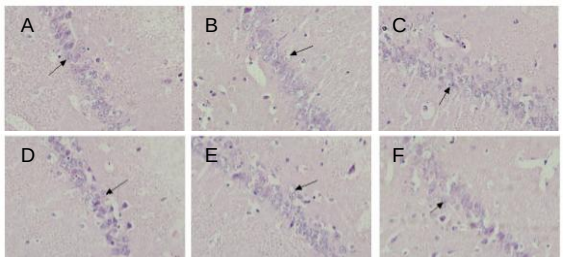

Figure 5 Effect of Lut treatment on the morphology of hippocampal neurons in rats with diabetic encephalopathy (hematoxylin-eosin staining, light microscopy, × 200).

In Con (A), Lut (B) and DMSO (C) groups, neurons were arranged orderly and the neuronal cytoplasm was lightly stained. The DM group showed a significantly reduced number of neurons with a disorderly arrangement (D). After Lut treatment for 15 days, orderly arranged cells were seen in the CA1 region of the hippocampus and the number of neurons was similar to that in the Con group (E), while findings in the DM+ Lut + W group were similar to those in the DM group (F).

Con: Control; Lut: luteolin; DMSO: dimethyl sulfoxide; DM: diabetes mellitus; W: the phosphatidylinositol 3-kinase inhibitor wortmannin.

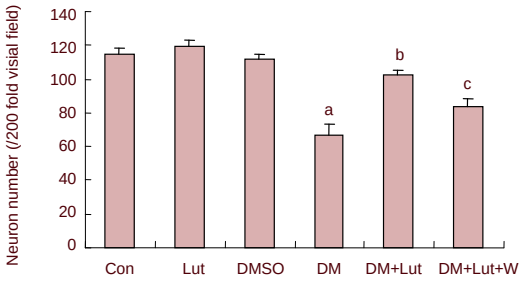

Figure 6 Effect of Lut treatment on the quantity of hippocampal neurons in rats with diabetic encephalopathy.

[a]*P* < 0.01, *vs*. Con, Lut and DMSO groups; [b]*P* < 0.01, *vs*. DM group; [c]*P* < 0.01, *vs*. DM + Lut group. Data are expressed as mean ± SD from five rats in each group. Differences between groups were compared using one-way analysis of variance and least significant difference *t*-test.

Con: Control; Lut: luteolin; DMSO: dimethyl sulfoxide; DM: diabetes mellitus; W: the phosphatidylinositol 3-kinase inhibitor wortmannin.

Influence of luteolin on streptozotocin-induced cell apoptosis in the hippocampi of diabetic encephalopathy rats

Apoptotic cells were detected by terminal deoxynucleotidyl transferase mediated dUTP nick end labelling (TUNEL) staining in hippocampal slices (Figures 7, 8). In control, luteolin and dimethyl sulfoxide groups, there were few TUNEL-positive cells in the hippocampus (Figures 7A–C). In contrast, significant numbers of TUNEL-positive cells appeared in the hippocampal CA1 area in the diabetes mellitus group

($P < 0.01$; Figure 7D). TUNEL-positive cells were also found in luteolin-treated diabetic rats (Figure 7E). There were significantly fewer TUNEL-positive cells in the diabetes mellitus + luteolin group compared with the diabetes mellitus and diabetes mellitus + luteolin + wortmannin group (Figure 7F).

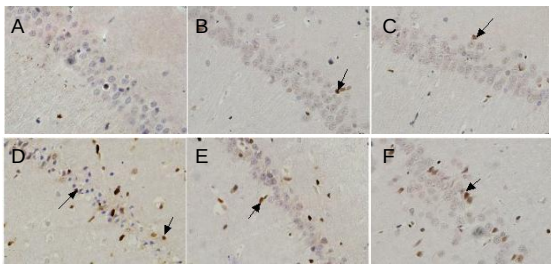

Figure 7 Effect of Lut treatment on cell apoptosis in the hippocampi of rats with diabetic encephalopathy (TUNEL staining, light microscopy, × 200).

There were fewer positive cells in the control, Lut and DMSO groups (A–C) than in the DM, DM + Lut and DM + Lut + W groups (D–F). In comparison, the number of TUNEL-positive cells was increased markedly in the DM group (D). Lut significantly reduced the number of TUNEL-positive cells (E), but there were more TUNEL-positive cells in the DM + Lut + W group (F).

Lut: Luteolin; DMSO: dimethyl sulfoxide; DM: diabetes mellitus; W: the phosphatidylinositol 3-kinase inhibitor wortmannin; TUNEL: terminal deoxynucleotidyl transferase mediated dUTP nick end labeling.

Influence of luteolin on the expression of apoptosis-related proteins

To further confirm the influence of luteolin treatment in hyperglycemia-induced apoptosis in hippocampal neuronal cells, a western blot assay was performed to detect caspase-8, caspase-3, apoptosis-inducing factor and Bax activities, as well as cytochrome c release from mitochondria. As shown in Figure 9, streptozotocin clearly up-regulated the activities of cleaved caspase-8, cleaved caspase-3, apoptosis-inducing factor and Bax, as well as cytochrome c release in the diabetes mellitus group compared with the control, luteolin and dimethyl sulfoxide groups ($P < 0.01$). Expression levels of hyperglycemia-induced apoptosis-related factors were attenuated by treatment with 10 mg/kg luteolin for 15 days. These findings indicate that luteolin inhibits apoptosis through the mitochondrial pathway.

Influence of luteolin on the streptozotocin-induced changes in the phosphatidylinositol 3-kinase/Akt signaling pathway

The phosphatidylinositol 3-kinase signaling pathway, which promotes cell survival, has been reported to be involved in apoptosis. It is reported that luteolin can inhibit apoptosis, so we next sought to determine whether the effects of luteolin on apoptosis in streptozotocin-induced rat brain disorder are mediated by this pathway. We first addressed whether apoptosis of hippocampal neuronal cells under streptozotocin-induced hyperglycemic conditions is related to Akt phosphorylation by luteolin. As shown in Figures 7B, C, dimethyl sulfoxide and luteolin alone did not cause apoptosis or death of hippocampal neuronal cells, while the apoptotic population among the diabetes mellitus group was significantly increased ($P < 0.01$) and luteolin treatment could effectively protect cells against streptozotocin-induced cell apoptosis in the diabetes mellitus group.

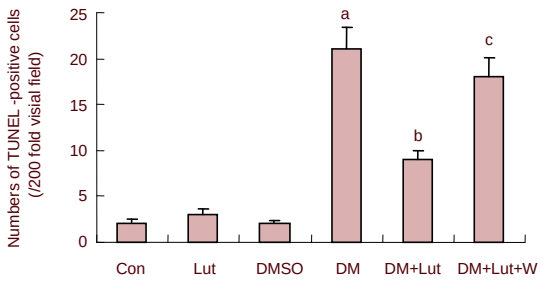

Figure 8 Effect of Lut treatment on the number of TUNEL-positive cells in the hippocampi of rats with diabetic encephalopathy.

[a]$P < 0.01$, *vs*. Con, Lut and DMSO groups; [b]$P < 0.01$, *vs*. DM group: [c]$P < 0.01$, *vs*. DM + Lut group. Data are expressed as mean ± SD from five rats in each group. Differences between groups were compared using one-way analysis of variance and least significant difference *t*-test.

Con: Control; Lut: luteolin; DMSO: dimethyl sulfoxide; DM: diabetes mellitus; W: the phosphatidylinositol 3-kinase inhibitor wortmannin; TUNEL: terminal deoxynucleotidyl transferase mediated dUTP nick end labeling.

However, when rats were pre-treated with both luteolin and wortmannin (a specific phosphatidylinositol 3-kinase inhibitor, 16 μg/kg), the protective effect of luteolin disappeared. We then determined whether luteolin could induce Akt phosphorylation in hippocampal neuronal cells under streptozotocin-induced hyperglycemic conditions by western blot assay. When the rats were treated with streptozotocin for 80 days, the level of Akt phosphorylation decreased compared with the control, luteolin and dimethyl sulfoxide groups ($P < 0.01$), whereas it was significantly increased after luteolin treatment ($P < 0.01$; Figure 10). However, co-treatment with wortmannin effectively inhibited Akt phosphorylation, indicating that the phosphatidylinositol 3-kinase/Akt pathway plays an important role in mediating the survival of hippocampal neuronal cells induced by luteolin.

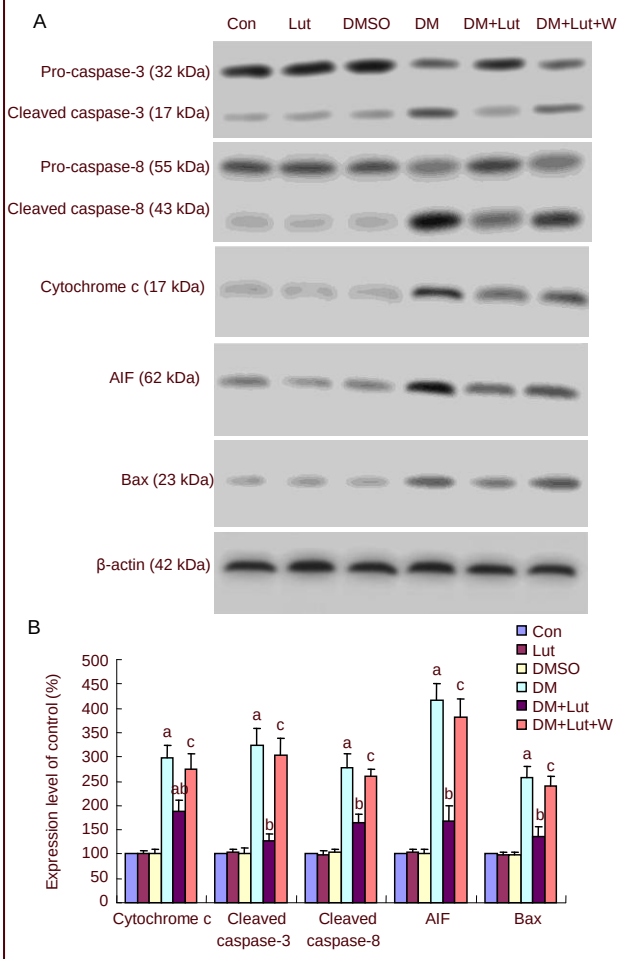

Figure 9 Effect of Lut on the expression of apoptosis-related proteins by western blot assay.

(A) Relative levels of caspase-3, caspase-8, apoptosis-inducing factor, Bax and cytochrome c were calculated and normalized to the loading control.

(B) Corresponding protein levels were assessed using densitometry and are expressed as relative intensities. $^{a}P < 0.01$, *vs*. Con group; $^{b}P < 0.01$, *vs*. DM group; $^{c}P < 0.05$, *vs*. DM + Lut group. Data were expressed as mean ± SD from five rats in each group. Differences between groups were compared using one-way analysis of variance and least significant difference *t*-test.

Con: Control; Lut: luteolin; DMSO: dimethyl sulfoxide; DM: diabetes mellitus; W: the phosphatidylinositol 3-kinase inhibitor wortmannin; AIF: apoptosis-inducing factor.

DISCUSSION

In the present study, streptozotocin treatment produced a significant increase in plasma glucose levels along with a reduction in body weight. Hyperglycemia and weight loss are the most important characteristics of diabetes mellitus, so elevated blood glucose level and body weight in diabetes mellitus are very important in determining whether diabetes is properly controlled at a specific time.

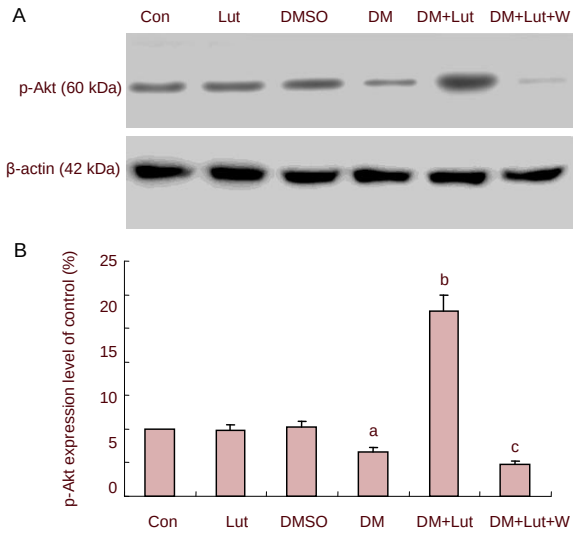

Figure 10 Effect of Lut on the phosphatidylinositol 3-kinase/Akt (p-Akt) pathway in diabetic encephalopathy rats.

(A) Western blot analysis using a p-Akt antibody was performed. β-Actin served as the loading control.

(B) Quantitative analysis was performed by measuring the fluorescence intensity relative to that in the Con group. $^{a}P < 0.01$, *vs*. Con group; $^{b}P < 0.01$, *vs*. DM group; $^{c}P < 0.05$, *vs*. DM + Lut group. Data are expressed as mean ± SD from five rats in each group. Differences between groups were compared using one-way analysis of variance and least significant difference *t*-test.

Con: Control group; Lut: luteolin; DMSO: dimethyl sulfoxide; DM: diabetes; Lut: luteolin; W: the phosphatidylinositol 3-kinase inhibitor wortmannin.

Blood glucose level and body weight were measured in all rats, and these results indicated that the administration of luteolin decreased the blood glucose concentration and increased body weight in diabetes mellitus rats. On the other hand, it was shown that the impairment of cognition in diabetic encephalopathy could be ameliorated significantly by luteolin treatment. Moreover, hyperglycemia promoted apoptosis of neurons in the CA1 region of the hippocampus, and a pivotal role of luteolin in protecting against apoptosis under hyperglycemia conditions was identified. Lastly, luteolin treatment strongly activated the phosphatidylinositol 3-kinase/Akt signaling pathway, suggesting that the suppression of hyperglycemia-induced apoptosis by luteolin may be a result of activation of an anti-apoptotic signaling pathway. These findings together with the fact that luteolin treatment improved learning and memory in the Morris water maze test in streptozotocin-induced diabetic rats, suggests that it may be a potential neuroprotective agent with

therapeutic value.

In the present study, the number of healthy neurons was markedly decreased and the number of TUNEL-positive cells was significantly increased after induction of diabetic encephalopathy. Treatment with luteolin significantly reduced the number of TUNEL-positive cells and rescued healthy neurons. The expression level of Bax has a close relationship with mitochondrial apoptosis, which plays an important role in the regulation of cell apoptosis. We found that hyperglycemia increased both the numbers of apoptotic cells and the expression of Bax, and that hyperglycemia-induced apoptosis is mediated by mitochondria. We also found that luteolin effectively inhibited cytochrome c release from mitochondria and the expression of Bax and apoptosis-inducing factor in rats treated with streptozotocin. In addition, luteolin strongly inhibited caspase-3 and caspase-8 activation under the same conditions. This suggests that the action of luteolin may be connected to molecular pathways downstream of mitochondria, involving activation of the caspase cascade.

In the present study, we used streptozotocin-induced diabetes, a well-documented model of experimental diabetes mellitus, because it provides a relevant example of endogenous chronic oxidative stress. We showed that the level of glutathione, a potent endogenous antioxidant that is the first line of defense against free radicals, decreased, while that of malondialdehyde, an important marker of lipid peroxidation, increased in the hippocampi of rats with diabetic encephalopathy. These results indicated that a disturbance in the endogenous anti-oxidant balance occurs in diabetic encephalopathy. After treatment of diabetes mellitus rats with luteolin, the level of glutathione increased while that of malondialdehyde decreased significantly. Our data suggest that hyperglycemia can induce oxidative stress in hippocampal neurons and that luteolin treatment modulated the hyperglycemia-induced activities of antioxidant enzymes, suggesting that luteolin may inhibit apoptosis by decreasing oxidative stress.

In the present study, we found that phosphorylation of Akt was decreased in streptozotocin-treated rats and that luteolin supplementation increased the level of phosphorylated Akt. Luteolin-mediated Akt activation and subsequent events were inhibited by a phosphatidylinositol 3-kinase inhibitor. These data suggest that luteolin activates survival signals through the phosphatidylinositol 3-kinase/Akt signaling pathway. Furthermore, a phosphatidylinositol 3-kinase inhibitor enhanced hyperglycemia-induced CA1 neuronal cell death, supporting the idea that activation of the phosphatidylinositol 3-kinase/Akt signaling pathway protects CA1 neuronal cells from hyperglycemia-induced apoptosis.

In conclusion, luteolin can prevent neuronal loss in the hippocampal CA1 region and improve learning and memory in rats with diabetic encephalopathy. Luteolin supplementation inhibited neuronal apoptosis by several independent mechanisms, including an indirect antioxidant effect, modulating glutathione and malondialdehyde production, and also by inhibiting hyperglycemia-induced activation of caspase-8, caspase-3, apoptosis-inducing factor and Bax, as well as cytochrome c release from mitochondria. Furthermore, luteolin activated the phosphatidylinositol 3-kinase/Akt pathway and directly inhibited hyperglycemia-induced apoptosis and diabetes-related brain disorders.

MATERIALS AND METHODS

Design

A randomized, controlled animal experiment.

Time and setting

This experiment was performed at the Central Laboratory, the First Affiliated Hospital of China Medical University, China between May 2011 and March 2012.

Materials

Animals

Male Sprague-Dawley rats at 10 weeks of age were bred in the Center of Experimental Animals, China Medical University, China (license No. SYXK (Liao) 2008-0013). Experimental protocols were in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[12].

Drugs

Luteolin (> 99% purity, analytical grade) was purchased from Sigma, St. Louis, MO, USA.

Methods

Animal treatment and intervention

Rats fasted for 12 hours were subjected to a single intraperitoneal injection of streptozotocin (Sigma; 55 mg/kg), which was freshly dissolved in 100 mM

sodium citrate buffer at pH 4.5. Control rats received citrate buffer only. Development of diabetes was confirmed by fasting blood glucose levels using a reagent kit. Rats with fasting blood glucose levels higher than 16.7 mM at 72 hours after streptozotocin injection were considered to be diabetic rats and fed for 65 days; then, rats were allocated into groups. Model success was assessed using behavioral testing.

Blood glucose testing and body weight measurements were repeated at 4, 9 weeks and 80 days after streptozotocin injection. Sixty-five days after streptozotocin administration, luteolin (10 mg/kg, 99% purity; Sigma) was given by tail vein injection twice daily for 15 days[13]. Wortmannin (a specific phosphatidylinositol 3-kinase inhibitor, 16 μg/kg, Sigma) was injected *via* the tail vein 5 minutes before luteolin injection (luteolin and wortmannin were both dissolved in dimethyl sulfoxide)[13]. The luteolin group and dimethyl sulfoxide group received the same volume of luteolin and dimethyl sulfoxide for 15 days. Following completion of the luteolin regimen, all experimental rats were assessed with a Morris water maze test from day 81 to day 84.

Effect of luteolin on the learning and memory capacity of diabetic rats detected by Morris water maze test

The experimental apparatus (Chinese Academy of Science, China) consisted of a circular water tank (diameter 130 cm, height 50 cm) containing water at 23 ± 2°C. The target platform (10 cm × 10 cm) was submerged 1 cm below the water surface and placed at the midpoint of one quadrant. The platform was fixed at a position equidistant from the center and the wall of the pool. The pool was placed in a test room containing various prominent visual cues. Rats were subjected to a spatial reference memory version of the water maze as described previously[14]. The water maze task was carried out for 5 consecutive days.

Learning test: A place navigation test was performed wherein the extent of learning was assessed. The rats received four consecutive daily training trials, with each trial having a ceiling time of 90 seconds and a trial interval of approximately 30 seconds. For each trial, each rat was put into the water at one of four start locations, the sequence of which was selected randomly. The rat had to swim until it climbed onto the platform submerged underneath the water. After climbing onto the platform, the animal remained there for 20 seconds before the commencement of the next trial. The escape platform was kept in the same position relative to the distal cues. If the rat failed to reach the escape platform within the maximally allowed time of 90 seconds, it was promptly allowed to climb onto the platform and to remain there for the same amount of time. The time to reach the platform (escape latency) was measured.

Memory test: The time spent in the target quadrant indicates the degree of memory after learning training. In the probe trial, the hidden platform was removed from the pool, and the rat was placed into the pool from the start location at the quadrant opposite the former platform quadrant. The number of times crossing the former platform and the percentage of time spent in the former platform quadrant were recorded for 90 seconds.

Estimation of biochemical parameters

Brain tissue preparation: The rats were decapitated under ethyl ether anesthesia. Brains were rapidly removed, and the temporal cortex and hippocampus were isolated. The whole hippocampus was quickly removed and cleaned with chilled normal saline on the ice. A 10% (w/v) homogenate of brain samples (0.03 M sodium phosphate buffer, pH 7.4) was prepared using an Ultra-Turrax T25 homogenizer (Sigma) at a speed of 9 500 r/min. The homogenized tissue preparation was used to measure malondialdehyde and glutathione levels.

Measurement of malondialdehyde levels: Malondialdehyde, which provides a measure of lipid peroxidation, was measured spectrophotometrically by the method of Colado *et al* [15], using 1,1,3,3-tetraethoxypropane as a standard. Malondialdehyde level is expressed in nanomoles per mg protein. The malondialdehyde-detecting kit was purchased from Nanjing Jiancheng Bio-Corporation (Nanjing, Jiangsu Province, China). Tissue homogenate (500 μL) in PBS (pH 7.4), 300 μL of 30% trichloroacetic acid, 150 μL of 5 N HCl and 300 μL of 2% (w/v) 2-thiobarbituric acid were combined, and then the mixture was heated for 15 minutes at 90°C. The mixture was centrifuged at 12 000 × *g* for 10 minutes. A pink supernatant was obtained, which was measured spectrophotometrically at 532 nm.

Measurement of glutathione levels: A glutathione-detecting kit was purchased from Nanjing Jiancheng Bio-Corporation. Glutathione level was determined by its reaction with 5,5'-dithiobis (2-nitrobenzoic acid; Ellman's reagent) to yield a yellow chromophore, which was measured spectrophotometrically[16]. Brain homogenates were mixed with an equal amount of 10% trichloroacetic

acid (Sigma) and centrifuged (Remi cold centrifuge) at 2 000 × *g* for 10 minutes at 4°C. The supernatant was used for glutathione estimation. Processed tissue sample (0.1 mL), 2 mL of PBS (pH 8.4), 0.5 mL of 5,5'-dithiobis (2-nitrobenzoic acid; Sigma) and 0.4 mL of double-distilled water were combined, and the mixture was shaken vigorously on a vortex. The absorbance was read at 412 nm within 15 minutes. Glutathione content is expressed in micromole per milligram of protein.

Hematoxylin-eosin and TUNEL staining for the detection of hippocampal neuronal morphology and apoptosis

Rats were anesthetized and fixed by perfusing 200–300 mL fixative (4% paraformaldehyde in 0.1 M PBS, pH 7.4) through the left ventricle of the heart. The hippocampus was removed and post-fixed in perfusion fixative for 2 hours at room temperature, immersed in 20% sucrose solution, then immersed in wax and embedded. The hippocampus was coronally sectioned at a thickness of 10 μm for hematoxylin-eosin and TUNEL staining. A hematoxylin-eosin staining kit was purchased from Boster Biological Technology (Wuhan, Hubei Province, China). A TUNEL staining kit was purchased from Nanjing KeyGEN Biotech Co., Ltd. (Nanjing, China). Hippocampal neuronal morphology and quantity were observed by hematoxylin-eosin staining. As a histochemical marker for apoptotic cells, sections of the hippocampus were processed for TUNEL staining. Neuronal numbers and TUNEL-positive cells on each section were counted under a microscope (Olympus, Tokyo, Japan). Three complete typical 200 × fields of view were randomly selected from each section to quantify neurons or TUNEL-positive cells.

Western blot analysis

The following primary antibodies (rabbit anti-rat monoclonal antibodies) were used in this study: anti-p-Akt antibody (1:400; Cell Signaling Technology, Boston, MA, USA), anti-caspase-3 antibody (1:400; Cell Signaling Technology), anti-caspase-8 antibody (1:400; Cell Signaling Technology), anti-apoptosis inducing factor (1:400; Cell Signaling Technology), anti-Bax antibody (1:400; Cell Signaling Technology), anti-cytochrome c (1:400; Cell Signaling Technology), and anti-β-actin antibody (1:400; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After incubation with primary antibodies, polyvinylidene difluoride membranes were washed with Tris-Buffered Saline with Tween 20 and incubated with appropriate horseradish peroxides-labeled goat anti-rabbit IgG (1:200; Santa Cruz Biotechnology). After two rinses and four washes with Tris-Buffered Saline with Tween-20, the membranes were incubated in enhanced chemiluminescence reagent for horseradish peroxidase (60 seconds) and exposed to autoradiography film for visualization of the bands. The EC3 Imaging System (UVP Inc. CA, USA) was used to detect specific bands and the absorbance of each band was measured using Image J software (NIH Image, USA). The ratio between the absorbance of proteins and that for actin in the same sample was calculated as relative content and expressed graphically.

Statistical analysis

Statistical analysis was performed using SPSS 18.0 software (SPSS, Chicago, IL, USA). Data are expressed as mean ± SD. Variance was homogenous for use of standard analysis of variance methodology and a *post hoc* least significant difference *t*-test. The level of significance was defined at $P < 0.05$.

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Conflicts of interest: None declared.

Ethical approval: The experiments were approved by the Animal Research Committee, China Medical University, China.

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