

Luteolin attenuates cognitive dysfunction induced by chronic cerebral hypoperfusion through the modulation of the PI3K/Akt pathway in rats

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Abstract

Introduction: In our study, we evaluated the beneficial effect of luteolin in the treatment of cognitive dysfunction in rat models induced by cerebral hypoperfusion by two-vessel occlusion (2-VO). **Material and Methods:** Seventy-five male Sprague Dawley rats were subjected to 2-VO surgery, in all but 15 (the sham group, group I) the ligation being permanent to impair cognitive abilities. The sham group rats received saline instead of a drug; 15 2-VO rats were not injected at all (the model group, group II); 15 2-VO rats were administered luteolin at 50 mg/kg b.w. (the lut 50 group, group III); to a further 15 luteolin was given at 100 mg/kg b.w. (the lut 100 group, group IV); and the final 15 received nimodipine at 16 mg/kg b.w. as positive controls (the nimodipine group, group V). Object recognition and Morris water maze tests were performed to investigate memory ability. A Western blot test was also conducted to assess expression of phosphatidylinositol 3-kinase (PI3K), its downstream target protein kinase B (Akt), and the phosphorylated form (P-Akt) in cerebral cortex and hippocampus tissue samples. **Results:** Significant variations in the discrimination index in the object recognition test, the escape latencies in the Morris water maze test, and expression levels of PI3K-p110 α and PI3K-p85 were observed three months after 2-VO surgery in both lut groups, with a significant change in the nimodipine group compared to the model group. P-Akt and Akt were expressed significantly higher in both lut groups and the nimodipine group than in the model group. **Conclusion:** Luteolin treatment of rats cognitively dysfunctional after experimental cerebral hypo perfusion was neuroprotective by activating the PI3K/Akt signals which inhibit neuronal death in the cerebral cortex and hippocampal region.

Keywords: luteolin, nimodipine, hypoperfusion, hippocampus.

Introduction

With the advancement of age, dogs experience a neurodegenerative disorder that has several correlations with age-linked cognitive dysfunction and human Alzheimer's disease. Besides Alzheimer's disease, memory dysfunction is also a prevalent disorder in the geriatric population and is due to underlying vascular dementia, characterised by multiple clinical disorders in the cerebrovascular areas. It is difficult to diagnose cognitive dysfunction syndrome (CDS) in dogs since it relies largely on the pet owner reporting symptoms which are disorientation, variations in social relationships with owners or other pets, changes in sleep-wake periods, commencement of house soiling or

changes in acquired habits and activity levels. Although the precise cause of cognitive impairment is not clear, some changes in the brains of elderly dogs and cats have been reported, including increased oxidative stress markers, decreased brain mass, increased ventricular size, glial changes, demyelination, meningeal calcification, decreased neurotransmission, neuroaxonal degeneration, and increased apoptosis, lipofuscin, and β -myelitis. A study by the American Heart Association (5) showed that 19% of dementia cases constitute vascular dementia, the mechanism of which derives from neurochemical actions close to those of Alzheimer's disease. However, the treatments of vascular dementia and Alzheimer's disease cannot be interchanged. Neuropsychological analysis revealed that

the possible contributory causes of cognitive impairment are certainly oxidative injury and elevated β -amyloid content in the canine brain. Several meta-analyses of the studies showed the significance of cholinesterase inhibitors in the treatment of vascular dementia. The latest findings (2) in dogs receiving cholinergic therapy often suggest that early impairments are likely to correlate with age-linked cholinergic failure, as is often observed in humans. Canine CDS therefore represents an important therapeutic goal, but in Canada and the United States, only two therapeutic agents are approved in pet animals: selegiline and a prescription diet. As ageing is synonymous with many physiological shifts, it is proposed that instead of dietetic monotherapy, a synergistic mix of ingredients, including fatty acids, important minerals, vitamins, enzymes, protective co-factors, and tropical nutritious fruits such as banana, pineapple, and avocado could better protect brain cell health and preserve memory ability (7–9). Since oxidative stress damage accumulates in step with the age of the dog, following many additional pathological outcomes, such a multi-modal synergistic strategy has centred the attention on canine clinical treatments (10–12, 14, 15).

At present, the treatment of vascular dementia focuses mainly on symptomatic management and significant brain injury reduction. In the brain tissue and blood vessels, antioxidants like Se and vitamins E and C protect the organ from oxidative injury and inflammation-induced degradation (17–22) in middle-aged and elderly cats (23). Approved treatments, however, are still limited in medical practice. Therefore, finding an effective treatment for vascular dementia is essential. Different studies have demonstrated the benefits of herbal medicine for the treatment of many diseases including cognitive dysfunction, as herbal therapy seems to have a significant beneficial impact on memory function and is well-tolerated in patients with vascular dementia. The research investigated a phosphatidylserine, Ginkgo biloba, vitamin E and pyridoxine proprietary nutraceutical supplement approved in Italy as a neuroprotective dietary supplement for pathophysiological brain ageing in older cats and dogs. In this research comprising three recent clinical studies, it was confirmed that phosphatidylserine or the particular proprietary supplement referred to above mitigated clinical signs consistent with CDS in pets (2, 12, 14, 15); however, the implications of such a nutritional supplement on the memory function of elderly dogs have not been reported. Nutritional and dietary strategies may boost antioxidant resistance, reducing the impact of free radicals. A senior diet for dogs makes the symptoms milder and delays the development of cognitive decline. In laboratory-aged dog and cat trials, *S*-adenosyl methionine (SAME) increased mental function measures and probably attention, indicating that therapy using SAME may be more effective at an early stage of disease development but rather in more seriously affected patients. As another

plant-derived potential therapeutic, curcumin should be taken into consideration. It is known to be a valuable antioxidant, anti-amyloid and anti-inflammatory and is present in spices such as turmeric and catechin.

Luteolin is a natural flavonoid found in fruits, vegetables and Chinese medicinal plants known for its anti-apoptotic and anti-inflammatory properties, attenuation and regulation of neuroinflammation in Parkinson's disease, and alleviation of amyloid β peptide-induced impaired cognition in Alzheimer's (13). Luteolin has been shown in studies to protect against infarction–reperfusion (IR) injury in a variety of pathological conditions, including myocardial and cerebral IR injury (6). In a previous study, luteolin was found to protect human umbilical vein endothelial cells from oxidative stress and inflammation caused by tumour necrosis factors (27, 29, 30). Various processes like the cell cycle and the regulatory mechanism of proliferation and differentiation involve the phosphatidylinositol 3-kinase/protein kinase B (PI3K–Akt) pathway, which in turn modifies the apoptotic and autophagic activity.

Two-vessel occlusion surgery (2-VO) is performed on rats by permanently ligating the bilateral common carotid arteries to experimentally cause chronic cerebral hypoperfusion and mimic the neurological disorder of vascular dementia. Rats with two vessels occluded have often been used to explore the biological processes associated with hypoperfusion that are implicated in cognitive impairment and identify the possible benefits of new drugs for the treatment of vascular dementia. The possible beneficial effects of luteolin on vascular dementia using 2-VO rat models were evaluated in our present study, and the underlying mechanisms of luteolin activity were subsequently investigated.

Material and Methods

Drug substances. Luteolin (purity $\geq 98\%$) and nimodipine were obtained from Sigma Aldrich Ltd (Shanghai, China). Nimodipine was used as a drug for positive control in our studies due to its effective role in cognitive dysfunction as a calcium inhibitor in the cerebrovascular smooth muscles.

Animals. Male Sprague Dawley rats aged eight weeks (and weighing 280–300 g) were provided by the Shanghai Laboratory Animal Centre (Shanghai, China), housed at a temperature controlled to $23 \pm 2^\circ\text{C}$ and allowed standard food and water access. Ethical approval was sought from the University Bioethics Committee, and the experimental procedures were carried out based on the recommendations provided by the University's Animal Ethics Committee. All studies were performed under bright light.

Animal grouping and treatment. Healthy male Sprague Dawley rats were divided into five groups with 15 rats in each ($n = 15$): a sham group (I), model group (II), luteolin-administered group at 50 mg/kg b.w. (III),

luteolin-administered group at 100 mg/kg b.w. (IV), and a nimodipine-administered group at 16 mg/kg b.w. (V). The rats were starved for 12 hours before being injected with anaesthetic. The middle of the cervical region was incised, exposing the two carotid arteries. Silk sutures were used to ligate them firmly. Except for the control group rats, the common coronary arteries of all rats were ligated after surgery. When the rats regained consciousness, they were kept in a warm environment. Six hours after the surgery, drug treatment was started with intragastric administration (10 mL/kg b.w.) of luteolin or nimodipine and continued once a day for three months until the end of the experiment. The rats from the sham (control) group were administered only saline. The overall survival percentage was 84.6 %, although few rats died during the experiments (1, 3).

Object recognition test (ORT). An ORT was conducted according to previous studies (16) to investigate non-spatial memory in the first and third months post-2-VO. The test was performed for three days: the first was a habituation day – the rats were positioned in the centre of an open field and permitted to move freely to explore for ten minutes; the second was a training day – two identical objects were kept in the field facing each other and the rats were positioned equidistant from the two objects in the centre of the field and allowed to move freely for ten minutes to explore; and the third was a testing day – one familiar object (experimentally verified) was replaced in the same location in the field by a new one. For ten minutes, the rats were again centrally positioned between the objects to identify them. After every individual trial, each item was carefully cleaned using 75 percent v/v ethyl alcohol. On the testing day, the time spent discovering the familiar and the new objects was recorded. These values were employed to compute the memory discrimination index (DI). The higher the DI, the greater the memory ability of rats. The formula for deriving the DI is

$$DI = (N - F) / (N + F)$$

where N = time taken to identify new objects and F = time taken to identify familiar objects.

Morris water maze test (MWM). The MWM test is the commonly used method to evaluate the spatial learning ability linked to the hippocampus in chronic cerebral hypoperfusion in 2-VO rats. This test was performed as previously reported (16) at one month and three months after 2-VO surgery. In brief, from the starting position with the head of an animal at the wall of the water pool, rats were lowered gently into the water. Every animal was observed through two trials with two separate entry positions each day including the side quadrant of the opposite and adjacent platform. The time for locating the hidden platform under 1cm of water was recorded as the escape latency. Rats were permitted to swim for 120 s at every trial to approach the hidden target. The test lasted for five days. A video monitoring system (ANY-maze, Stoelting Co, Wood Dale, IL, USA) measured the escape latency, swimming distance and swimming speed automatically.

Western blot. After randomisation, the brains of four rats from every group were carefully extracted as soon as the animals were sacrificed after the behavioural tests. Next the hippocampal and cerebral cortex regions were homogenised in a mixture of protein lysis buffer and protease inhibitor (Roche, Mannheim, Germany). Total concentration of proteins was measured with an RC-DC Protein Assay Kit (Cell Signaling Technology, Danvers, MA, USA), following the instructions given by the manufacturer. Separation of proteins was performed on an SDS-PAGE gel and the proteins were electrophoretically moved on a PVDF membrane, later they were embedded in nonfat milk (5%) for an hour at room temperature, after which primary antibody probing at 4°C was carried out during the night. These antibodies were rabbit-anti-PI3K p85, rabbit-anti-PI3Kp110 α , rabbit-anti-Akt, and rabbit anti-phospho-Akt (1:1000, Cell Signaling Technology). The next day, their respective secondary antibodies conjugated with horseradish peroxidase underwent incubation for an hour. Chemiluminescence was used to visualise the bands and band intensities were compared to β -actin bands to analyse protein band blots.

Statistical analyses. The values of our results were expressed as mean \pm SEM. The outcomes of the MW test were analysed through repeatedly measured two-way ANOVA, while another test analysis was performed using one-way ANOVA followed by Dunnett's test. GraphPad Prism v 8.0 (GraphPad, San Diego, CA, USA) was used for statistical analysis. Significant differences ($P < 0.05$) were evaluated by comparing the means of five groups using the least significant difference (LSD) procedure.

Results

Luteolin effects on escape latency in the Morris water maze test. One month after 2-VO surgery, no significant differences in the duration of escape latencies were noticed when all three treatment groups were compared with the sham and 2-VO model groups over the five test days. Three months after 2-VO surgery, the duration of escape latencies from day 2 to 5 had decreased when compared with day 1 escape latencies in all groups. On day 5, the escape latencies were significantly longer in the model group (37.37 ± 6.07 s; $P < 0.01$) than in the sham group (6.21 ± 2.2 s) and significantly shorter in the luteolin treatment groups (5.77 ± 2.3 s for the lut 50 group and 6.52 ± 3.02 s for the lut 100 group) than in the model group. Also on the fifth day, no significant disparity was recorded between the nimodipine group (34.48 ± 5.85 s) and the 2-VO model group (37.37 ± 6.07 s). However, the latencies for the luteolin treatment groups were little different to those of the sham group, thus suggesting that luteolin showed accumulated dose-dependent mitigation of the impairment of memory caused by chronic 2-VO and restoration of normal values (Tables 1 and 2).

Table 1. Escape latencies in the Morris water maze test in all groups of rats at 1 month after 2-VO surgery (seconds)

Group	N	Day 1	Day 2	Day 3	Day 4	Day 5
Sham	12	91.18 ± 5.87	52.32 ± 6.03	33.38 ± 5.67	20.09 ± 4.83	22.34 ± 2.1
Model	12	93.2 ± 8.53	53.62 ± 6.04	34.58 ± 5.61	20.89 ± 4.98	24.26 ± 4.56
Lut 50	13	100.9 ± 5.65	55.26 ± 5.52	32.75 ± 5.49	30.63 ± 3.08	24.71 ± 4.98
Lut 100	12	94.35 ± 5.99	60.45 ± 2.76	37.57 ± 6.13	26.48 ± 6.15	23.08 ± 3.36
Nimodipine	12	105.4 ± 5.49	64.91 ± 3.4	39.98 ± 9.33	24.78 ± 6.65	25.28 ± 4.94

Data are expressed as mean ± SEM

Sham – group subjected to sham two-vessel occlusion surgery; Model – group subjected to two-vessel occlusion surgery without drug treatment; Lut 50 – group subjected to two-vessel occlusion surgery and administered luteolin at 50 mg/kg b.w.; Lut 100 – group subjected to two-vessel occlusion surgery and administered luteolin at 100 mg/kg b.w.; Nimodipine – group subjected to two-vessel occlusion surgery and administered nimodipine at 16 mg/kg b.w.

Table 2. Escape latencies in the Morris water maze test in all groups of rats at 3 months after 2-VO surgery (seconds)

Group	N	Day 1	Day 2	Day 3	Day 4	Day 5
Sham	12	19.6 ± 5.85	10.08 ± 4.81	9.367 ± 4.51	8.49 ± 4.34	6.21 ± 2.22
Model	12	44.34 ± 5.88	37.96 ± 5.59 ^{##}	27.61 ± 5.55 ^{##}	35.3 ± 5.40 ^{##}	37.37 ± 6.07 ^{##}
Lut 50	13	20.6 ± 5.40	8.553 ± 4.16 ^{**}	7.66 ± 3.52 ^{**}	6.93 ± 3.33 ^{**}	5.77 ± 2.36 ^{**}
Lut 100	12	26.76 ± 5.84	10.76 ± 4.56 ^{**}	10.18 ± 4.77 ^{**}	9.89 ± 5.07 ^{**}	6.52 ± 3.02 ^{**}
Nimodipine	12	56.24 ± 5.76	35.33 ± 5.60	35.52 ± 5.82	29.72 ± 7.65	34.48 ± 5.85

Data are expressed as mean ± SEM. Sham – group subjected to sham two-vessel occlusion surgery; Model – group subjected to two-vessel occlusion surgery without drug treatment; Lut 50 – group subjected to two-vessel occlusion surgery and administered luteolin at 50 mg/kg b.w.; Lut 100 – group subjected to two-vessel occlusion surgery and administered luteolin at 100 mg/kg b.w.; Nimodipine – group subjected to two-vessel occlusion surgery and administered nimodipine at 16 mg/kg b.w.

Table 3. Swimming speed of rats on the fifth day in the Morris water maze test (cm/s)

Group	N	1 month after 2-VO	3 months after 2-VO
Sham	12	14.25 ± 4.71	16.64 ± 5.70
Model	12	13.12 ± 3.26	15.31 ± 5.46
Lut 50	13	16.42 ± 5.09	17.18 ± 6.07
Lut 100	12	15.07 ± 5.24	16.09 ± 5.93
Nimodipine	12	13.25 ± 3.87	16.21 ± 6.01

Data are expressed as mean ± SEM

Sham – group subjected to sham two-vessel occlusion surgery; Model – group subjected to two-vessel occlusion surgery without drug treatment; Lut 50 – group subjected to two-vessel occlusion surgery and administered luteolin at 50 mg/kg b.w.; Lut 100 – group subjected to two-vessel occlusion surgery and administered luteolin at 100 mg/kg b.w.; Nimodipine – group subjected to two-vessel occlusion surgery and administered nimodipine at 16 mg/kg b.w.

Luteolin effects on swimming speed in the Morris water maze test. Our results did not demonstrate any significant differences between the sham, 2-VO-model and all three treatment groups in the swimming speed in the Morris water maze test one month after 2-VO surgery. Three months after 2-VO surgery, a similar trend was observed (Table 3). These findings showed that the swimming speed in the Morris water maze test did not affect the motor function involved in escape latencies, thereby indicating the ameliorative effects of luteolin on 2-VO induced spatial learning impairment.

Effects of luteolin on discrimination index. The discrimination index in the sham group was 0.31 ± 0.03

one month after 2-VO surgery (Figs 1a and 1b), which decreased significantly to 0.05 ± 0.01 in the model group and increased significantly in the two luteolin treatment groups (0.45 ± 0.04 for the lut 50 group and 0.49 ± 0.03 for the lut 100 group) relative to the model group. There were no significant differences between the nimodipine (0.11 ± 0.01) group and the model group. Significant reductions in the discrimination index in the model group (0.17 ± 0.03) relative to the sham group (0.41 ± 0.02) were seen three months after 2-VO surgery. Compared with the model group, the differentiation index was significantly increased in the luteolin treatment groups (0.59 ± 0.04 for the lut 50 group and 0.36 ± 0.06 for the lut 100 group) and the nimodipine group.

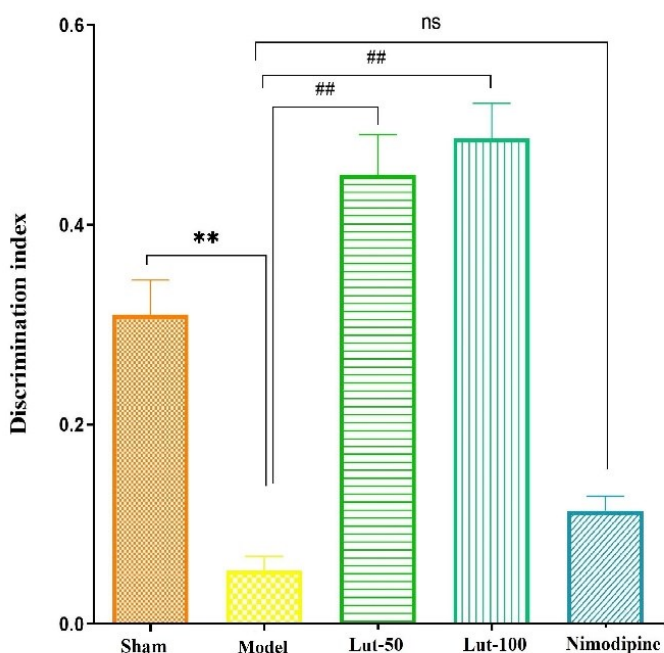


Fig. 1a. Effects of luteolin on learning and memory impairment in 2-VO rats evaluated by the object recognition test one month after 2-VO surgery. Discrimination index was measured at one month after 2-VO surgery. Data are expressed as mean ± SEM. n = 15 in each group
 Sham – group subjected to sham two-vessel occlusion surgery; Model – group subjected to two-vessel occlusion surgery without drug treatment; Lut 50 – group subjected to two-vessel occlusion surgery and administered luteolin at 50 mg/kg b.w.; Lut 100 – group subjected to two-vessel occlusion surgery and administered luteolin at 100 mg/kg b.w.; Nimodipine – group subjected to two-vessel occlusion surgery and administered nimodipine at 16 mg/kg b.w.; ** – P < 0.01, model group vs. sham group; ## – P < 0.01, luteolin-treated groups vs. model group; ns – non-significant

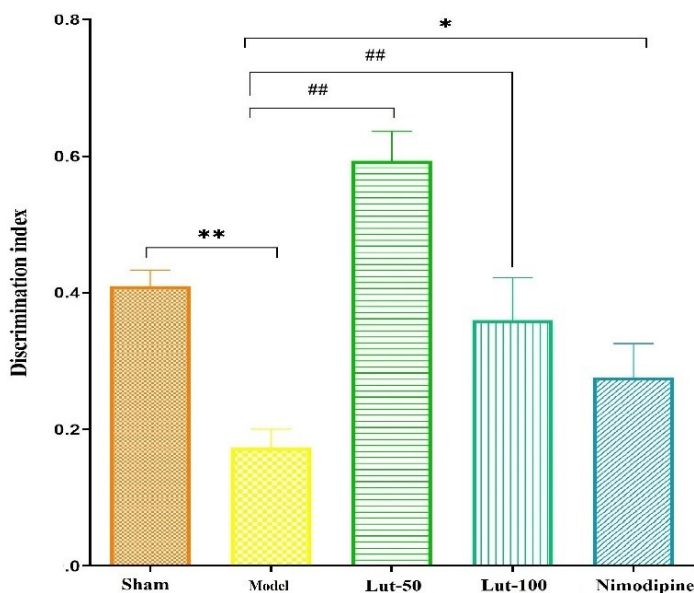


Fig. 1b. Effects of luteolin on learning and memory impairment in 2-VO rats evaluated by the object recognition test three months after 2-VO surgery. Discrimination index was measured at three months after 2-VO surgery. Data are expressed as mean ± SEM. n = 15 in each group
 Sham – group subjected to sham two-vessel occlusion surgery; Model – group subjected to two-vessel occlusion surgery without drug treatment; Lut 50 – group subjected to two-vessel occlusion surgery and administered luteolin at 50 mg/kg b.w.; Lut 100 – group subjected to two-vessel occlusion surgery and administered luteolin at 100 mg/kg b.w.; Nimodipine – group subjected to two-vessel occlusion surgery and administered nimodipine at 16 mg/kg b.w.; * – P < 0.05, ** – P < 0.01, model group vs. sham group; * – P < 0.05, all three treatment groups vs. model group; ## – P < 0.01, luteolin-treated groups vs. model group

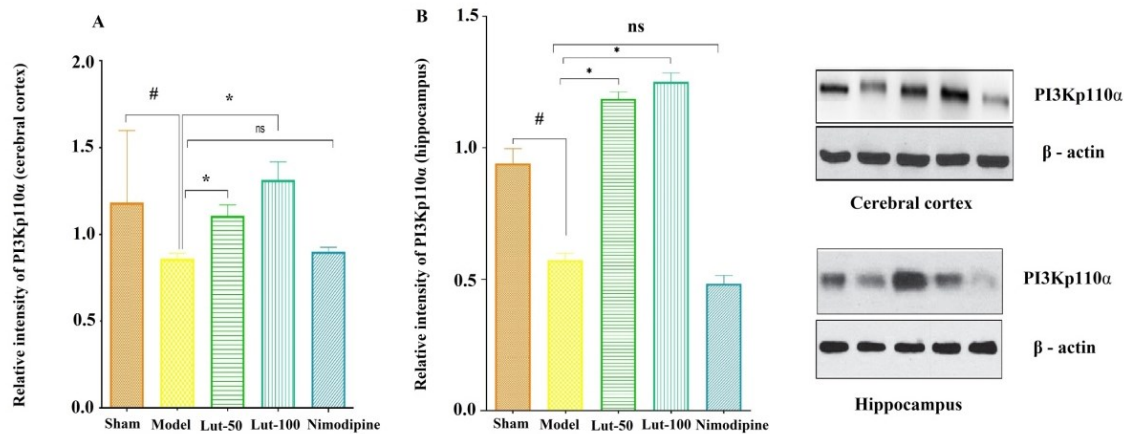


Fig. 2. Effects of luteolin on the PI3K/Akt pathway in the cerebral cortex (A) and hippocampus (B) of 2-VO rats shown in the relative intensity of PI3K subunits of p110 α and representative Western blot images of important factors in the PI3K pathway three months after 2-VO surgery. Data are expressed as mean \pm SEM. $n = 4$ in each group

Sham – group subjected to sham two-vessel occlusion surgery; Model – group subjected to two-vessel occlusion surgery without drug treatment; Lut 50 – group subjected to two-vessel occlusion surgery and administered luteolin at 50 mg/kg b.w.; Lut 100 – group subjected to two-vessel occlusion surgery and administered luteolin at 100 mg/kg b.w.; Nimodipine – group subjected to two-vessel occlusion surgery and administered nimodipine at 16 mg/kg b.w.; PI3K – phosphatidylinositol 3-kinase; β -actin – total protein amount normaliser; # – $P < 0.05$, model group vs. sham group; * – $P < 0.05$, luteolin-treated groups vs. model group; ns – non-significant

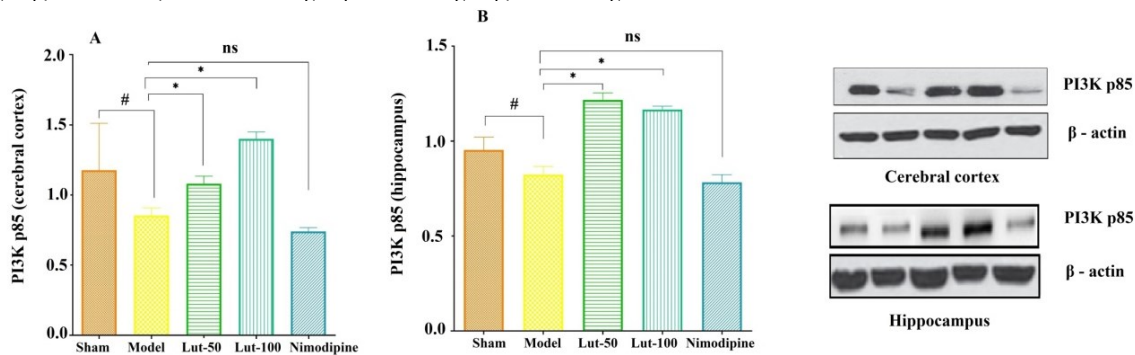


Fig. 3. Effects of Luteolin on the PI3K/Akt pathway in the cerebral cortex (A) and hippocampus (B) of 2-VO rats shown as the relative intensity of PI3K subunits of p85 and representative Western blot images of important factors in the PI3K pathway three months after 2-VO surgery. Data are expressed as mean \pm SEM. $n = 3$ in each group

Sham – group subjected to sham two-vessel occlusion surgery; Model – group subjected to two-vessel occlusion surgery without drug treatment; Lut 50 – group subjected to two-vessel occlusion surgery and administered luteolin at 50 mg/kg b.w.; Lut 100 – group subjected to two-vessel occlusion surgery and administered luteolin at 100 mg/kg b.w.; Nimodipine – group subjected to two-vessel occlusion surgery and administered nimodipine at 16 mg/kg b.w.; PI3K – phosphatidylinositol 3-kinase; β -actin – total protein amount normaliser; # – $P < 0.05$, model group vs. sham group; * – $P < 0.05$, luteolin-treated groups vs. model group; ns – non-significant

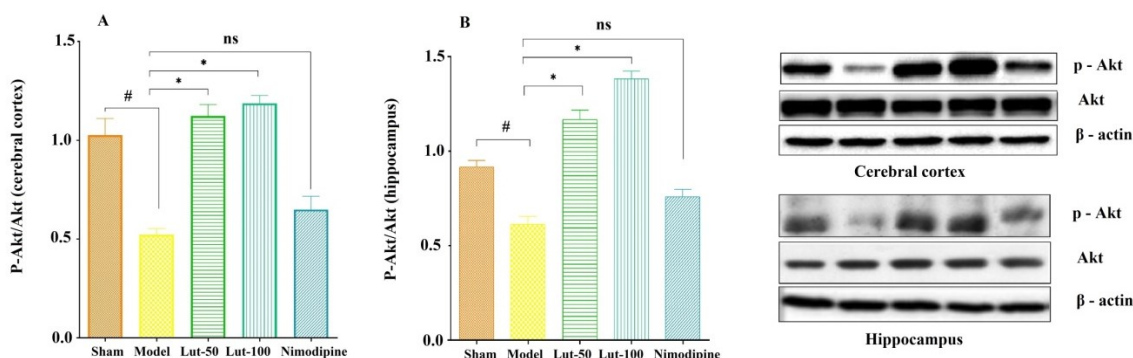


Fig. 4. Effects of luteolin on the P-Akt/Akt pathway in the cerebral cortex (A) and hippocampus (B) of 2-VO rats shown as the relative intensity of P-Akt and total Akt and representative Western blot images of important factors in the Akt pathway three months after 2-VO surgery. Data are expressed as mean \pm SEM. $n = 3$ in each group

Sham – group subjected to sham two-vessel occlusion surgery; Model – group subjected to two-vessel occlusion surgery without drug treatment; Lut 50 – group subjected to two-vessel occlusion surgery and administered luteolin at 50 mg/kg b.w.; Lut 100 – group subjected to two-vessel occlusion surgery and administered luteolin at 100 mg/kg b.w.; Nimodipine – group subjected to two-vessel occlusion surgery and administered nimodipine at 16 mg/kg b.w.; P-Akt – phosphorylated protein kinase B; Akt – protein kinase B; β -actin – total protein amount normaliser; # – $P < 0.05$, model group vs. sham group; * – $P < 0.05$, luteolin-treated groups vs. model group; ns – non-significant

Effects of luteolin on PI3K-p110 α expression in brain tissue. One month after 2-VO surgery, no significant variations were found between the sham, model and three treatment groups in the PI3K-p110 α expression levels in the cerebral cortex and hippocampus of the brain. The PI3K-p110 α expression levels in these regions of the brain were substantially decreased ($P < 0.05$) in the model group (0.86 ± 0.03 in the cortex and 0.63 ± 0.02 in the hippocampus) relative to the sham group (1.18 ± 0.41 in the cortex and 0.94 ± 0.06 in the hippocampus) three months after 2-VO surgery. Cerebral cortex and hippocampus expression levels of this protein were significantly ($P < 0.05$) upregulated in the lut 50 (1.15 ± 0.02 in the cortex and 1.18 ± 0.02 in the hippocampus) and lut 100 (1.31 ± 0.10 in the cortex and 1.25 ± 0.03 in the hippocampus) groups three months after 2-VO surgery. However, no major differences were found in its expression levels in the nimodipine group (0.90 ± 0.02 in the cortex and 0.61 ± 0.03 in the hippocampus) (Fig. 2) at this time compared to the model group.

Effects of luteolin on PI3K-p85 expression in brain tissue. No significant variations between the sham, model and three treatment groups were noticed in the levels of PI3K-p85 expression in the cerebral cortex and hippocampus one month after 2-VO surgery. The PI3K-p85 expression levels in these tissues were significantly downregulated in the model group (0.85 ± 0.05 in the cortex and 0.82 ± 0.04 in the hippocampus) when compared to the sham group (1.17 ± 0.33 in the cortex and 0.95 ± 0.06 in the hippocampus) three months after 2-VO surgery. Significant upregulation of this protein in the brain samples of both the lut 50 (1.13 ± 0.03 in the cortex and 1.2 ± 0.02 in the hippocampus) and lut 100 (1.4 ± 0.04 in the cortex and 1.17 ± 0.02 in the hippocampus) treatment groups was noted relative to the model group. However, no major differences between the sham (0.9321 ± 0.02) and nimodipine (0.7819 ± 0.04) groups were found in the PI3K-p85 expression levels of the cerebral cortex and hippocampus (Fig. 3) relative to the model group.

Effects of luteolin on phosphorylated protein kinase B and protein kinase B (P-Akt/Akt) expression in brain tissue. No major differences between the sham, model and three treatment groups were noticed one month after 2-VO surgery in the P-Akt/Akt expression levels in the cerebral cortex and hippocampus. The levels of P-Akt/Akt expression in the cerebral cortex in the sham group (1.027 ± 0.08) decreased substantially relative to the model group (0.61 ± 0.03) three months after 2-VO surgery. However, in the Lut 50 and 100 groups (1.12 ± 0.06 and 1.19 ± 0.04 , respectively), P-Akt/Akt expression levels were substantially higher relative to those of the model group. No major differences between the nimodipine group (0.65 ± 0.06) and the model group were observed. The levels of P-Akt/Akt expression in the sham group (0.95 ± 0.06) decreased substantially relative to the model group (0.61 ± 0.03) three months after 2-VO

surgery. However, expression levels of this protein in this brain region were significantly increased in the Lut 50 and 100 (1.12 ± 0.05 and 1.38 ± 0.04 , respectively) treatment groups compared to the model group (0.79 ± 0.04) three months after 2-VO surgery. Nevertheless, there was no substantial reduction in P-Akt/Akt expression levels in the nimodipine group (0.88 ± 0.03) (Fig. 4) when compared to the model group.

Discussion

There are different factors in the aetiology of vascular dementia, while the most frequent pathogenic mechanism is *via* hypoperfused conditions, occlusion of the vasculature or hypoxia causing ischaemic injury in different brain regions and subsequent memory and cognition dysfunction. Further pathological changes in severe cerebral ischaemic disorders may involve lesions leading to neuronal degeneration and other damage to neuronal pathways, as dilation of the vessel cannot be regarded as a significant cause of impaired cognition, memory or learning abilities. Two-vessel occlusion surgery (2-VO) was performed in rats by permanently ligating the bilateral common carotid arteries to mimic chronic cerebral hypoperfusion and thereby induce vascular dementia. We used the 2-VO rat model in our study to evaluate the influence of luteolin on cognitive dysfunction. Our findings showed that three months after 2-VO surgery in rats, the memory-building capacity, recognition memory and spatial learning abilities were significantly damaged. These outcomes were consistent with the results of previous studies using this model. The findings of a previous study showed that cerebral hypoperfusion increased the expression of β -site amyloid precursor protein cleaving enzyme (BACE1), elevated the amyloid beta (A) levels in the cortex and hippocampus, and activated nuclear factor-B (NF-B). Long-term administration of luteolin, on the other hand, greatly reduced the expression of NF-B and BACE1 and the deposition of these proteins (16).

Our findings demonstrated that luteolin treatment at 50 and 100 mg/kg b.w. significantly improved the cognitive dysfunction in 2-VO rats, greatly increasing the subjects' memory-building capacity, recognition memory and spatial learning abilities. Nimodipine treatment, however, demonstrated no noticeable changes in our 2-VO rat model, which may be because nimodipine is a calcium channel blocker of the cerebral vascular smooth muscle; thus, vasodilation itself may not be effective in improving learning and memory function. In agreement with our present findings, earlier studies suggested that nutraceutical supplementation comprising phosphatidylserine, ginkgo Biloba, vitamin E, and pyridoxine significantly enhanced canine short-term memory function. Nutraceuticals can provide higher amounts of active ingredients compared to dietary supplementation, particularly of ingredients which are difficult to develop in foods. Both the aforementioned

enhanced antioxidant diet and the latest nutraceutical intervention have been reported to enhance the clinical indications correlated with canine CDS, thus validating the application of the experimental canine cognitive model in the evaluation of treatments for both dogs and humans. Consistently with the cognitive benefits, phosphatidylserine supplementation enhanced social relationships, memory recall, and movement in humans and rodents, and Ginkgo biloba promoted short-term spatial memory retention (5) and enhanced cognitive performance in humans and aged animals.

Our recent findings were also compatible with previous studies investigating the antioxidant-enriched diet, in that some cognitive performance improvement might occur shortly after initiation of therapy (1). Among the major factors in brain ageing and dementia are oxidative stress and inflammation. A natural blend diet containing fruits, berries, cereals, nuts, legumes, and vegetables among the main ingredients and also offering fatty fish oils potentiates anti-oxidation and anti-inflammatory activities. These improvements may help to reduce the harm caused by oxidative stress and reduce inflammation in the entire body, including the brain. The findings offer more evidence for the usage of dietary intervention strategies based on the ingestion of a nutrient mix to modulate the age-related deterioration in brain activity. For instance, by incorporating additional nutrients and/or bio-actives, it may be possible to develop a more successful nutritional intervention. We have noticed that medium-chain triacylglyceride-supplemented diets often possess cognition-enhancing abilities, likely by supplying additional energy to the brain. The neuro-developmental consequences of a diet supplemented with a sialic acid-rich whey component were studied in preterm pigs, and it was observed that many sialyllactose-supplemented animals exceeded the learning needs in a T-maze, comparing favourably to control preterm pigs and reaching the cognitive performance of equivalent full-term animals. Sialyllactose supplements additionally succeeded in regulation of genes linked to the metabolism of sialic acid, myelination, and biosynthesis of ganglioside. Although in naturally raised full-term pigs, growth and hippocampal structural indicators were stronger, in intensively reared counterparts clinical indicators and growth were poorer, while cognitive efficiency was nevertheless retained. Basic nutrients such as sialic acid have been shown to increase brain growth, learning, and memory in animals.

The major pathophysiologic modification in the later stages of long-term cerebral ischaemia is a degenerative neuronal lesion (24). Our study showed that PI3K/Akt signalling was triggered, leading to significant changes in the promotion of neuron formation, differentiation and regeneration, along with synaptic plasticity, neuron survival and enhancement of neuronal growth (28). Phosphatidylinositol 3 kinase is a heterodimer composed of p85 and p110 subunits with a regulatory and catalytic role and belongs to the phospholipid kinase family (25).

Luteolin stimulates PI3K, which is processed into inositol diphosphate (PIP₂) and contributes to the binding of PIP₂ to Akt (or PKB-protein kinase B) by an unknown mechanism. In the presence of 3-phosphate inositol-dependent protein kinase-1 (PDK-1), luteolin induces the phosphorylation of Akt, resulting in its activation. Akt is the primary downstream PI3K molecule, and its phosphorylation might have a protective effect on cells being injured in sustained ischaemia. The findings of our current research indicate that 2-VO caused a reduction in the brain expression levels of PI3Kp110 α , PI3Kp85 and p-Akt in rats, suggesting the downregulation of the pathways of PI3K/Akt (4).

In the cerebral cortex and hippocampus of 2-VO rats, luteolin substantially improved PI3Kp110 α and PI3Kp85 expression and stimulated the phosphorylation of Akt. These results demonstrate that luteolin may preserve the PI3K/Akt pathway while shielding neurons and synapses from chronic cerebral hypoperfusion-induced damage. This may be the reason why luteolin in 2-VO rats enhanced learning and reduced cognitive impairment (26).

The findings of our research confirmed the neuroprotective effect of luteolin on chronic cerebral hypoperfusion-induced cognitive dysfunction, indicating that luteolin could be a novel candidate for vascular dementia treatment. Further studies are warranted using luteolin, however, to demonstrate the other underlying pathways implicated in vascular dementia and how luteolin interacts with them when proposed as treatment.

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