Combined treatment with celecoxib and sevoflurane after global cerebral ischaemia has no additive neuroprotective effects in rats

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Cerebral ischaemia is caused by various clinical conditions, and restoration of cerebral blood flow often exacerbates ischaemic brain damage.¹ Cerebral ischaemia–reperfusion (IR) injury is mediated by multiple mechanisms, and inflammation plays an important role in the ensuing brain damage.² Proinflammatory cytokines, such as tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), are crucial factors in the inflammatory process, leading to the exacerbation of cerebral IR injury. Consequently, blockade of these cytokines can reduce IR-induced brain damage.³ ⁴

Cyclooxygenase-2 (COX-2) is an enzyme involved in inflammatory processes. Celecoxib is a COX-2 inhibitor that is widely used for clinical management of inflammation and pain, and has neuroprotective effects against different types of brain injury.⁵–⁷

Volatile anaesthetics are known to have neuroprotective effects and anaesthetic properties.⁸–¹² Diverse mechanisms, such as inhibition of glutamate neurotoxicity, antioxidant effects, and regulation of intracellular calcium concentration, have been proposed to explain the neuroprotection induced by volatile anaesthetics.⁸ Administration of sevoflurane, after IR insults, is known as sevoflurane post-conditioning and provides neuroprotection both in vivo and in vitro.⁹–¹³

There is no single drug that completely protects against neuronal damage after cerebral IR injury. However, combined application of two or more drugs with different
mechanisms might produce additive or synergistic effects. Therefore, we hypothesized that the combined administration of celecoxib and sevoflurane immediately after cerebral ischaemia would have better neuroprotective effects than the administration of each drug alone. To verify the hypothesis, we examined the histopathological outcomes in the hippocampus and measured serum levels of TNF-α and IL-1β after transient global cerebral ischaemia in rats.

Methods

Animal preparation

All animal experiments and care were performed according to the Guide for the Care and Use of Laboratory Animals. This study was approved by Seoul National University Hospital Institutional Animal Care and Use Committee (IACUC No. 10-0098). Male Sprague–Dawley rats aged 10–16 weeks and weighing 350–380 g were used in the study. The rats were housed in an animal room under a 12-h day–night cycle at room temperature and observed until they had fully recovered from anaesthesia, determined as the points at which the rat could right itself with its four paws touching the floor and make coordinated movements.

Experimental protocols

Anaesthesia was induced by i.p. administration of zoletil (a mixture of zolazepam and tiletamine) 20 mg kg$^{-1}$ and xylazine 5 mg kg$^{-1}$ into the intraperitoneal cavity. After tracheal intubation, mechanical ventilation was performed with 60% nitrogen and 40% oxygen, and tidal volume and respiratory rate were adjusted to maintain normocarbia. A rectal probe was inserted and rectal temperature was maintained at 37.0 (0.1) °C using a heating pad. A 22-gauge needle thermometer was implanted s.c. beneath the right temporalis muscle adjacent to the skull, and pericranial temperature was monitored and servoregulated (model TCAT-2 Temperature Controller; Harvard Apparatus, Holliston, MA, USA) at 37.5 (0.1) °C by surface heating or cooling.

Rats were placed in the supine position, and the hair in the neck and right inguinal area was shaved. The right femoral artery was catheterized for continuous monitoring of mean arterial pressure (MAP) and for the periodic analyses of arterial blood gases, haemoglobin, and glucose. The right femoral vein was also catheterized for drug administration. Anaesthesia was maintained by continuous infusion of zoletil 10 mg kg$^{-1}$ h$^{-1}$ and intermittent administration of xylazine 5 mg kg$^{-1}$ via the venous catheter. After performing a frontal midline cervicotomy, the bilateral common carotid arteries (CCAs) were exposed and isolated carefully from the surrounding connective tissue. The right jugular vein was cannulated with a silicone catheter for blood withdrawal.

After anaesthesia was established, rats were allowed to stabilize physiologically for 30 min. MAP, temperature at rectum and temporalis muscle, arterial blood gases, haemoglobin, and glucose were measured and 50 U of heparin was administered i.v. 5 min before ischaemic insults. Reversible global cerebral ischaemia was produced by bilateral CCA occlusion with systemic hypotension. The heparinized rats were exsanguinated from the right jugular vein to reduce MAP to 26–30 mm Hg and then bilateral CCAs were clamped using surgical clips. MAP was maintained at 26–30 mmHg during the ischaemic period. After 8 min of ischaemia, CCAs were unclamped and blood was reinfused slowly whilst monitoring vital signs. After 30 min of reperfusion, MAP, arterial blood gases, and haemoglobin were measured once more.

After completion of the experimental protocol, all catheters were removed from the vessels, 0.5% bupivacaine was injected around the incision site to minimize pain, and incision sites were sutured. Rats were then placed in a cage at room temperature and observed until the rat could right itself with its four paws touching the floor and make coordinated movements.

Group assignments

Rats were randomly assigned to one of the five groups using computer-generated random numbers (Fig. 1). Specifically, four ischaemic groups (total, 40 rats) were randomized into five blocks of eight rats each, and the sham procedure (n=4) was performed at intervals of each block. In the sham group, the same surgical procedure was performed as in the other groups, but ischaemia was not induced. In the control group (n=10), global cerebral ischaemia was induced, but no drugs were administered after reperfusion. In the celecoxib group (n=10), celecoxib 2 mg kg$^{-1}$, dissolved in 10% dimethyl sulfoxide and normal saline to 1 mg ml$^{-1}$, was i.v. injected at the onset of reperfusion. The equal volume (2 ml kg$^{-1}$) of vehicle solution was also administered after reperfusion in the other groups apart from the sham group. In the sevoflurane group (n=10), sevoflurane 2.4% [1 minimum alveolar concentration (MAC) for rats$^{16}$] was inhaled for 5 min after reperfusion, washed out for 10 min, and then administered for another 5 min to achieve post-conditioning.$^{11}$ In the celecoxib+sevoflurane group (n=10), both drugs were simultaneously administered in the same manner as in the celecoxib and sevoflurane alone groups.

Histopathological evaluations

Histopathological evaluation of the hippocampus was performed 7 days after the experimental procedure. After anaesthetizing rats with i.p. of zoletil 20 mg kg$^{-1}$, the brains were fixed in situ by intra-aortic infusion of buffered 10% formalin and then removed carefully from the skull and stored in 10% formalin for 24 h. Brains were sliced into coronal blocks, embedded in paraffin, and serial coronal 5-μm thick sections, including bilateral cerebrum, hippocampus, and brainstem, were obtained en bloc. Sections were stained with haematoxylin and eosin (H&E) for examination of necrotic neurones, which were characterized by pyknotic or karyolytic nuclei and cytoplasmic shrinkage.
For the detection of DNA fragmentation, terminal deoxynucleotidyl transferase-mediated deoxyuracil triphosphate biotin in situ nick end labelling (TUNEL) analysis was performed with an Apoptag Peroxidase In Situ Apoptosis Detection Kit S7100 (Millipore Corporation, Billerica, MA, USA) according to the manufacturer’s instructions. TUNEL-positive cells with brown-stained nuclei were identified as apoptotic cells.

An investigator who was unaware of the group assignments examined four brain tissue slides (two H&E and two TUNEL staining) per animal via light microscopy. Three fields of view, in the medial, intermediate, and lateral sections, in each of the left and right hippocampal Cornu Ammonis area 1 (CA1) were investigated under high-power magnification (×400). The total number of cells and the numbers of necrotic and apoptotic cells were counted in each field of view. The mean percentages of necrotic or apoptotic cells were calculated as the ratio of the number of necrotic or apoptotic cells to the total cell number in each field, respectively.

### Measurements of proinflammatory cytokine levels in the plasma

Serum levels of TNF-α and IL-1β were measured 2 h, 3 and 7 days after reperfusion. i.p. injection of zoletil 20 mg kg⁻¹, blood samples were obtained from the retro-orbital plexuses of the rats. The samples were centrifuged at 2000 g for 10 min and the separated serum samples were stored at −80°C until analyses. The serum levels of TNF-α and IL-1β were measured using MILLIPLEX MAP Rat Cytokine/Chemokine Panel (Millipore Corporation, Billerica, MA, USA) according to the manufacturer’s instructions.

**Statistical analysis**

A previous study documented that the percentage of TUNEL-positive cells was 49 (14)% in the hippocampal CA1 of rats with no treatment after global cerebral ischaemia. In our study, a 40% decrease in the percentage of TUNEL-positive cells resulting from post-ischaemic treatment with celecoxib and sevoflurane was considered significant. Assuming a type-I error protection of 0.05 and a power of 0.80, 10 rats were needed in each group.

All data are expressed as median (interquartile and full range). Physiological variables were analysed by repeated-measures analysis of variance. The mean percentages of necrotic and apoptotic cells in the hippocampal CA1 and the plasma levels of TNF-α and IL-1β were analysed using the Kruskal–Wallis test followed by the Mann–Whitney U test. SPSS software (version 18.0, SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. P-values of <0.05 were considered statistically significant.

**Results**

Two of the total 46 rats died both from the control group: one during the ischaemic period and the other immediately after reperfusion. The remaining 44 rats fully recovered from anaesthesia within 2 h after reperfusion. Physiological variables, such as MAP, temperature at rectum and temporalis muscle, arterial blood gases, haemoglobin, and glucose, were not significantly different in any experimental groups before ischaemia and 30 min after reperfusion (Supplementary Table).

**Histopathological findings**

The size of the rat brain sections varied from 10 × 6 to 15 × 10 mm. In the H&E stained slide, many atrophic neurones with
shrunken cytoplasm and damaged nuclei were observed in the control group, whereas no apparent morphological changes were detected in the sham group (Fig. 2A). The median [range] percentage of necrotic neurones in the hippocampal CA1 was significantly higher in the control group (66.5 [40–96]%) than in the sham group (1.5 [0–3.0]%, P<0.001; Fig. 2B). The mean percentages of necrotic neurones were significantly lower in the celecoxib (44.1 [18.0–62.0]%) and sevoflurane (38.0 [12.9–63.0]%) groups when compared with the control group (P<0.05). The mean percentage of necrotic cells in the celecoxib + sevoflurane group (39 [16–60]%) was significantly lower than that in the control group (P<0.05), but did not differ from the celecoxib and sevoflurane groups.

Proinflammatory cytokines

Serum TNF-α and IL-1β were measured 2 h, 3 and 7 days after IR injury. TNF-α levels in the sham group remained constant at all three time points after reperfusion (Fig. 4A). TNF-α levels in the control group was markedly increased at 2 h and then decreased at 3 and 7 days after reperfusion. Two hours after reperfusion, TNF-α levels were significantly lower in the celecoxib, sevoflurane, and celecoxib + sevoflurane groups than in the control group (P<0.05), but comparable among the former three groups. At 3 and 7 days after ischaemia, there were no significant differences in the TNF-α levels between any of the groups.

The findings for IL-1β were similar to those for TNF-α. In the sham group, IL-1β was barely detected in the plasma.
2 h, 3 and 7 days after reperfusion (Fig. 4B). IL-1β levels of the control group were highest at 2 h and then markedly decreased at 7 days after reperfusion. Two hours after reperfusion, IL-1β levels were significantly lower in the celecoxib, sevoflurane, and celecoxib + sevoflurane groups when compared with the control group (P<0.05), but did not differ among the three groups treated with celecoxib or sevoflurane. At 3 and 7 days after ischaemia, the plasma IL-1β levels were comparable among the four groups, apart from the sham group.

Discussion

This study demonstrated that post-ischaemic administration of celecoxib and sevoflurane attenuated neuronal necrosis and apoptosis in the rat hippocampus after global cerebral ischaemia. Both drugs significantly decreased serum levels of TNF-α and IL-1β after ischaemia. However, the combined administration of both drugs neither improved the histological outcome in the hippocampus nor reduced the proinflammatory cytokine levels compared with treatment with each drug alone. Neuroprotective processes after IR injury are mediated by multiple molecular pathways, and each neuroprotective agent has different mechanisms to attenuate cell damage. Although the major neuroprotective mechanisms of COX-2 inhibitors and volatile anaesthetics are anti-inflammatory and anti-excitotoxic, respectively, both agents seem to share some processes involved in neuroprotection. Selective COX-2 inhibitors, such as volatile anaesthetics, may protect neuronal cells by suppressing glutamate-induced excitotoxicity: it protects cerebellar neurons from glutamate-mediated cell death, and provides neuroprotection in cortical cell culture by reducing N-methyl-D-aspartate receptor activation. Volatile anaesthetics, such as COX-2 inhibitors, also have anti-inflammatory properties that confer neuroprotection: sevoflurane improves neurological performance and decreases infarct volume after focal cerebral ischaemia of rats by attenuating inflammatory processes. Moreover, there are
Neuroprotection by celecoxib and sevoflurane

Fig 4 Serum TNF-α (a) and IL-1β (b) 2 h, 3 and 7 days after transient global cerebral ischaemia in rats. Results are median, interquartile, and full ranges (n=4 in the sham group and n=10 in the other groups). *P<0.001 compared with the sham group 2 h after ischaemia. †P<0.05 compared with the control group 2 h after ischaemia. ‡P<0.05 compared with the sham group 3 or 7 days after ischaemia. TFN-α, tumour necrosis factor-α; IL-1β, interleukin-1β.

several lines of evidence to suggest that both agents act on similar molecular pathways to diminish IR injury. These drugs inhibit activation of caspase-3, a pivotal protein in the apoptotic process, and enhance phosphoinositide-3-kinase/Akt signalling pathway, which plays a central role in cell growth and survival after cerebral ischaemia. They also attenuate IR-induced brain oxidative injury, decreasing lipid peroxidation levels and enhancing antioxidant enzyme activities. As in our findings, the suppression of post-ischaemic proinflammatory cytokines by treatment with these drugs is an example of such a shared mechanism. Therefore, the similar neuroprotective mechanisms of both drugs might be a reason why combined treatment did not provide additive neuroprotection.

Proinflammatory cytokines, such as TNF-α and IL-1β, are produced by many cell types and induce inflammatory reactions that exacerbate brain damage. These cytokines contribute to neuronal cell death by being involved in apoptosis, and in inflammation. Large amounts of TNF-α and IL-1β are produced about 1–6 h after IR injury, triggering subsequent cytokine cascades and inflammatory processes. Our study indicated that the administration of celecoxib and sevoflurane immediately after reperfusion effectively suppressed the production of proinflammatory cytokines at the most critical period in the post-ischaemic inflammatory processes. Thereby, both drugs seem to provide neuroprotection by suppressing TNF-α and IL-1β after cerebral ischaemia.

Numerous strategies have been proposed to reduce ischaemic brain damage. Short ischaemic stimuli, oxidative stress, and volatile anaesthetics, which are applied before ischaemic events, provide neuroprotection against IR injury. Although these prophylactic treatments are effective for attenuating ischaemic brain damage, their clinical uses are limited because ischaemic events are usually unpredictable. However, the onset of reperfusion after ischaemia is often predictable; thus, post-ischaemic neuroprotective interventions are clinically more applicable. Our findings suggest a neuroprotective effect of post-treatment with celecoxib and sevoflurane after IR injury.

COX-2 is induced by various physiological stresses and provokes extensive inflammatory reactions, which up-regulates nitric oxide, prostaglandin E2, and proinflammatory cytokines, including TNF-α and IL-1β. COX-2 is produced not only by inflammatory cells but also by neurones of the central nervous system. Selective COX-2 inhibitors attenuate cerebral inflammation and oedema in intracerebral haemorrhage and have beneficial effects on focal cerebral ischaemia in rats. Post-conditioning with volatile anaesthetics, in addition to preconditioning, provides neuroprotection against IR injury. Phosphoinositide-3-kinase/Akt pathway, glycogen synthase kinase 3β, and mitochondrial KATP channel are associated with the neuroprotective effects of sevoflurane post-conditioning. Sevoflurane, when compared with isoflurane or enflurane, had greater suppressive effects on the release of TNF-α and IL-1β from blood mononuclear cells.

The neuroprotective effect of selective COX-2 inhibitors may depend on the dose administered. A previous study documented that celecoxib at 2 mg kg⁻¹, but not at the 0.5 or 20 mg kg⁻¹, completely prevented methylmalonate-induced seizures, by decreasing prostaglandin E2 production in rats. Such a biphasic effect of celecoxib may be explained by a lack of selective COX-2 inhibition at the higher dose. Non-selective inhibition of COX, including COX-1, may offset the neuroprotective effect of a selective COX-2 inhibitor. Our study showed that celecoxib 2 mg kg⁻¹ was an appropriate dosage for neuroprotection and cytokine inhibition after IR.
Therefore, in our study, sevoflurane 1.0 MAC was administered twice for 5 min with a 10-min washout, which was beneficial to post-ischaemic neuroprotection and suppression of cytokines. Based on these previous findings, higher doses or chronic inflammatory phases, it might be necessary to verify near-complete cerebral ischaemia during bilateral two-vessel occlusion model is well known to induce near-complete ischaemia in the rat hippocampus, and neuroprotection. Moreover, both COX-2 inhibitors and volatile anaesthetics provide neuroprotection in the ischaemic hippocampus.

Pyramidal cells in the hippocampal CA1 are most vulnerable to global cerebral IR injury. For this reason, the hippocampus has been widely used to assess cerebral ischaemia and neuroprotection. Moreover, both COX-2 inhibitors and volatile anaesthetics provide neuroprotection in the ischaemic hippocampus.

Post-ischaemic benzodiazepine administration is neuroprotective against cerebral ischaemia. We used zoletil, which contains zolazepam, for anaesthesia and this might have affected the ischaemic outcomes in this study, although its neuroprotective effects are not known. However, zoletil was administered to all rats, so any effect of zolazepam would be the same in all groups.

Our study has several limitations. First, we measured the cytokine levels only during the acute inflammatory phase. To evaluate the anti-inflammatory effects during sub-acute or chronic inflammatory phases, it might be necessary to measure, for example, IL-6 or IL-8, which are induced later. Furthermore, we studied neuronal damage at 7 days after ischaemia and long-term neurological outcomes were not evaluated. Additionally, no isoelectric electroencephalography changes or cerebral blood flow were observed to verify near-complete cerebral ischaemia during bilateral CCAs occlusion with systemic hypotension. However, the two-vessel occlusion model is well known to induce near-complete ischaemia in the rat hippocampus, and electroencephalography does not always demonstrate isoelectric maintenance during carotid occlusion. Finally, the temperature of the rats was not measured during the recovery period, so undetected hypothermia could have attenuated IR injury. However, because all rats were maintained at normothermia throughout the experimental procedure and then recovered at room temperature, body temperature during recovery should not have differed between the groups.

In conclusion, celecoxib and sevoflurane attenuated neuronal damage in the rat hippocampus after transient global cerebral IR injury when both drugs were administered after reperfusion. However, the combined administration of both drugs had no additive neuroprotection compared with treatment with each drug alone. A potential mechanism of such neuroprotection seems to be associated with the suppression of proinflammatory cytokines including TNF-α and IL-1β after IR injury.

**Supplementary material**

Supplementary material is available at *British Journal of Anaesthesia* online.

**Declaration of interest**

None declared.

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