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Protective effects of catalpol on oligodendrocyte death and myelin breakdown in a rat model of chronic cerebral hypoperfusion

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ABSTRACT

Chronic cerebral hypoperfusion is thought to induce white matter lesions (WMLs) with oligodendrocyte (OLG) death and myelin breakdown. Although apoptosis is believed to be involved in the pathologic process of WMLs, effective therapies for such remain lacking. In the present study, we investigated whether catalpol, an iridoid glycoside, could act on oligodendrocytes (OLGs) and myelin sheaths in a rat chronic hypoperfusion model, and whether transcription factor cAMP-responsive element binding protein (CREB) phosphorylation is involved in the resulting neuroprotection. A rat model of chronic cerebral hypoperfusion was prepared by bilateral common carotid artery ligation. On the 30th day after hypoperfusion, OLG loss and myelin disruption in the ischemic white matter were more severe and evident than in the sham control. Spatial memory was also more seriously impaired in rats after hypoperfusion. Treatment with catalpol significantly suppressed diminished OLGs and myelin breakdown, and promoted the recovery of cognitive decline. The expression of Bcl-2 and phosphorylated CREB (p-CREB) was also significantly increased by catalpol treatment. In conclusion, catalpol could protect against hypoperfusion-induced WMLs and cognitive impairment through the p-CREB signaling pathway leading to downstream upregulation of Bcl-2. Our results suggest that catalpol may be a useful approach for treating cerebrovascular WMLs.

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Cerebral white matter contains oligodendrocytes (OLGs) and myelin sheaths resulting from OLGs, which are sensitive to various adverse stimuli including ischemia. White matter lesions (WMLs) are frequently observed in human cerebrovascular diseases such as vascular dementia, Binswanger's disease, and Alzheimer's disease [13]. WMLs are believed to be associated with cognitive impairment and may be caused by chronic cerebral hypoperfusion, which can be experimentally induced by permanent occlusion of both common carotid arteries in rats [3]. Thus, the permanent occlusion of both common carotid arteries is proposed as a model for vascular dementia and WMLs [3,7]. Previous studies using this model have demonstrated that chronic cerebral hypoperfusion can initiate a wide array of neuropathological white matter changes, including vacuolation, rarefactions, axonal damage, and OLG loss [16,17]. It has been reported that apoptosis is an important mechanism involved in the WMLs induced by hypoperfusion [19]. Therefore, reducing apoptosis may protect white matter against damage. However, no effective therapy has yet been reported for this.

Rehmannia is a genus of six species of flowering plants in the order Lamiales, which is endemic to China. It is frequently prescribed in China and has been reported to have a wide range of biological and pharmacological activities, including anti-tumor, purgative, sedative, liver protective, and anti-aging activities [26]. There is increasing evidence that the extracts from *Rehmannia* is of neuroprotection [24]. In these extracts, catalpol, an iridoid glycoside extracted from the root of *Rehmannia*, has been shown to attenuate hippocampal CA1 neuron apoptosis by modulating the expression of Bcl-2 and Bax in a transient global ischemia model [9], and protect primary cultured astrocytes from ischemia-induced damage [10]. Based on the above reports, we hypothesized that catalpol may ameliorate WMLs in the hypoperfused white matter by regulating the expression of the anti-apoptotic protein of Bcl-2.

Experiments were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996. Male Wistar rats (250–300 g) were obtained from the Experimental Animal Center of The Third Military Medical University. Rats were divided into three groups ($n = 28$ for each group), i.e., sham-control group (Sha group), vehicle-treated group (Veh group), and catalpol-treated group (Veh + Cat group). Rats in the Veh group and Veh + Cat group

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were subjected to chronic cerebral hypoperfusion induced by permanent occlusion of both bilateral common carotid arteries, as previously described [7]. In these groups, the saline or catalpol (dissolved in saline) (5 mg/kg) were intra-peritoneally injected, once a day for 10 days after surgery, respectively. In the Sha group, the same surgical procedures were performed except for carotid ligation. The rats in this group received no manipulations during the first 10 days post-operation.

The spatial memory test was performed in an eight-arm radial maze. The rats received a habituation session for one day prior to training. They were then trained continually for 7 days, and rats with less than two total errors were selected for further testing. On the 23rd day after hypoperfusion, the rats performed an eight-arm radial maze test. Pellets were then placed only in the four food wells for the test. Arms with pellets were called working arms, while those without were called reference arms. The rats were placed on the center of the maze. The test phase was ceased either when each rat had eaten the four food pellets or after 10 min had elapsed. Working memory errors, which occurred when the rats entered the same working arm for the second time, were recorded. The rats completed one trial per day for 7 successive trials.

All animals were sacrificed 30 days after operation. The coronal brain blocks containing the corpus callosum from Bregma 0.20 mm to Bregma −0.30 mm were used for the following analysis. Twenty-micrometer thick free-floating coronal sections were cut on a cryostat microtome.

For immunohistochemical staining, sections were incubated with the primary antibody to the adenomatous polyposis coli (APC) (1:100, Calbiochem, Gibbstown, NJ, USA), myelin basic protein (MBP) (1:100, Santa Cruz, CA, USA), or caspase-3 (1:100, Santa Cruz, CA, USA) at 4 °C overnight after incubation in 3% H_2O_2 followed by 5% normal goat serum. The sections were then rinsed with phosphate-buffered saline (PBS) and subsequently incubated with biotinylated secondary antibodies (1:100, Zhongshan, Beijing, China) at 37 °C for 3 h. Finally, the immunoreaction products were visualized with a chromogen solution containing 0.05% 3,3′-diaminobenzadine (DAB) (Zhongshan, Beijing, China). Negative controls were performed by using PBS or polyclonal rabbit immunoglobulin G (Santa Cruz) instead of the primary antibodies.

Double immunofluorescence staining was performed to determine the apoptosis of OLGs. After incubation in the blocking solution of 5% normal goat serum, the sections were incubated with APC antibody (1:100, Calbiochem) at 4 °C for 24 h, followed by Cy3-conjugated goat anti-mouse IgG (1:100, Abcam, Cambridge, UK) at 37 °C for 3 h. They were further reacted with caspase-3 antibody (1:100, Santa Cruz) at 4 °C for 24 h, followed by incubation with FITC-conjugated goat anti-rabbit IgG (1:100, Abcam, Cambridge, UK) at 37 °C for 3 h. Negative controls were prepared as described earlier.

TUNEL staining was performed using an In Situ Cell Death Detection Kit, POD (Roche, Mannheim, Germany) according to the manufacturer's instructions. The sections were reacted with TUNEL reaction mixture for 60 min at 37 °C, and incubated with peroxidase streptavidin conjugate for 30 min at 37 °C. The slides were further visualized with DAB solution. Negative control was set up using Label Solution instead of TUNEL reaction mixture.

For Western blot analysis, the whole corpus callosum, including the median and paramedian regions, was homogenized. Extracts were separated onto 12% SDS-polyacrylamide gels and then transferred to a polyvinylidene difluoride membrane. Immunoblotting was performed using the primary antibody to MBP (1:500, Santa Cruz), caspase-3 (1:500, Santa Cruz), Bcl-2 (1:500, Santa Cruz), or p-CREB (1:1000, Cell Signaling, Beverly, MA, USA) for 3 h at 37 °C, and then left at 4 °C overnight. After washing with PBS containing 0.05% Tween-20, the membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000, Santa Cruz) at 37 °C for 3 h. Protein bands were detected by an enhanced chemiluminescence method (ECL kit, Amersham, Buckinghamshire, UK). The optical densities of the specific bands of MBP, caspase-3 and Bcl-2 were normalized based on the β-actin band (Santa Cruz), and p-CREB was normalized based on the CREB band (Cell Signaling).

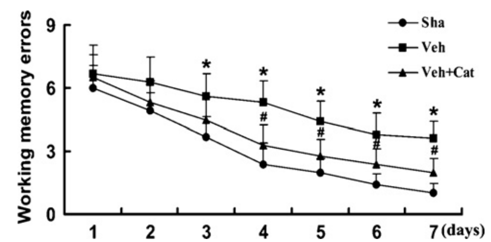

Fig. 1. Catalpol protected against the hypoperfusion-induced cognitive impairment as shown by the eight-arm radial maze test. The working memory errors of Sha, Veh, and Veh + Cat groups were recorded. Values are expressed as mean ± SEM. N = 10/group. *P < 0.05 vs. Sha; #P < 0.05 vs. Veh.

For electron microscopy (EM) analysis, the corpus callosum containing samples were diced into 1 mm^3 fragments and fixed in 2.5% glutaraldehyde at 4 °C for 24 h. The samples were post-fixed in 1% osmium tetroxide for 2 h, dehydrated through a graded series of cold acetone, and embedded in Araldite epoxy resin. Semi-thin sections were cut and stained with toluidine blue. Ultra-thin sections stained with uranyl acetate and lead citrate were examined using a Philips TECNAI10 electron microscope.

The number of immunostained and TUNEL-positive cells within a 0.25 mm^2 area of the corpus callosum adjoining the lateral ventricle was counted. For EM analysis, the percentage of axons with myelin breakdown within a 500 μm^2 area of the corpus callosum adjoining the lateral ventricle was counted. For MBP immunohistochemical staining and Western blot analysis, the mean optical density (OD) was measured using Image-Pro-Plus 5.0 software. All values were expressed as mean ± SEM. One-way analysis of variance was performed, followed by Tukey's multiple comparison tests. The significance level was set at P < 0.05.

The eight-arm radial maze test was used to evaluate the effects of catalpol on cognitive function. Rats in the Veh group committed more working memory errors from the third day (3rd day, 5.6 ± 1.08, P < 0.05) than those in the Sha group (3rd day, 3.7 ± 0.95). Catalpol treatment significantly reduced the number of working memory errors from the fourth day (4th day, 3.3 ± 0.95, P < 0.05) compared with the Veh group (4th day, 5.3 ± 1.06) (Fig. 1).

Chronic cerebral hypoperfusion caused a conspicuous decrease in the number of APC-positive OLGs in the corpus callosum in the Veh group (126.33 ± 18.85, P < 0.01) compared with the Sha group (280.34 ± 31.26, P < 0.05). Following catalpol treatment, the number of OLGs was significantly increased (209.22 ± 22.30) (Fig. 2A and D). Also, MBP immunoreactivity was lower in the Veh group (0.145 ± 0.018, P < 0.01) compared with the Sha group (0.312 ± 0.041), and catalpol treatment significantly improved the MBP immunoreactivity (0.244 ± 0.029, P < 0.05) when compared with the Veh group (Fig. 2B and D). Western blot analysis showed a significant decrease in MBP expression (both the 21.5 and 18 kDa isoforms) in the Veh group (0.487 ± 0.052, P < 0.01). In the Veh + Cat group, MBP expression (0.820 ± 0.085) was comparable to that in the Sha group (0.964 ± 0.091) (Fig. 4).

Under EM, marked changes were seen in the myelin sheaths and the wrapped axons of rats from the Veh group, including thinner myelin sheath, myelin sheath loss, and loss and disappearance of microtubules in axons. Incomplete axonal membrane was even seen in some axons, suggesting the presence of dead axons (Fig. 2C). The quantitative analysis of myelin sheath ultrastructure showed that the percentage of axons with myelin breakdown was significantly increased in the Veh group (51.00% ± 6.38%, P < 0.01)

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