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The modified 2VO ischemia protocol causes cognitive impairment similar to that induced by the standard method, but with a better survival rate

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Abstract

Permanent bilateral occlusion of the common carotid arteries (2VO) in the rat has been established as a valid experimental model to investigate the effects of chronic cerebral hypoperfusion on cognitive function and neurodegenerative processes. Our aim was to compare the cognitive and morphological outcomes following the standard 2VO procedure, in which there is concomitant artery ligation, with those of a modified protocol, with a 1-week interval between artery occlusions to avoid an abrupt reduction of cerebral blood flow, as assessed by animal performance in the water maze and damage extension to the hippocampus and striatum. Male Wistar rats (N = 47) aged 3 months were subjected to chronic hypoperfusion by permanent bilateral ligation of the common carotid arteries using either the standard or the modified protocol, with the right carotid being the first to be occluded. Three months after the surgical procedure, rat performance in the water maze was assessed to investigate long-term effects on spatial learning and memory and their brains were processed in order to estimate hippocampal volume and striatal area. Both groups of hypoperfused rats showed deficits in reference ($F_{(8,172)} = 7.0951$, $P < 0.00001$) and working spatial memory [2nd ($F_{(2,44)} = 7.6884$, $P < 0.001$), 3rd ($F_{(2,44)} = 21.481$, $P < 0.00001$) and 4th trials ($F_{(2,44)} = 28.620$, $P < 0.0001$)]; however, no evidence of tissue atrophy was found in the brain structures studied. Despite similar behavioral and morphological outcomes, the rats submitted to the modified protocol showed a significant increase in survival rate, during the 3 months of the experiment ($P < 0.02$).

Key words: Chronic cerebral hypoperfusion; Spatial memory; Hippocampus; Striatum; Water maze

Introduction

Disorders of the cerebral circulation are associated with neurological and psychiatric illnesses. Clinical evidence supports the hypothesis that chronic cerebral hypoperfusion is associated with cognitive decline, both in aging and in neurodegenerative disorders (1-3); in fact, there is a correlation between the severity of memory dysfunction and the decline in cerebral blood flow in Alzheimer's disease, vascular dementia and post-stroke hypoperfusion (4,5).

Permanent bilateral occlusion of both common carotid arteries in rats (2-vessel occlusion, 2VO) has been used to model chronic cerebral hypoperfusion (6); the main findings include histopathological damage and impaired spatial learning function (6-8). This cognitive impairment may be related to progressive loss of hippocampal pyramidal neurons, an association often observed in human aging and dementia states (6).

The hippocampus is highly vulnerable to ischemic insults (7), particularly the CA1 pyramidal cell layer (7,8). Ischemia-induced neuronal degeneration is also observed in other structures, such as the striatum, cerebral cortex and thalamus (7-9). In this model, there is an abrupt reduction of whole brain blood flow, ranging from approximately 35-45% in the cortical area to 60% in the hippocampus compared to control levels (4,10-12). This hypoperfusion is believed to sustain a chronic state of moderate hypoglycemia, a pathophysiological condition closely resembling that of reduced cerebral blood flow present in human aging and dementia (6).

Adaptations of the 2VO protocol have been explored in order to refine the experimental model by avoiding the abrupt reduction of cerebral blood flow. A modified procedure permitting the gradual establishment of cerebral hypoperfusion

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(13) has been proposed, with a 1-week interval between the occlusion of the two common carotid arteries. Consequent cognitive dysfunction was demonstrated regarding object recognition and Y-maze tests (14), but no direct comparison has been made between the two surgical protocols.

However, the conventional 2VO model, although producing interesting results, has a survival rate well below that induced by other lesion methods frequently used in our laboratory (4-vessel occlusion ischemia and neonatal hypoxia-ischemia) (15,16). Additionally, high mortality rates of about 50% have been reported for rats undergoing conventional 2VO (17-19). In order to minimize the number of animals for future experiments and to optimize research efforts, we decided to directly compare survival rates and long-term cognitive and morphological outcomes after applying the standard and modified 2VO protocols, as assessed by reference and working memory tasks in the water maze and damage extension to the hippocampus and striatum.

Material and Methods

Animals

Male Wistar rats aged 3 months were obtained from the Central Animal House of the Institute of Basic Health Sciences, Universidade Federal do Rio Grande do Sul. They were maintained in a temperature-controlled room ($21 \pm 2^\circ\text{C}$) on a 12/12-h light/dark cycle, with food and water available *ad libitum*. All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals adopted by the National Institute of Health (USA) and with the Federation of Brazilian Societies for Experimental Biology (FESBE), and were approved by the Research Ethics Committee of Universidade Federal do Rio Grande do Sul.

Animals were randomly assigned to different experimental groups: sham-operated control with manipulation of both carotids at the same time - standard protocol (N = 12); sham-operated control with manipulation of the two carotids with a 1-week interval between procedures - modified protocol (N = 12); ischemic group submitted to occlusion of both carotids at the same surgical time (N = 10), and ischemic group submitted to carotid occlusion with a 1-week interval between procedures (N = 13). Unpaired *t*-tests indicated no significant differences between the two control groups.

2VO procedure

Rats were anesthetized with halothane; a neck ventral midline incision was made and the common carotid arteries were then exposed and gently separated from the vagus nerve. Rats (N = 10) assigned to the standard 2VO protocol had both arteries concomitantly occluded with 5-0 silk suture. In the modified protocol (N = 13), carotids were occluded with a 1-week interval between interventions, the right common carotid being the first to be assessed and the left one

being occluded 1 week later. Sham-operated controls (N = 24) received the same surgical procedures without carotid artery ligation. Animals were randomly assigned to sham or 2VO groups so as to avoid any litter effect.

Morris water maze

Three months after surgery, the rats were submitted to behavioral testing for spatial memory in the Morris water maze. The maze consisted of a black circular pool 200 cm in diameter filled with water (temperature about 23°C , 40 cm in depth) situated in a room with visual cues on the walls. A black platform 10 cm in diameter was submerged in the water (2 cm below the water surface). The pool was conceptually divided into four quadrants and had four points designed as starting positions (N, S, W, or E). Two behavioral protocols, for reference and working memory, were used.

Reference memory protocol

In this task, rats received five training sessions (one session/day) and a probe trial on the 6th day. Each session consisted of four trials with a 15-min intertrial interval. A trial began when the rat was placed in the water at one of the four starting positions, chosen at random, facing the wall. The order of the starting position varied in each trial and any given sequence was not repeated on acquisition phase days. The rat was given 60 s to locate the platform; if the animal did not succeed it was gently guided to the platform and left on it for 10 s. Rats were dried and returned to their home cages after each trial. The latency to find the platform was measured in each trial and the mean latency for each training day was calculated. The probe consisted of a single trial, as described before, with the platform removed. Here, the latency to reach the original platform position, the number of crossings over that place and the time spent on the target, as well as in the opposite quadrants, were measured (20). Sessions were recorded with a video acquisition system. Videotapes were used by a trained observer using a dedicated software (ANY-maze®). Videos were subsequently placed in randomized order in a separate ANY-maze protocol to be scored by a trained observer blind to the experimental condition using a keyboard-based behavioral tracking system.

Working memory protocol

This protocol consisted of four trials/day on 4 consecutive days, with the location of the platform being changed daily. Each trial was conducted as described in the reference memory protocol, with a 5-min intertrial interval. Latency to find the platform was measured in each trial and the mean latency for each trial along the 4 days was calculated, permitting the observation of the ability of the animals to locate the novel platform position each day (20).

Morphological analysis

Rats were sacrificed 1 day after the completion of the

behavioral study. They were anesthetized with chloral hydrate (30%, 10 mL/kg, *ip*) and submitted to transcardiac perfusion with 0.9% saline followed by 4% formaldehyde. Brains were removed and maintained in formaldehyde solution. For the morphological analysis, brains were cryoprotected with a 30% sucrose solution for 2 days and sectioned, and coronal 50- μ m thick sections were obtained using a cryostat (Leica).

Hippocampal volume

The volume of the hippocampus was estimated as described below. The 50- μ m sections covering the whole hippocampus were mounted on gelatinized glass slides and stained with hematoxylin and eosin; the Image J program (NIH, USA) was used to delineate and estimate the hippocampal and dentate gyrus area (21). The volume of the hippocampus was calculated by the sum of areas multiplied by the section interval according to the Cavalieri method (22). The Ammon's horn volume was calculated as the difference between the volume of the entire hippocampus and the volume of the dentate gyrus (22).

Striatal area

Striatal atrophy was also estimated at the +1.20-mm level from the bregma according to Paxinos and Watson (23); one slice per rat was used. The Image J program (NIH, USA) was used to delineate and estimate the striatal area at that level (21).

Statistical analysis

Behavioral performance in reference memory was analyzed by one-way repeated-measures analysis of variance (ANOVA) with lesion as the independent variable and session as the repeated measure. Data regarding working memory, hippocampus volume and striatum area at the +1.20-mm level from the bregma according to Paxinos and Watson (23), were analyzed by one-way ANOVA. The *post hoc* Duncan test for multiple comparisons was applied when indicated. Fisher exact tests were applied to contingency tables (2 x 2) for comparison of mortality rates. Unpaired *t*-tests indicated no significant differences between sham-operated control groups submitted to the standard or modified protocols, and therefore only 3 groups will appear in the figures: control, ischemic group-standard protocol and ischemic group-modified protocol.

Data are reported as means \pm SEM. Probability values of less than 5% were considered to be significant. All statistical analyses were performed using the Statistica® software package running on a compatible personal computer.

Results

Survival rate

The survival rates obtained after the conventional and modified 2VO protocols were 60 and 92%, respectively,

when assessed 24 h after surgery (Table 1). Fisher exact tests revealed a significant decrease of lethality in the group exposed to the modified 2VO protocol compared to the group exposed to the standard protocol ($P < 0.02$).

Behavioral effects

Rats receiving either standard or modified protocols of bilateral common carotid artery occlusion showed a significant impairment of reference memory performance in the water maze compared to control (one-way ANOVA for repeated measures, $F_{(8,172)} = 7.0951$, $P < 0.00001$). Interestingly, the Duncan test for multiple comparisons indicated no significant differences between the standard and modified protocol groups (Figure 1).

One-way ANOVA revealed significant differences in the escape latencies of 2VO animals in the 2nd ($F_{(2,44)} = 7.6884$, $P = 0.00137$), 3rd ($F_{(2,44)} = 21.481$, $P = 0.00001$)

Table 1. Survival rate of rats submitted to standard (both arteries concomitantly occluded) and modified protocols (carotids were occluded with a 1-week interval) of bilateral occlusion of the common carotid arteries (2VO).

2VO protocol	Operated rats	Surviving rats	Survival rate	Time of survival
Standard protocol	20	12	60%	3 months
Modified protocol	20	18	90%*	3 months

* $P < 0.05$ compared to the standard protocol (Fisher exact test).

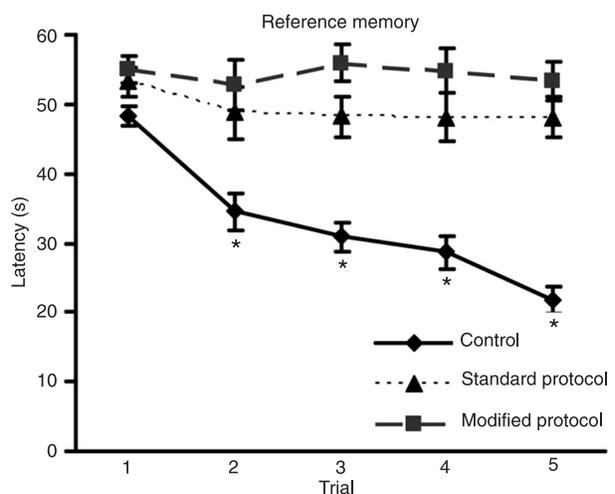


Figure 1. Reference memory performance in the water maze. Data are reported as means \pm SEM. * $P < 0.05$ control group ($N = 24$) compared to standard (both arteries concomitantly occluded, $N = 10$) and modified (carotids were occluded with a 1-week interval, $N = 13$) 2VO protocol groups (repeated measures ANOVA).

and 4th trials ($F_{(2,44)} = 28.620$, $P = 0.0001$) of the working memory task compared to control. There were also no differences between the standard and modified protocols in this task (Figure 2).

Permanent occlusion of the bilateral common carotid arteries did not cause any motor deficit; the mean swimming speed was 26 cm/s for control animals and 24.5 cm/s for ischemic rats.

Lesion extension in the hippocampus and striatum

There were no differences between the control and 2VO groups regarding the total hippocampus (Figure 3A, B and C) or the striatum area at the +1.20-mm level from the bregma according to Paxinos and Watson (23) (Figure 3D, E and F).

Discussion

The present study reports, for the first time, the effects of conventional and modified 2VO protocols on survival rates, spatial water maze memory performance and brain damage in adult Wistar rats. It is shown that the modified protocol, with a 1-week interval between each carotid occlusion, significantly reduced the mortality rate compared with the standard model (Table 1). High mortality rates after the standard procedure have been previously reported. Farkas and colleagues (17) showed a survival

rate of 69.23% for the group submitted to hypoperfusion compared to 82% for the control group. Other authors have reported a survival rate of 66.66% (18) and 50% (19) for

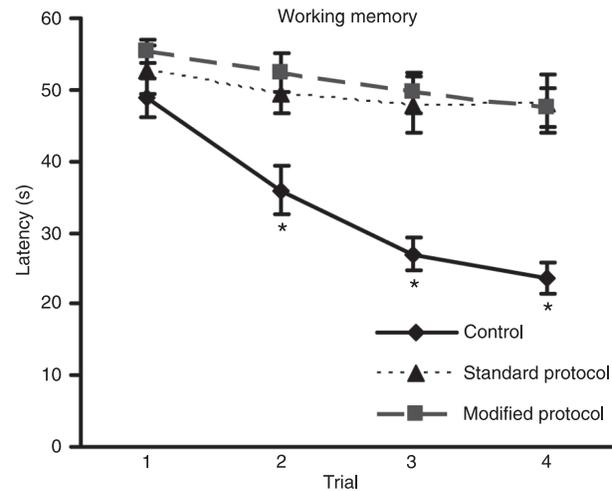


Figure 2. Working memory performance in the water maze. Data are reported as means \pm SEM. * $P < 0.05$ control group ($N = 24$) compared to standard (both arteries concomitantly occluded, $N = 10$) and modified (carotids were occluded with a 1-week interval, $N = 13$) 2VO protocol groups (repeated measures ANOVA followed by the Duncan test).

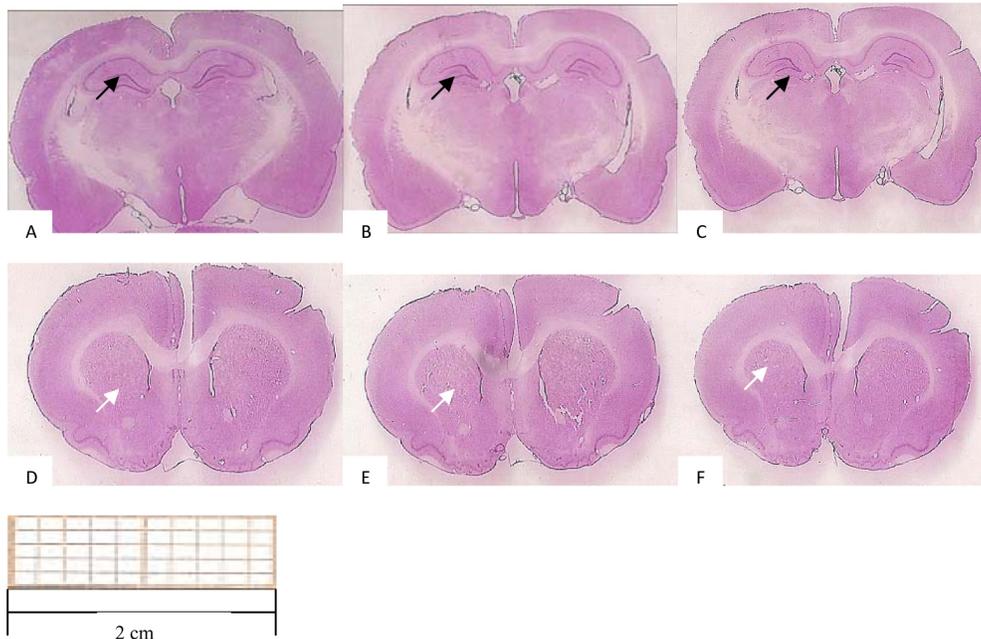


Figure 3. Representative photomicrographs of the rat brain. The hippocampus (black arrows) appears in Panels A (control), B (standard protocol), and C (modified protocol). The striatum (white arrows) appears in Panels D (control), E (standard protocol), and F (modified protocol).

ischemic groups. However, Institoris et al. (19) managed to significantly improve survival with the administration of a selective cyclooxygenase (COX-2) inhibitor.

Both protocols impaired cognitive function similarly as assessed by the use of spatial memory, hippocampus-dependent, tasks in the water maze (Figures 1 and 2), run 3 months after surgery, and failed to produce measurable lesions in the hippocampus and striatum (Figure 3). Previous studies have reported that 2VO occlusion provokes short- and long-term memory impairments using passive avoidance, Y-maze, eight-arm radial maze tasks (24) and the Morris Water Maze task (25,26).

The reports of morphologic outcomes after 2VO ischemia are conflicting. Some studies have found a direct correlation between cerebral hypoperfusion-induced memory deficit and CA1 cell damage (26-28), and an association of impaired learning performance 3 weeks after the procedure with a significant pyramidal cell loss in the CA1 region (29). However, others have reported no correlation or only a weak one between diminished CA1 neuron number and performance in the Morris maze (30-32). In addition, Murakami et al. (33) reported that mice with chronic cerebral hypoperfusion exhibit learning impairments in the water maze without marked histological alterations in the hippocampus. In summary, a direct link between 2VO ischemia-induced memory failure and the appearance of neuronal damage in the hippocampus has not been established (19).

It is possible that delayed cell death processes were still ongoing in our study, since Farkas et al. (6) reported that

CA1 neurons begin to degenerate after several weeks of reduced blood perfusion, and Ni et al. (25) demonstrated that 2VO rats showed loss of cells (30%) and increased glial fibrillary acidic protein (GFAP) density in CA1 only 150 days after surgery. On the other hand, acquisition and performance of both Morris and radial arm mazes might be impaired by dysfunction of neurochemical systems in many brain regions (34,35). The neurobehavioral consequences of chronically reduced cerebral blood flow could provide an insight into the role of reduced cerebral energy metabolism in Alzheimer's dementia, which is characterized by decreased brain glucose metabolism (36,37). It is important to remember that physiological levels of endogenous glutamate can become excitotoxic whenever neuronal energy production is compromised (38). Along this line, we have recently described a significant increase of S100B and GFAP levels, as well as a decrease in glutamate uptake in the hippocampus, in 2VO rats (39).

We suggest that the use of a modified 2VO protocol, with 1 week of interval between the occlusion of each carotid, will allow brain hypoperfusion to gradually develop (13) and result in higher survival rates, as compared with the standard method of concomitant artery occlusion. In addition, Kaliszewski et al. (40) showed that gradual vessel occlusion produces less severe tissue ischemia due to a more effective development of collateral circulation. In conclusion, the modified 2VO protocol may be more useful and reliable, with similar induced cognitive deficits and lower mortality rates, than the standard 2VO procedure.

References

- de la Torre JC. Impaired brain microcirculation may trigger Alzheimer's disease. *Neurosci Biobehav Rev* 1994; 18: 397-401.
- Kalaria RN. The role of cerebral ischemia in Alzheimer's disease. *Neurobiol Aging* 2000; 21: 321-330.
- Sopala M, Danysz W. Chronic cerebral hypoperfusion in the rat enhances age-related deficits in spatial memory. *J Neural Transm* 2001; 108: 1445-1456.
- Ohta H, Nishikawa H, Kimura H, Anayama H, Miyamoto M. Chronic cerebral hypoperfusion by permanent internal carotid ligation produces learning impairment without brain damage in rats. *Neuroscience* 1997; 79: 1039-1050.
- Komatani A, Yamaguchi K, Sugai Y, Takanashi T, Kera M, Shinohara M, et al. Assessment of demented patients by dynamic SPECT of inhaled xenon-133. *J Nucl Med* 1988; 29: 1621-1626.
- Farkas E, Luiten PG, Bari F. Permanent, bilateral common carotid artery occlusion in the rat: a model for chronic cerebral hypoperfusion-related neurodegenerative diseases. *Brain Res Rev* 2007; 54: 162-180.
- Pulsinelli WA, Brierley JB, Plum F. Temporal profile of neuronal damage in a model of transient forebrain ischemia. *Ann Neurol* 1982; 11: 491-498.
- Smith ML, Auer RN, Siesjo BK. The density and distribution of ischemic brain injury in the rat following 2-10 min of forebrain ischemia. *Acta Neuropathol* 1984; 64: 319-332.
- Freund TF, Buzsaki G, Leon A, Baimbridge KG, Somogyi P. Relationship of neuronal vulnerability and calcium binding protein immunoreactivity in ischemia. *Exp Brain Res* 1990; 83: 55-66.
- Otori T, Katsumata T, Muramatsu H, Kashiwagi F, Katayama Y, Terashi A. Long-term measurement of cerebral blood flow and metabolism in a rat chronic hypoperfusion model. *Clin Exp Pharmacol Physiol* 2003; 30: 266-272.
- Tsuchiya M, Sako K, Yura S, Yonemasu Y. Cerebral blood flow and histopathological changes following permanent bilateral carotid artery ligation in Wistar rats. *Exp Brain Res* 1992; 89: 87-92.
- Ulrich PT, Kroppenstedt S, Heimann A, Kempfski O. Laser-Doppler scanning of local cerebral blood flow and reserve capacity and testing of motor and memory functions in a chronic 2-vessel occlusion model in rats. *Stroke* 1998; 29: 2412-2420.
- Sarti C, Pantoni L, Bartolini L, Inzitari D. Cognitive impairment and chronic cerebral hypoperfusion: what can be learned from experimental models. *J Neurol Sci* 2002; 203-204: 263-266.
- Sarti C, Pantoni L, Bartolini L, Inzitari D. Persistent impair-

- ment of gait performances and working memory after bilateral common carotid artery occlusion in the adult Wistar rat. *Behav Brain Res* 2002; 136: 13-20.
15. Pereira LO, Nabinger PM, Strapasson AC, Nardin P, Gonçalves CA, Siqueira IR, et al. Long-term effects of environmental stimulation following hypoxia-ischemia on the oxidative state and BDNF levels in rat hippocampus and frontal cortex. *Brain Res* 2009; 1247: 188-195.
 16. Valentim LM, Michalowski CB, Gottardo SP, Pedroso L, Gestrich LG, Netto CA, et al. Effects of transient cerebral ischemia on glial fibrillary acidic protein phosphorylation and immunocontent in rat hippocampus. *Neuroscience* 1999; 91: 1291-1297.
 17. Farkas E, Timmer NM, Domoki F, Mihaly A, Luiten PG, Bari F. Post-ischemic administration of diazoxide attenuates long-term microglial activation in the rat brain after permanent carotid artery occlusion. *Neurosci Lett* 2005; 387: 168-172.
 18. Farkas E, Institoris A, Domoki F, Mihaly A, Bari F. The effect of pre- and posttreatment with diazoxide on the early phase of chronic cerebral hypoperfusion in the rat. *Brain Res* 2006; 1087: 168-174.
 19. Institoris A, Farkas E, Berczi S, Sule Z, Bari F. Effects of cyclooxygenase (COX) inhibition on memory impairment and hippocampal damage in the early period of cerebral hypoperfusion in rats. *Eur J Pharmacol* 2007; 574: 29-38.
 20. Netto CA, Hodges H, Sinden JD, Le Pellet E, Kershaw T, Sowinski P, et al. Effects of fetal hippocampal field grafts on ischaemic-induced deficits in spatial navigation in the water maze. *Neuroscience* 1993; 54: 69-92.
 21. Pereira LO, Strapasson AC, Nabinger PM, Achaval M, Netto CA. Early enriched housing results in partial recovery of memory deficits in female, but not in male, rats after neonatal hypoxia-ischemia. *Brain Res* 2008; 1218: 257-266.
 22. Rodrigues AL, Arteni NS, Abel C, Zylbersztein D, Chazan R, Viola G, et al. Tactile stimulation and maternal separation prevent hippocampal damage in rats submitted to neonatal hypoxia-ischemia. *Brain Res* 2004; 1002: 94-99.
 23. Paxinos G, Watson C. *The rat brain in stereotaxic coordinates*. 2nd edn. Sydney: Academic Press; 1986.
 24. Zhao Q, Murakami Y, Tohda M, Watanabe H, Matsumoto K. Preventive effect of chotosan, a Kampo medicine, on transient ischemia-induced learning deficit is mediated by stimulation of muscarinic M1 but not nicotinic receptor. *Biol Pharm Bull* 2005; 28: 1873-1878.
 25. Ni J, Ohta H, Matsumoto K, Watanabe H. Progressive cognitive impairment following chronic cerebral hypoperfusion induced by permanent occlusion of bilateral carotid arteries in rats. *Brain Res* 1994; 653: 231-236.
 26. Pappas BA, de la Torre JC, Davidson CM, Keyes MT, Fortin T. Chronic reduction of cerebral blood flow in the adult rat: late-emerging CA1 cell loss and memory dysfunction. *Brain Res* 1996; 708: 50-58.
 27. De Jong GI, Farkas E, Stienstra CM, Plass JR, Keijser JN, de la Torre JC, et al. Cerebral hypoperfusion yields capillary damage in the hippocampal CA1 area that correlates with spatial memory impairment. *Neuroscience* 1999; 91: 203-210.
 28. Nunn J, Hodges H. Cognitive deficits induced by global cerebral ischaemia: relationship to brain damage and reversal by transplants. *Behav Brain Res* 1994; 65: 1-31.
 29. Xiong Z, Liu C, Wang F, Li C, Wang W, Wang J, et al. Protective effects of breviscapine on ischemic vascular dementia in rats. *Biol Pharm Bull* 2006; 29: 1880-1885.
 30. Jaspers RM, Block F, Heim C, Sontag KH. Spatial learning is affected by transient occlusion of common carotid arteries (2VO): comparison of behavioural and histopathological changes after '2VO' and 'four-vessel-occlusion' in rats. *Neurosci Lett* 1990; 117: 149-153.
 31. Lyeth BG, Jenkins LW, Hamm RJ, Dixon CE, Phillips LL, Clifton GL, et al. Prolonged memory impairment in the absence of hippocampal cell death following traumatic brain injury in the rat. *Brain Res* 1990; 526: 249-258.
 32. Olsen GM, Scheel-Kruger J, Moller A, Jensen LH. Does neuronal damage of CA1 relate to spatial memory performance of rats subjected to transient forebrain ischemia? *Acta Neurol Scand* 1994; 89: 204-209.
 33. Murakami Y, Zhao Q, Harada K, Tohda M, Watanabe H, Matsumoto K. Choto-san, a Kampo formula, improves chronic cerebral hypoperfusion-induced spatial learning deficit via stimulation of muscarinic M1 receptor. *Pharmacol Biochem Behav* 2005; 81: 616-625.
 34. Brandeis R, Brandys Y, Yehuda S. The use of the Morris Water Maze in the study of memory and learning. *Int J Neurosci* 1989; 48: 29-69.
 35. McNamara RK, Skelton RW. The neuropharmacological and neurochemical basis of place learning in the Morris water maze. *Brain Res Brain Res Rev* 1993; 18: 33-49.
 36. Kalaria RN, Harik SI. Reduced glucose transporter at the blood-brain barrier and in cerebral cortex in Alzheimer disease. *J Neurochem* 1989; 53: 1083-1088.
 37. Simpson IA, Chundu KR, Davies-Hill T, Honer WG, Davies P. Decreased concentrations of GLUT1 and GLUT3 glucose transporters in the brains of patients with Alzheimer's disease. *Ann Neurol* 1994; 35: 546-551.
 38. Henneberry RC, Novelli A, Cox JA, Lysko PG. Neurotoxicity at the N-methyl-D-aspartate receptor in energy-compromised neurons. An hypothesis for cell death in aging and disease. *Ann N Y Acad Sci* 1989; 568: 225-233.
 39. Vicente E, Degerone D, Bohn L, Scornavaca F, Pimentel A, Leite MC, et al. Astroglial and cognitive effects of chronic cerebral hypoperfusion in the rat. *Brain Res* 2009; 1251: 204-212.
 40. Kaliszewski C, Fernandez LA, Wicke JD. Differences in mortality rate between abrupt and progressive carotid ligation in the gerbil: role of endogenous angiotensin II. *J Cereb Blood Flow Metab* 1988; 8: 149-154.