

The effects of two-stage carotid occlusion on spatial memory and pro-inflammatory markers in the hippocampus of rats

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Abstract The purpose of this study was to evaluate the effects of cerebral hypoperfusion on cognitive ability, TNF α , IL1 β and PGE2 levels in both hippocampi in a modified two-vessel occlusion model. Both common carotid arteries of adult male Wistar rats were permanently occluded with an interval of 1 week between occlusions. Learning and memory were significantly decreased after 1 month. This reduction was not significant after 2 months, which may be attributed to blood flow compensation. The TNF α level was significantly increased after 3 h and 1 day. IL1 β was significantly increased after 1 day. After a week there was no significant difference in pro-inflammatory

levels. Furthermore, there was no difference between right and left hippocampi. It is possible that TNF α and IL1 β elevation initiates pathologic processes that contribute to memory impairment.

Keywords Cerebral hypoperfusion · Inflammation · Interleukins · Spatial memory · Hippocampus

Introduction

Reduction of cerebral blood flow has been shown to contribute to the physiopathological mechanisms of cognitive impairment in age-related disease [1–4], but the role of cerebrovascular pathology in the development of neural dysfunction has not yet been clearly defined [5]. Recently, inflammation, particularly pro-inflammatory cytokines and eicosanoids such as PGE2, has attracted attention for its role in neurodegenerative disorders [5–10].

The pro-inflammatory cytokines have important roles in regulation of synaptic function, and particularly TNF α , IL1 β and IL-18 elevation have all been shown to suppress LTP (long-term potentiation) [11]. In studies on postoperative memory deficit, IL1 β and TNF α have been involved in a failure to consolidate new spatial memory following an inflammatory surgical injury [12, 13]. IL1 β plays a key role in CNS inflammatory response to CNS insults and TNF α has been consistently involved in memory impairment [14, 15]. A number of mechanisms for pro-inflammatory cytokines have been elucidated that may contribute to a neuronal injury [5], including: oxidative stress, abnormal neurotransmission, induction of nitric oxide, and apoptosis. For instance, much evidence suggests that TNF α plays an important role in the disruption of vascular circulation both in vivo and ex vivo. The enhanced expression of TNF α

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induces reactive oxygen species (ROS) production, leading to endothelial dysfunction in various pathophysiological conditions [16].

Cyclooxygenases (COXs) are the essential enzymes in the formation of prostaglandins from arachidonic acid [12]. COX2 is a well-known mediator of neuroinflammation [17, 18]. It is considered to be an important enzyme implicated in brain injury following cerebral ischemia [19]. COX2 inhibitors demonstrate neuroprotective effects in neurodegenerative models, suggesting PGE2, one of the COX products, can contribute to the neurodegeneration processes [20–22]. PGE2 is involved in inflammation and the apoptosis of neural cells [18, 23, 24]. There is evidence that pathologically elevated PGE2 can induce memory dysfunction in fear conditioning [25].

Cell-culture experiments have also demonstrated that pro-inflammatory cytokines, $\text{TNF}\alpha$ and $\text{IL1}\beta$, enhance COX2 expression and PGE2 in microglia, astrocytes and neurons. On the other hand, the enhanced expression of PGE2 and COX2 elevates the production of prostanoids, cytokines and also mediators of oxidative stress, which in turn leads to increased cell loss in different culture models [8, 26]. Furthermore, it has been indicated that prostaglandins and cytokines are directly involved in memory impairment in stressful conditions [27]. Nevertheless, it has been reported that the inhibition of particular inflammatory pathways can enhance amyloid deposition in a mouse model of Alzheimer's disease (AD) [10, 28, 29]. It appears that there are conflicting data about the role of inflammation in age-related diseases. For instance, in CSF of individuals with AD, both increased and decreased levels of PGE2 have been observed [26, 30, 31]. Therefore, it seems further study is required to clarify the role of inflammation in cognitive dysfunction.

Chronic cerebral hypoperfusion can be induced in the rat by permanent occlusion of the two common carotid arteries (2-vessel occlusion, 2VO), simultaneously. Reperfusion injury does not happen and the induced cerebral hypoperfusion is global [32]. It seems that the chronic phase of the model resembles more closely the cerebral hypoperfusion observed in age-related disease [32]. A modified method permitting the two-stage establishment of cerebral hypoperfusion [32, 33] has been suggested, with a 1-week interval between the occlusion of both common carotid arteries. Kaliszewski et al. [34] showed that gradual vessel occlusion induces less severe tissue injury due to a more efficient development of collateral circulation. In confirming Cechetti et al.'s studies [33], our previous experience showed that survival of animals using the modified method was much greater than with the 2VO method. There are few studies that have applied this technique on rats in order to reduce blood flow to the brain. As far as we know, this study

investigates for the first time the alterations in inflammation markers in both hippocampi following cerebral hypoperfusion produced by the modified 2VO method. Therefore, the current study was designed to investigate spatial learning and memory impairment following chronic cerebral hypoperfusion induced by the modified 2VO method. Furthermore, alterations in the inflammatory markers, as potential candidates leading to cognitive dysfunction, and their temporal pattern in both hippocampi were investigated after inducing cerebral hypoperfusion.

Materials and methods

Animals

Male Wistar rats (weight 250–300 g) were accommodated four per cage and kept on a 12–12 h light–dark cycle in an air-conditioned room and maintained at $(23 \pm 1^\circ\text{C})$ for at least 10 days before any experimental procedure. Food and water were provided ad libitum. Experimental procedures were conducted in accordance with the Guidelines for Reporting Animal Research [35].

Surgery

The animals were divided into two groups. In the first group ($n = 36$), cerebral hypoperfusion was induced by modified 2VO, the second group served as sham-operated controls (sham, $n = 36$). The animals were anesthetized with 400 mg/kg chloral hydrate i.p. Briefly, after a ventral midline neck incision, carotid arteries were exposed, gently freed from their sheath, then double ligated just below the bifurcation with 4–0 silk sutures. At first, the right common carotid was occluded, and then the left one was occluded 1 week later. The animals in the sham group underwent all the surgical procedures except ligation of the carotid arteries. Lidocaine (1 %) was applied as local anesthetic. During the operation, their body temperature was maintained at $37 \pm 0.5^\circ\text{C}$ by means of a heating lamp. Then the animals in both groups were divided into five groups, the first group after 3 h, the second and third groups after 1 day and 1 week, respectively, were deeply anesthetized with chloral hydrate, the brains were removed and the samples were quickly taken from the areas of the hippocampus. Then the samples were quickly frozen (until further processing) to assess inflammatory markers ($\text{IL1}\beta$, $\text{TNF}\alpha$ and PGE2) by ELISA technique later. In the fourth group (after 1 month) and fifth group (after 2 months), cognitive function was evaluated using the Morris water maze test (MWM) [36].

Testing of spatial learning and memory in the Morris water maze

The rats were trained in a standard MWM task [36]. The maze consisted of a black circular pool, 200 cm in diameter, filled with water (temperature around 23 °C, depth 40 cm) located 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) and the pool was conceptually divided into four quadrants and had four points designed as beginning starting positions (N, S, W or E) [36]. The rats performed four trials per day for 4 consecutive days. In the trials, each individual rat was released gently into the water at a randomly chosen quadrant. The rat swam and learned how to reach the hidden platform within 60 s. Upon arrival, the rat was permitted to stay on the platform for 15 s and was then taken back into the cage. The rats were placed on the platform, if they could not escape to the platform within 60 s by themselves, their escape latency was accepted as 60 s. During the inter-trial intervals, animals were kept in a dry home cage for 60 s. The time to reach the platform (latency), the length of traveled path, and the swim speed were recorded semi-automatically with a video tracking system. Twenty-four hours after the last day of training, subjects were examined on a probe trial, during which the platform was removed and the time spent in the target quadrant was measured for a 60-s trial.

Determination of pro-inflammatory markers

For the assessment of cerebral cytokine levels, hippocampus samples were homogenized in Tris HCl buffer (0.02 M) containing DTT (0.1 mM), EDTA (0.1 mM), sucrose (0.25 M), pH 7.5 and 10 µl of 10 % Triton X-100 at 0 °C. Protein concentration was measured by the method of Bradford [37] in which bovine serum albumin (BSA) was used as the standard. ENZO life sciences ELISA test kits (USA) were utilized for measurement of rat IL1 β , TNF α and PGE2 concentrations. The assays were conducted in accordance with the manufacturer's protocol. All ELISA measurements were conducted in duplicate, and the means of the two readings for each sample were utilized in the statistical analysis.

IL1 β determination

The IL1 β (rat) kit (ENZO life sciences, USA) used an antibody to rat IL1 β fixed on a microtiter plate to attach the rat IL1 β in the sample or standards. The extra sample or standard was rinsed, after a short incubation, and a biotinylated antibody to rat IL1 β was included. This antibody attached to the rat IL1 β captured on the plate. After a

short incubation the extra antibody was rinsed and streptavidin conjugated to horseradish peroxidase (HRP) was added, which attached to the biotinylated rat IL1 β antibody. Extra conjugate was rinsed and substrate was added. The enzyme reaction was stopped after a short incubation and the color developed was read at 450 nm. The determined optical density was directly proportional to rat IL1 β concentration in samples or standards [38, 39]. IL1 β concentration was expressed in pg/mg of protein.

TNF α determination

In this protocol, samples and standards were added to wells coated with a monoclonal antibody specific for rat TNF α . The plate was then incubated, then rinsed, leaving only attached rat TNF α on the plate. A solution of biotinylated polyclonal antibody to TNF α was added to bind the TNF α attached on the plate. The plate was then incubated. The excess antibody was removed by washing the plate. A solution of HRP conjugate was added to the wells, attaching to the rat TNF α polyclonal. The plate was then incubated, and after that the plate was washed to remove extra HRP conjugate. TMB substrate solution was added. The blue color generated was stopped by adding stop solution. The color generated was read at 450 nm. The measured signal was directly proportional to the TNF α level in the sample. TNF α concentration was expressed in pg/mg of protein.

PGE2 determination

The PGE2 high sensitivity kit (ENZO life sciences, USA) used a monoclonal antibody to PGE2 to attach the PGE2 in the sample or an alkaline phosphatase molecule which has PGE2 covalently bound to it, in a competitive manner. After a simultaneous incubation at 4 °C the extra reagents were rinsed away and substrate was added. After incubation at 37 °C, the enzyme reaction was stopped and the resulting color was measured on a microplate reader at 405 nm. The intensity of the color was inversely proportional to the PGE2 level in either samples or standards. For calculating PGE2 concentration, the measured optical density was used [38, 39]. PGE2 concentration was expressed in pg/mg of protein.

Statistical analysis

Statistical analysis of data was performed by applying one-way analysis of variance (ANOVA) followed by a Tukey test for biochemical parameters and behavioral tests at different time intervals, unpaired *t*-test for behavioral and biochemical comparison between two groups and paired *t*-test for comparing between both

hippocampi. A P value of <0.05 was considered statistically significant. To present the data, mean \pm standard error of the mean (SEM) was applied.

Results

Behavioral results

There were no differences between sham and non-operated groups (data are not shown); therefore, the cognitive performance of the hypoperfusion groups was compared to that of the sham operated groups, referred to as control groups. Figure 1a shows the effects of cerebral hypoperfusion on learning memory performance of rats measured 1 month after occluding both common carotids. In the first session of the escape latency trial, wherein the latency to find the platform was assessed, the statistical analysis did

not reveal any significant difference between the two groups ($p > 0.05$). As shown in Fig. 1a, escape latency decreased significantly during training ($p < 0.01$) in the control groups and there was a significant difference between two groups after the 2nd day ($p < 0.01$). Analysis of the swim path distance (traveled distance) (Fig. 2a) revealed a statistical difference between the groups ($p < 0.05$), again after the 2nd day, indicating a cognitive impairment within the hypoperfusion group: rats swam longer distances in the tank before they found the platform compared to the controls ($p < 0.05$). This discrepancy continued throughout the whole training period. To determine whether the difference between two groups was due to differences in swimming ability, swim speed was measured for each group, there was no significant difference between the two groups (Fig. 3a). In the probe trials, as shown in Fig. 4, there was a significant difference in time spent in the target quadrant between the two groups ($p < 0.05$). As shown in Figs. 1, 2, 3, and 4b, 2 months after surgery, there were no significant differences between the two groups ($p > 0.05$).

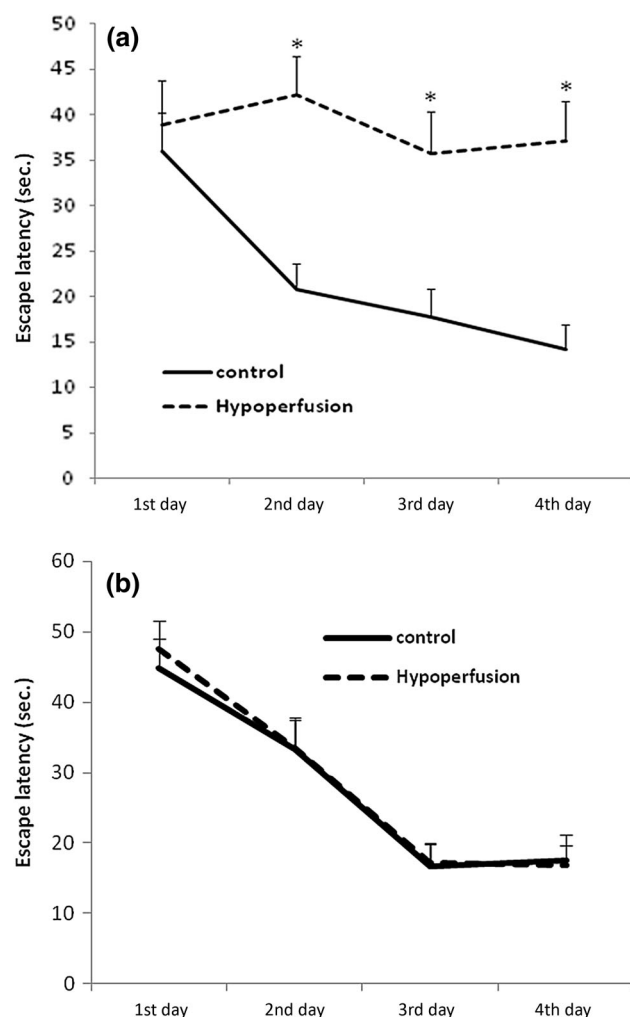


Fig. 1 The effect of cerebral hypoperfusion on escape latency. **a** 1 month after surgery. **b** 2 months after surgery. * $p < 0.05$ in comparison to the control

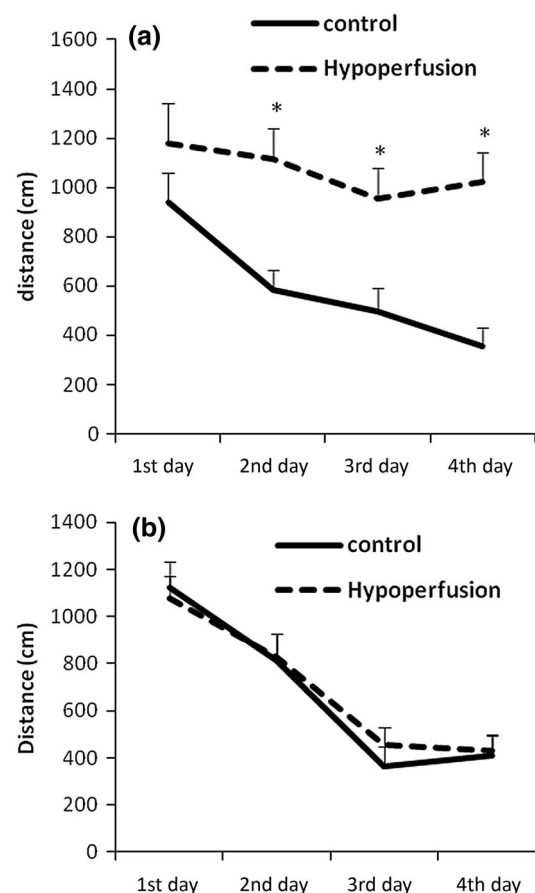


Fig. 2 The effect of cerebral hypoperfusion on traveled distance in MWM test. **a** 1 month after surgery. **b** 2 months after surgery. * $p < 0.05$ in comparison to the control

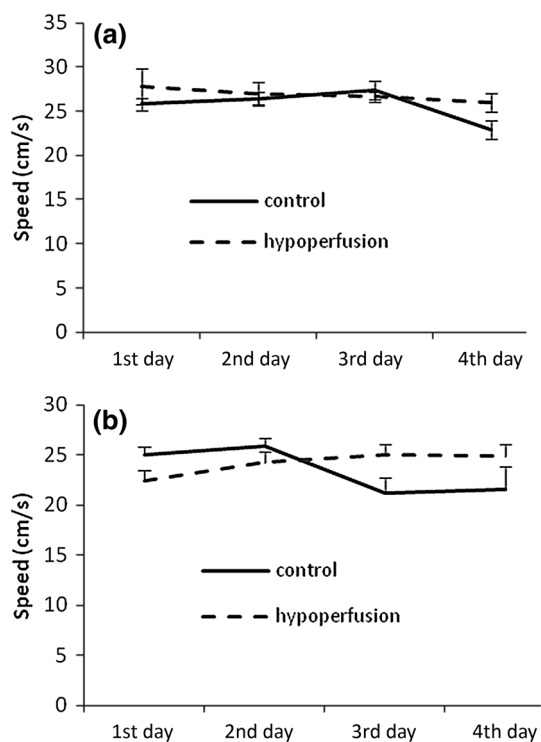


Fig. 3 The effect of cerebral hypoperfusion on swimming speed in MWM test. **a** 1 month after surgery. **b** 2 months after surgery

Inflammatory markers

Since the two common carotids were occluded separately with a 1-week interval, we measured the inflammatory marker concentrations in both hippocampi. The results showed that there was no significant difference between the left and right hippocampi ($p > 0.05$) (Table 1). In the present study, for convenience we considered only the right hippocampus in the statistical analysis.

TNF α

As shown in Fig. 5, the TNF α levels in the hypoperfusion group reached their peak 3 h after surgery, and were significantly elevated as compared with the control groups ($p < 0.001$), then markedly decreased by 1 day, but still were significantly elevated in comparison to the control group ($p < 0.01$, Fig. 5). There was no significant difference between both hypoperfusion and control groups after a week ($p > 0.05$).

IL1 β

The IL1 β level in the hippocampus of the hypoperfusion group tended to increase significantly 1 day after surgery as compared with that in the control group ($p < 0.05$), then the significant difference was abolished 1 week after surgery

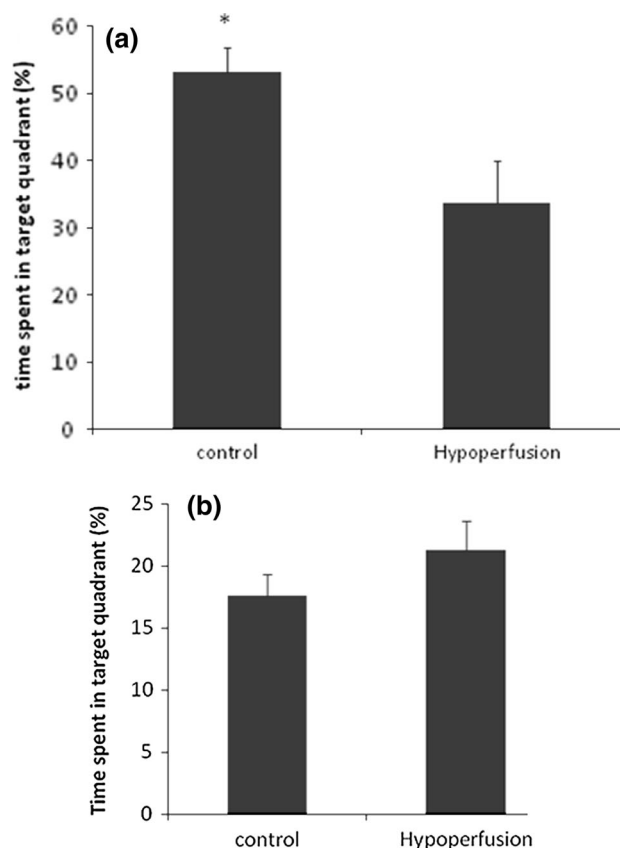


Fig. 4 The effect of modified two-vessel occlusion on time spent in target quadrant (%). **a** 1 month after surgery. **b** 2 months after surgery. * $p < 0.05$ in comparison to the hypoperfusion group

(Fig. 6). There were no significant differences in IL1 β concentrations between the different time intervals ($p > 0.05$).

PGE2

The hippocampus PGE2 level in the hypoperfusion group was not significantly different as compared with that in the control group (Fig. 7). Although the PGE2 concentration in both hippocampi increased after a week as compared to the control group, this difference was not significant.

Survival rate

In this study, we lost only one animal from the total animals studied (74 animals), and the survival rate was 98.64 %.

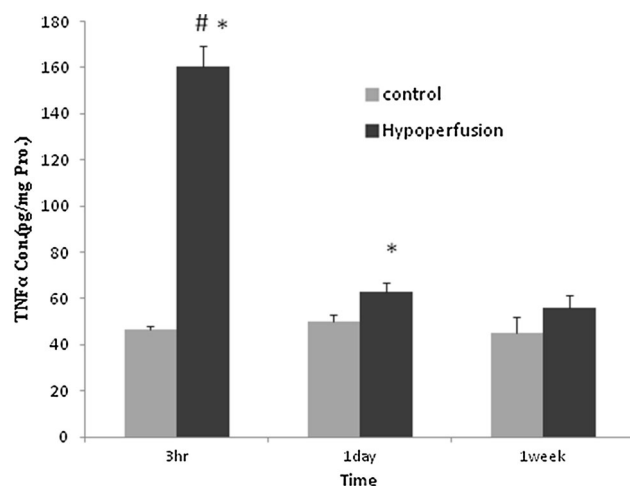
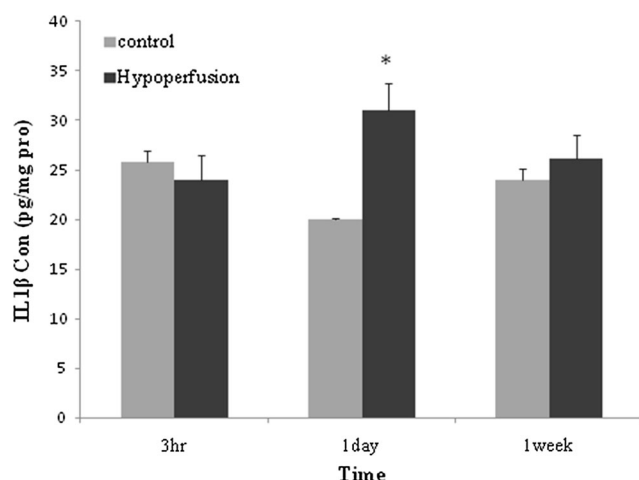
Discussion

Chronic cerebral hypoperfusion is an early feature of age-related diseases including AD, mild cognitive impairment (MCI), and vascular dementia (VD) [40–42]. The obtained

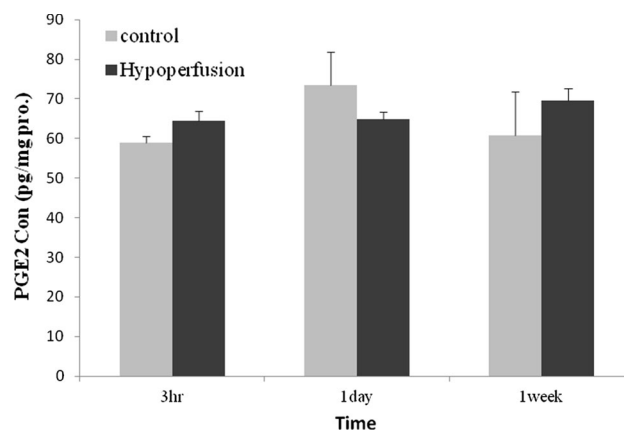
Table 1 The effect of cerebral hypoperfusion on pro-inflammatory markers in both rat hippocampi

| Time after 2VO | TNF (pg/mg protein) | | IL1 β (pg/mg protein) | | PGE2 (pg/mg protein) | |
|----------------|---------------------|---------------------|-----------------------------|--------------------|----------------------|--------------------|
| | R | L | R | L | R | L |
| 3 h | 160.5 \pm 8.76 | 161.05 \pm 11.482 | 23.982 \pm 2.438 | 29.550 \pm 2.567 | 64.401 \pm 2.575 | 68.768 \pm 4.691 |
| 1 day | 62.685 \pm 4.384 | 57.473 \pm 7.890 | 30.715 \pm 3.039 | 23.891 \pm 2.085 | 64.815 \pm 1.882 | 70.145 \pm 4.592 |
| 1 week | 55.827 \pm 5.765 | 57.713 \pm 10.87 | 26.122 \pm 2.340 | 19.635 \pm 2.267 | 69.655 \pm 2.929 | 68.138 \pm 4.399 |

R, right hippocampus; L, left hippocampus

**Fig. 5** The effect of cerebral hypoperfusion on TNF α concentration in the hippocampus. * $p < 0.05$ in comparison to the control. # $p < 0.05$ in comparison to other time intervals (1 day and 1 week)**Fig. 6** The effect of cerebral hypoperfusion on IL1 β concentration in right hippocampus. * $p < 0.05$ in comparison to control

results showed that 1 month postoperatively (the modified 2VO), learning and memory in MWM were significantly impaired. The data showed TNF α and IL1 β levels were elevated in the hypoperfusion group. However, the levels

**Fig. 7** The effect of cerebral hypoperfusion on PGE2 concentration in the hippocampus

returned to basal levels within 1 week and there were no significant changes in the PGE2 levels of the hypoperfusion group in comparison with the control groups.

Several animal models have been developed to investigate the effects of chronic cerebral hypoperfusion [32, 43–45]. In association with cerebral hypoperfusion, there are neuronal loss, progressive cognitive dysfunction, astrogliosis, and cholinergic dysfunction in the hippocampus [45–47]. Consistent with the published reports, data presented in Figs. 1, 2, 3, and 4 show spatial learning and memory impairments 1 month after the modified 2VO, as evaluated in the MWM [36]. Synaptic and neuronal loss in the hippocampus reasonably impairs animal performance in the MWM [12].

Liu et al. [42] have shown that 2VO rats in a MWM task performed significantly worse than the control group 4 weeks after 2VO. In addition, cognitive ability of the rats significantly deteriorated as time passed. However, our behavioral results were partly in contrast with their results. After 2 months, learning and memory improved to the control level, potentially due to the establishment of collateral blood supply, as found in chronic vascular occlusion studies, particularly in the modified 2VO [48]. Cerebral blood flow was significantly lower than the control values 4 weeks after 2VO occlusion. Between 8 weeks and

3 months, no significant reduction was reported [32, 49]. In the modified 2VO, a stronger compensatory system is expected because there is more time to compensate for the reduced blood flow. Furthermore, in this study, we used 3-month-old animals, which are considered young animals. The cardiovascular and inflammatory potential of young animals are particularly stronger than old animals. Occluding the first artery possibly produces a type of cerebral preconditioning that reduces the damage of occluding the second artery. Taken together, it appears that the young age of animals and more subtle approach caused the complete recovery of learning and memory to the control level [32, 43].

A strong connection between neuroinflammation, mainly mediated by pro-inflammatory cytokines, and both AD and VD has been demonstrated [5, 50]. Our results showed that $\text{TNF}\alpha$ concentration was significantly increased 3 h postoperatively and this elevation persisted for 1 day. It has been reported that 20 min after 2VO operation (acute phase), the $\text{TNF}\alpha$ and $\text{IL1}\beta$ levels in the cortex and hippocampus of rat were increased significantly in comparison with sham groups. But over chronic time, 3 weeks after permanent vessel occlusion, rat brain cytokine concentrations were completely recovered [51]. Zhu et al. [7] have shown that $\text{TNF}\alpha$ mRNA levels in mice reached the first peak at 3 h in the hippocampus after a transient 2VO operation. The peak levels of IL1 mRNA were observed 12 h after transient global ischemia and declined with time. This is similar to our data on $\text{TNF}\alpha$ and $\text{IL1}\beta$ at the protein level in rats. The pro-inflammatory cytokine mRNA expressions measured in the study returned to control levels 72 h after ischemia [7].

$\text{TNF}\alpha$ has been shown to be the main inducer of $\text{IL1}\beta$ [52, 53] and there is a temporal link between the two pro-inflammatory cytokines. Our results confirmed this temporal relationship. Brain damage induces inflammation with high levels of brain IL1 , simultaneously with learning and memory impairment [27]. This adverse effect was observed in both in vivo and ex vivo studies [27]. Furthermore, the pro-inflammatory cytokines can regulate COX2 in the brain. During pathologic conditions, the high production of prostaglandins aggravates neuroinflammation [54].

A brief induction of COX2 after various transient brain injuries has been shown in numerous studies [19, 24, 54, 55]. Following transient global ischemia, the elevation of COX2 expression was observed for up to 48 h, which then returned to normal levels [55]. Alteration in PG concentration is intimately related to the severity of brain damage and its outcome [56]. In addition, it has been shown that the initial elevation of COX2 is adaptive, while the sustained augmentation seen after injury is pathological [57]. In this study, this alteration was not significant even though PGE2 concentration was increased (Fig. 7). It appears that brain

damage caused by this method was not severe enough to elevate the PGE2 concentration significantly and consistently for 1 week. Because $\text{IL1}\beta$ and $\text{TNF}\alpha$ can suppress LTP in the hippocampus, the elevation of these cytokines alone may be enough to impair neuronal function. The results also showed the elevated inflammatory cytokine concentration returned to baseline level within a week after the occlusion. It has been reported that a single inflammatory insult has long-term effects on memory and cognition. Likewise, in animal models, a single inflammatory event impairs later memory and leads to long-term alterations in gene expression [15]. Adult neurogenesis and epigenetic modifications are two mechanisms which mediate these long-lasting effects. These processes are modulated by immune signaling [15, 58].

Additionally, pro-inflammatory cytokines mediate neurotoxic processes including enhancement of apoptosis, and impairments of synaptic function such as the inhibition of long-term potentiation [59]. Interruption in the neuron-glia signaling may also contribute to synaptic dysfunction in the injured CNS [60, 61]. In separate experiments, we observed the enhancement of apoptosis after 1 week in both hippocampi (unpublished data). Kitamura et al. [62] implanted an ameroid constrictor device on the common carotid arteries (CCAs). They showed that by gradually narrowing the CCAs (2-vessel gradual occlusion; 2VGO), MAG-immunopositive regions (indicating the degree of axon-glia integrity) declined in both 2VGO and 2VO groups when compared with sham, and the most obvious difference was at 28 days. The density of microglial and astrocyte cells and also white matter damage was significantly more in both 2VGO and 2VO groups compared with the sham. Spatial working memory was significantly impaired in both models. Comparing the modified 2VO method with the methods mentioned above, and regarding the timing of carotid occlusion, it is not surprising to observe memory deficits in the MWM test at 1 month after the second carotid occlusion.

In summary, this study showed that both (right and left) hippocampi reacted similarly to the separated occlusion of the two common carotids, regarding the pro-inflammatory cytokines. Moreover, the present study showed that the modified 2VO method resulted in a reduction in learning and memory. However, this effect was not sustained in the 2nd month. The obtained data confirmed a temporal relationship among these mediators of inflammatory damage; $\text{TNF}\alpha$ appears to be the inducer of $\text{IL1}\beta$. It is possible that $\text{TNF}\alpha$ and $\text{IL1}\beta$ elevation initiate pathologic processes that contribute to memory impairment. However, further studies, particularly with longer times, are required to clarify the role of inflammatory factors in memory impairment. Using old animals for future studies is recommended.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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