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Combination treatment with normobaric hyperoxia and cilostazol protects mice against focal cerebral ischemia-induced neuronal damage better than each treatment alone

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a) **Running title:** Oxygen & cilostazol therapy protects brain against ischemia

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d) **The list of nonstandard Abbreviations**: cerebral blood flow (CBF), carboxymethyl cellulose sodium salt (CMC), 8-hydroxy-2'-deoxyguanosine (8-OHdG), endothelial nitric oxide synthase (eNOS), middle cerebral artery (MCA), Normobaric hyperoxia (NBO), phospholyrated-eNOS (P-eNOS), regional cerebral blood flow (rCBF), terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL), 2,3,5-triphenyltetrazolium chloride (TTC)

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Abstract

Normobaric hyperoxia (NBO) and cilostazol (a selective inhibitor of phosphodiesterase 3) have each been reported to exert neuroprotective effects against acute brain injury after cerebral ischemia in rodents. Here, we evaluated the potential neuroprotective effects of combination treatment with NBO and cilostazol against acute and subacute brain injuries after simulated stroke. Mice subjected to 2 h filamental middle cerebral artery (MCA) occlusion were treated with NBO (95% O_2, during the ischemia) alone, with cilostazol (3 mg/kg, intraperitoneally after the ischemia) alone, with both of these treatments (combination), or with vehicle. The histological and neurobehavioral outcomes were assessed at acute (1 day) or subacute (7 days) stages after reperfusion. We measured regional cerebral blood flow (rCBF) during and after ischemia by laser-Doppler flowmetry, and recovery (versus vehicle) in the combination-therapy group just after reperfusion. Mean acute and subacute lesion volumes were significantly reduced in the combination group, but not in the two monotherapy groups. The combination therapy increased endothelial nitric oxide synthase (eNOS) activity in lesion area after ischemia versus vehicle. Combination therapy with NBO plus cilostazol protected mice subjected to focal cerebral ischemia by improvement of rCBF after reperfusion in partly association with eNOS activity.

Introduction

Recent experiments in animals have shown that normobaric hyperoxia (NBO) exerts beneficial effects when administered during transient cerebral ischemia without increasing markers of oxidative stress (Singhal et al. 2002a; b). Shin et al. reported that administration of 100% O_2 prevented the expansion of the zone with a severe cerebral blood flow deficit occurring during distal middle cerebral artery (MCA) occlusion in mice (Shin et al. 2007). In practice, oxygen treatment has the advantage of minimal technique, and it can be delivered in an ambulance or even at the patient's home following a stroke. A phase II study to assess the therapeutic potential of NBO given within 9 h of stroke onset is underway in the USA (Massachusetts General Hospital and National Institute of Neurological Disorders and Stroke).

Cilostazol, an antiplatelet drug used to treat intermittent claudication, increases the intracellular level of cyclic AMP by inhibiting its hydrolysis by type III phosphodiesterase. Its principal reported actions include inhibition of platelet aggregation (Kimura et al. 1985; Kohda et al. 1999), antithrombosis in feline cerebral ischemia, and vasodilation via an increased cyclic AMP level (Tanaka et al. 1989). Recently, it has been reported that cilostazol exerts neuroprotective effect against ischemic brain injury (Choi et al. 2002; Lee et al. 2003), and this neuroprotective potential has been ascribed to its anti-inflammatory and antiapoptotic effects mediated

by scavenging hydroxyl radicals, decrease the formation of tumor necrosis factor-α, and inhibition of poly(ADP-ribose) polymerase activity (Lee et al. 2004; Lee et al. 2007; Lee et al. 2008). We further reported that cilostazol provided neuroprotection against the permanent, filamental MCA occlusion-mediated increases in metallothionein-1 and -2 (Wakida et al. 2006). In addition, increasing evidence has shown that cilostazol may also have endothelial protection mediated by inhibition of lipopolysaccharide-induced apoptosis (Kim et al. 2002) and induced nitric oxide (NO) production by endothelial NO synthase (eNOS) activation (Hashimoto et al. 2006). NO produced by eNOS may play an important role in improving cerebral blood flow (CBF) and a neuroprotective role after cerebral ischemia (Hua et al. 2008; Murphy 2000; Lo et al. 1996).

If combining two low-dose therapies with different neuroprotective actions were found to increase the target effects and reduce the side effects (compared to single therapy at a regular dose), such combination therapy would be beneficial for stroke patients. For the present study, we chose NBO and cilostazol, both of which have proved to be safe and effective therapies against ischemia in animals, and for which the experimental evidence indicates different mechanisms of action, and we evaluated the therapeutic effects of combination treatment. Therefore the purpose of this study was to clarify whether this combination therapy has neuroprotection and to clarify the mechanisms in

postischemic processes.

To clarify an effect of the combination therapy, we examined the potential neuroprotective effects of concurrent treatment with insufficient time of NBO and a suboptimal dose of cilostazol on the cerebral infarct size and the neurobehavioral deficits present after subjecting mice to a 2 h occlusion of MCA followed by a 22 h or 7 days reperfusion. Concomitantly, we performed terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining to identify apoptotic cells, evaluated eNOS activity in lesion area after ischemia using Western blot analysis and immunohistochemical analysis for a marker of oxidative damage by using 8-hydroxy-2'-deoxyguanosine (8-OHdG), and. In addition, we measured regional CBF (rCBF) by a laser-Doppler flowmetry before and after the ischemia.

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Methods

Animals and drugs

The experimental designs and all procedures were in accordance with the Animal Care Guidelines of the Animal Experimental Committee of Gifu Pharmaceutical University. Male ddY mice (body weight, 24-28 g; Japan SLC Ltd., Shizuoka, Japan) were housed at controlled room temperature (24.5-25.0 °C), with a 12/12-h light/dark cycle. Mouse food pellets and tap water were provided ad libitum.

Cilostazol {6-[4-(1-cyclohexy-1H-tetrazol-5-yl)butoxyl]-3,4-dihydro-2-(1H)-quinolinone} was kindly gifted by Otsuka Pharmaceutical Co. Ltd. (Tokushima, Japan). Mice of the cilostazol and combination groups were treated with intraperitoneal (i.p.) injection of cilostazol at 3 mg/kg or 10 mg/kg body weight once: immediately after completion of MCA occlusion. Mice of the vehicle and NBO groups received i.p. injection of 8 ml/kg body weight 0.5% carboxymethyl cellulose sodium salt (CMC) without cilostazol at the timing and volume similar to that used in the cilostazol and combination groups.

Experiment protocol

This study consisted of two different experiments: experiment 1 aimed to investigate the neuroprotective potential of each single therapy NBO or cilostazol, and experiment

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2 aimed to investigate the neuroprotective effect of combination therapy with NBO and cilostazol.

Experiment 1: In order to investigate the effecting time of NBO exposing, mice were randomly assigned to three groups; normoxia, 2-h NBO, and 3-h NBO groups (Figure 1A). 2-h NBO and 3-h NBO groups received NBO during MCA occlusion. 3-h NBO group received additional 1 h NBO treatments after reperfusion. Each group breathed room air after NBO treatment. Normoxia group breathed room air throughout this procedure.

In order to investigate the effecting dose of cilostazol, mice were randomly assigned to three groups, vehicle, cilostazol 3, and cilostazol 10, which were administered vehicle (CMC), cilostazol 3 mg/kg, and cilostazol 10 mg/kg, respectively, just after the induction of ischemia (Figure 1B).

Experiment 2: In order to investigate the effect of combination therapy with NBO and cilostazol, mice were randomly assigned to four groups, vehicle, 2-h NBO, cilostazol 3, and combination (Figure 1C). And the effects of these treatments were assessed by neurobehavioral and histopathological evaluation until subacute phase (Figure 1D).

Transient focal cerebral ischemia

Mice were anesthetized with 1.0 to 1.5% isoflurane (Merck Hoei Co. Ltd. Osaka,

Japan) in air (21% O_2) via a face mask (Soft Lander; Sin-ei Industry, Saitama, Japan). Focal cerebral ischemia was induced by introducing an 8-0 monofilament (Ethicon, Somerville, NJ, USA) coated with silicone hardener mixture (Xantopren, Bayer Dental, Osaka, Japan) into the left common carotid artery and advancing it along the internal carotid artery until the tip occluded the proximal stem of the middle cerebral artery, as described by Hara et al. (Hara et al. 1996) Two hours after ischemia, animals were briefly reanesthetized with isoflurane and the filament was withdrawn to allow reperfusion through the common carotid artery. In all animals during surgery, the body temperature was maintained between 37.0 and 37.5°C with the aid of a heating lamp and heating pad. Any mice with an intracranial hemorrhage, pulmonary insufficiency, asphyxia and/or without an ischemic brain infarct were excluded. Sham mice underwent the same operation, but did not undergo ischemia and received neither NBO nor an injected of cilostazol or vehicle.

Normobaric hyperoxia (NBO)

The hyperoxia groups (2-h NBO, 3-h NBO, and combination groups) were exposed to 95 % O_2 in the chamber [regulated by an oxygen controller (PRO-OX110; Reming Bioinstruments Co., Redfield, USA)] after receiving an i.p. injection of either vehicle or cilostazol following completion MCA occlusion. The oxygen concentration inside the

chamber was continuously monitored using the oxygen controller, and carbon dioxide was cleared using soda lime (Nakarai Tesque Co. Kyoto, Japan) placed on the bottom of the chamber. After 2 h ischemia, the mouse was taken out from the chamber, and underwent reperfusion, all in room air (21% O_2) without 3-h NBO group that received additional 1 h NBO treatment. The room-air groups (sham, vehicle, cilostazol 3, and cilostazol 10) were exposed to room-air equivalent gas.

Physiological and rCBF monitoring

Mice were anesthetized by the same way of MCA occlusion and the left femoral artery was cannulated and blood pressure was measured during the preparation. In all animals during surgery and monitoring, the body temperature was maintained between 37.0 and 37.5°C with the aid of a heating lamp and heating pad. The temperatures were measured before, during (every 30 min), and after ischemia. Mean systemic arterial blood pressure (Power Lab; AD Instrument, Nagoya, Japan) was measured for three 3 min periods (starting at 30 min before and just after MCA occlusion and at 1 h after reperfusion). Arterial blood gases were sampled just after these vital measurements. Blood samples of 50 μl were taken 30 min before and just after the induction of ischemia, and at 1 h after reperfusion, and analyzed for pH, oxygen (PaO_2), and carbon dioxide ($PaCO_2$) (i-SAT 300F; i-SAT Corporation, NJ, USA). The

normoxia (sham, vehicle, and cilostazol 3) groups received air (21% O_2) via a face mask, and the hyperoxia (2-h NBO and combination) groups received 95% O_2 (0.75 l/min of 100% O_2 kept O_2 95% in the center of the face mask), with keeping % of isoflurane for anesthetizing during 2 h ischemia.

The rCBF was determined by laser-Doppler flowmetry (Omega-flow flo-N1; Omegawave Inc, Tokyo, Japan) using a flexible 0.5-mm fiberoptic extension to the masterprobe, as previously described (Kotani et al. 2008). Steady-state baseline values were recorded just before MCA occlusion, and further rCBF values were obtained during MCA occlusion and 30 min after reperfusion, and expressed as a percentage of the baseline value. The area under the rCBF curve was calculated for the period during ischemia and for the period after reperfusion. Mice that did not demonstrate either a significant reduction (to less than 40% baseline rCBF values) during MCA occlusion or a rapid restoration of the rCBF signal during reperfusion were excluded.

Neurobehavioral assessments

(a) Neurological deficit scoring: Mice were tested for neurological deficits just before reperfusion, 22 h after reperfusion, and every day until 7 days after the ischemia (Day 7), by an observer (Y.N.) who was blind to the animal groups. Scoring was done using the following scale: 0, no observable neurological deficits; 1, failure to extend the right

forepaw; 2, circling to the contralateral side; 3, loss of walking or righting reflex, as described by Hara and colleagues (Hara et al. 1996).

(b) Behavioral assessment: To assess the effects of combination treatment with NBO and cilostazol on functional changes after ischemia in mice, behavioral assessment was performed. A rotarod apparatus (Model BRR-MO5; Bio Medica Ltd., Japan) was used to assess motor coordination, strength, and balance (Jones and Roberts 1968). Each mouse received three trials on the day before surgery (Day -1) and again on Day 5. In each trial, we noted the number of mice that were able to walk on a rod rotating at 8 rpm for more than 600 sec before falling, with any moribund mice being excluded. In addition, we assessed locomotor activity in a novel environment. To this end, mice were individually placed in a transparent acrylic cage (length 175 × wide 245 × height 125 mm) with sawdust bedding on the floor, and locomotion was measured every 5 min for 120 min using digital counters with infrared sensors (NS-ASS01; Neuroscience, Inc, Tokyo, Japan). This test was performed once per animal on Day 7.

Assessment of acute brain injury

(a) Infarct volume measurement: At 22 h after reperfusion, mice were given an overdose of pentobarbital sodium (Dainippon Sumitomo Pharma. Osaka, Japan) and then

decapitated. The brain was cut into 2-mm-thick coronal block slices. These slices were immersed for 30 min in a 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma-Aldrich Co. St Louis, MO, USA) in normal saline at 37°C, then fixed in 10% phosphate-buffered formalin at 4°C. TTC reacts with intact mitochondrial respiratory enzymes to generate a bright red color that contrasts with the pale color of the infarction. The caudal face of each slice was photographed (COOLPIX 4500; Nikon, Tokyo, Japan). The unstained areas of the total, cortical, and subcortical infarctions were measured using Image J (http://rsb.info.nih.gov/ij/download/), and the infarction volume was calculated as in a previous report (Hara et al. 1997).

(b) Histopathological examination: Mice under anesthesia were perfused transcardially with saline followed by 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4) at 22 h after reperfusion. Then, the brains were post-fixed overnight in the same fixative, and immersed in 30% sucrose solution in the phosphate buffer. The brains were frozen and cut into 20-μm thick coronal sections from 0.4 to 1.0 mm anterior to bregma (through the anterior commissure) using a cryostat (Leica Instruments, Nussloch, Germany). To detect degenerated neurons, the sections were stained with TUNEL, as previously described (Kotani et al. 2008). The TUNEL assay was performed according to the manufacturer's instructions (Roche Molecular Biochemicals Inc.,

Mannheim, Germany). Immunohistochemical analysis for 8-OHdG was performed according as follows. Sections were rinsed three times in PBS, incubated in 3% H_2O_2 in methanol for 30 min, then placed in PBS and blocked with 1% mouse serum for 30 min. A monoclonal antibody against 8-OHdG was applied to sections overnight at 4°C. Secondary antibody (M.O.M. biotinylated anti-mouse) was applied for 10 min. The avidin/biotinylated horseradish peroxidase complex (ABC Elite kit; Vector Laboratories, United Kingdom) was applied for 30 min, and the sections were allowed to develop chromagen in 3, 3-diaminobenzidine + nickel solution (Sigma-Aldrich) for 2 min. To quantify the number of TUNEL and 8-OHdG-positive cells in the ischemic boundary zones within the cortex and subcortex were counted (Figure 4H). The regions of interest for the micrographs were periphery region between 100-500 μm from the lesion boundary. These were done in two high-power fields (× 400) within each lesion by masked investigators (Y.N. and K.H.). Each count was expressed as number/mm^2 (n = 5).

(c) Western blot analysis: Mice in ischemia groups were deeply anesthetized and decapitated at 10 min after reperfusion. As control group, no-surgery and no-treated mice were used. The brains were quickly removed and an 8-mm coronal section cut from the left hemisphere (between 2 and 10 mm from the frontal end of the forebrain).

Moreover, brain samples were divided into cortex and subcortex, and cortex was homogenized in 10 ml/g tissue ice-cold lysis buffer [50 mM Tris-HCl (pH8.0) containing 150 mM NaCl, 50 mM EDTA, 1% Triton X-100, and protease/phosphatase inhibitor mixture] and centrifuged at 14,000 × g for 10 min at 4˚C. An aliquot of 5 μg of protein was subjected to 5-20%-gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SuperSep Ace; Wako, Osaka, Japan), with separated protein being transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Billerica, MA, USA). For immunoblotting, the following primary antibodies were used: polyclonal antibody to nitric oxide synthase (endothelial) (1:1000 dilution; Funakoshi, Tokyo, Japan), phospholyrated-eNOS (P-eNOS) (Ser1177) antibody (1:1000 dilution; Cell Signaling, Danvers, MA, USA), monoclonal anti-β-actin (1:1000 dilution; Sigma Aldrich, St. Louis, MO, USA). The secondary antibody was anti-rabbit HRP-conjugated IgG (1:2000 dilution) and anti-mouse HRP-conjugated IgG (1:2000 dilution). The immunoreactive bands were visualized using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, Waltham, MA, USA). The band intensity was measured using a Lumino Imaging Analyzer (FAS-1000; Toyobo, Osaka, Japan) and Gel Pro Analyzer (Media Cybernetics, Atlanta, GA, USA).

Assessment of subacute brain injury in experiment 2

Perfused and fixed brains were removed at 7 days after ischemia (Day 7), and photographed using a digital camera (COOLPIX 4500; Nikon, Tokyo, Japan) to allow us to observe the changes on the brain surface. The brains were then cut into 2-mm-thick coronal slices. Coronal brain sections (20 μm per 2 mm) were cut on a cryostat, and stained with cresyl violet for histological measurements at a late time after ischemia, as previously described in rats (Iizuka et al. 1990). The various sections were used to detect lesion area, and hypo-cellular or neutrophil infiltration area was judged lesion area. In addition, whole brain volume minus ventricle volume was measured to allow us to assess brain atrophy by means of an indirect method that assesses the difference between the remaining healthy ipsilateral hemisphere and the contralateral one (Grabowski et al. 1993).

Statistical analysis

Data are presented as means ± S.E.M. Statistical comparisons were made using an analysis of variance (ANOVA) followed by a Student's *t*-test or a Dunnett's test or a Student-Newman-Keuls (SNK) test or a Mann-Whitney *U*-test with Bonferroni correction or a Chi square test. $P<0.05$ was considered to indicate statistical significance.

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Results

Physiological parameters and mortality

In the two hyperoxia (2-h NBO and combination) groups, arterial PaO_2 values increased approximately 3.5-fold during the administration of 95% oxygen. The other physiological parameters were not significantly different among the four groups (Supplemental Table 1).

The mortality of mice was approximately 10% (range from 4.5 to 10.0%) 24 h after ischemia and 20% (range from 12.5 to 25.0%) 7 days after ischemia in each group of experiment 1 and 2, and there were no significant differences among groups (P>0.05, Chi square test; data not shown). The excluded mice because of death, or intracranial hemorrhage, and so on were approximately 15% (range from 13.6 to 20.0%) 24 h after ischemia and there were no significant differences among groups (P>0.05, Chi square test; data not shown.) And all mice without death assessed subacute brain injury in experiment 2 were evaluated.

Experiment 1

Figure 2 A and B show the comparison of exposing 2-h with 3-h NBO on the infarct area and volume. Expanding 3-h NBO, but not 2-h NBO, showed a significant suppression of infarct size as compared to normoxia. Figure 2 C and D show the

comparison of 3 mg/kg with 10 mg/kg cilostazol on the infarct area and volume. Cilostazol, 10 mg/kg, but not 3 mg/kg, showed a significant suppression of infarct size as compared to vehicle.

Experiment 2

Cerebral blood flow

After the ischemic onset, rCBF dropped to approximately 30% of the preischemic baseline in all animals, and there was no significant difference either in the rCBF value at a given time-point or in the average rCBF value among the four groups during the ischemia (Figure 3A, B). Almost immediately after reperfusion, the rCBF of the combination group recovered almost to the pre-ischemic value, while the vehicle group exhibited incomplete recovery (to approximately 70% of baseline). These values indicated more-or-less successful reperfusion of previously ischemic tissue. However, there were significant differences in rCBF values at 2, 4, and 30 min after reperfusion between the vehicle and combination groups, with the average rCBF values obtained after reperfusion being 70.4 ± 7.1% and 96.8 ± 6.1% of baseline, respectively ($P<0.05$, Figure 3A, C).

Acute brain injury

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The ischemic mice showed apparent neurological deficits just before and at 22 h after ischemia. There was no significant difference in neurological deficit score among the four groups just before reperfusion (data not shown). In contrast, at 22 h after reperfusion the combination group exhibited significantly reduced neurological deficit scores. (P<0.05, combination versus vehicle, Figure 4D. This data added the mice that were assessed subacute brain injury.)

The infarct tissue was visualized by TTC staining in the ischemic hemisphere (Figure 4A), and combination treatment with 2-h NBO plus cilostazol 3 significantly reduced both the infarct area and infarct volume (versus vehicle) (Figure 4B, C). An ischemic lesion was consistently identified affecting the cortex and subcortex of the left cerebral hemisphere. A combination treatment-induced reduction of infarct volume could be seen both in the cortex and the subcortex (Figure 4E). In the subcortex, cilostazol 3 alone significantly reduced the infarct volume, while in the cortex 2-h NBO alone tended (non-significantly) to reduce the infarct volume (Figure 4E).

A large number of cells showed strong TUNEL-positive staining on the ischemic side of the brain, but not on the contralateral side. The numbers of TUNEL-positive degenerated cells in the ischemic boundary zones within the cerebral cortex (Figure 4F) and subcortex (Figure 4G) showed increases at 22 h after reperfusion. These numbers were significantly reduced by the combination treatment cortex and subcortex (Figure

4I and 4J, respectively). The numbers of TUNEL-positive cells were also significantly reduced by 2-h NBO treatment in the cerebral cortex (Figure 4I) and by cilostazol 3 treatment in the cerebral subcortex (Figure 4J).

P-eNOS and eNOS were almost 140 kDa. P-eNOS was increased after MCAO (Figure 5A). It was more prominent in all therapeutic groups than in control. On the other hands, eNOS was not different between all groups. In quantitative analysis, P-eNOS was significantly greater at NBO, cilostazol and combination groups than control group, but not at vehicle group (Figure 5B). Moreover, P-eNOS was significantly greater in combination group than in vehicle group. However, eNOS was not different between all groups (Figure 5C). There was a significant difference in the ratio of P-eNOS/eNOS between control and combination groups (Figure 5D).

At 22 h after reperfusion, strong 8-OHdG-immunoreactivity was detected in cortex and subcortex on the ischemic side of the brain, but not on the contralateral side in each group (Supplemental Figure 1A and 1B). Cilostazol group and combination group significantly reduced the number of 8-OHdG positive cells compared to vehicle group. Importantly, the number of 8-OHdG-positive cells was significantly reduced by the combination treatment in cortex (versus vehicle, 2-h NBO, and cilostazol groups, Supplemental Figure 1C) and in subcortex (versus vehicle group, Supplemental Figure 1D).

Subacute brain injury

The neurological deficit score, which was maximal just before reperfusion, was reduced within 3 days, and remained at its reduced level until 7 days after reperfusion (Day 7). There was no significant difference in neurological deficit score between each group just before reperfusion. However, from Day 1 onwards, the combination group displayed a significantly lower score than the vehicle group ($P<0.05$, Figure 6A).

Rotarod performance: The rotarod test has been used before to assess motor coordination, strength, and balance alterations after ischemic brain injury in rodents (Jones and Roberts 1968; Rogers et al. 1997). At one day before surgery (Day -1), no difference between the vehicle and combination groups was observed in rotarod performance (data not shown). On Day 5, the combination group contained a significantly greater proportion of mice [64.3% (9/14) $P<0.05$] who could stay on the rod for more than 600 sec [versus the vehicle group: 16.7% (3/18)](Figure 6B).

Locomotor activity: Locomotor activity data are shown in Figure 6C and D. The 2 h observation period was divided into 10 min intervals in order to display the time-dependency of the behavior. Figure 6C shows that all mice moved less as time passed. By comparison with the sham group, the vehicle group showed a significantly lower total activity count over the first 1 h (0 to 60 min, Figure 6D). Combination and

2-h NBO therapy prevented this decrease in novel envelopment activity after ischemia in the subacute phase. From the results of behavioral experiments, combination therapy prevented neurobehavioral function even until subacute phase. These neurobehavioral findings are consistent with the observed morphological outcomes.

Assessment of brain injury: On Day 7, obvious atrophy in the ischemic hemisphere could be seen in the gross photographs of whole brains (arrows in Figure 7A) and in brain sections stained with cresyl violet (Figure 7B). The shrinkage (percentage of the contralateral corresponding brain region) due to brain atrophy was significantly different between vehicle and the combination groups ($P<0.05$, Figure 7C). In the 4, 6, and 8 mm coronal slices, lesion area was significantly smaller in the combination group than in the vehicle group ($P<0.05$ at each level, Figure 7D). Lesion volume at 7 days after ischemia was significantly reduced by combination therapy ($P<0.01$ versus vehicle, Figure 7E).

Discussion

We found that 3-h NBO treatment reduced infarct volume more than 2-h NBO treatment. Shinghal et al. have reported similar results and demonstrated that NBO treatment against brain ischemia did not increase oxidative stress (Singhal et al. 2002b).

Moreover, cilostazol 10 mg/kg, but not 3 mg/kg, administered i.p. just after the induction of ischemia showed significant suppression of infarct size, and similar report has been published (Choi et al. 2002). Our study, for the first time to our knowledge, demonstrated that combination therapy with insufficient time of 2-h NBO and a suboptimal dose (3 mg/kg, i.p.) of cilostazol had neuroprotective effects.

Furthermore, we examined another experiment whether optimal treatment with 3-h NBO plus cilostazol 10 could reduce infarct volume at 22 h after 2 h ischemia most or not. The infarct volume of combination therapy with 3-h NBO plus cilostazol 10 was 31.4 ± 7.2 mm^3 (n = 4), significant ($P<0.05$) difference compared with vehicle (64.8 ± 6.0 mm^3, n = 17), and a little less (but not significant) than that with 2-h NBO plus cilostazol 3 (36.1 ± 4.3 mm^3, n = 11). This result might be caused from the limitation of protection in our model of focal cerebral ischemia in mice.

In this study, monotherapy with 2-h NBO protected the cortex rather than the subcortex, as indicated by our assessment of TTC and TUNEL staining. Singhal et al. showed previously in the rat that NBO significantly reduced cortical infarct volumes,

and moreover that the neuroprotective effects of NBO were most evident in the cerebral cortex itself (Singhal et al. 2002a; b). Shin et al. found that NBO improves CBF during ischemia of the cerebral cortex (by means of real-time, two-dimensional laser speckle flowmetry) (Shin et al. 2007). Combination therapy in present study demonstrated a more complete recovery of rCBF than the vehicle group immediately after reperfusion. Ischemia is particularly germane to CBF, and it is possible that improving rCBF after reperfusion may be a therapeutic potential to save salvageable ischemic brain.

eNOS is an enzyme that increase the activity just after ischemia (Zhang et al. 1993), and NO produced by eNOS plays an important role in improving CBF and a neuroprotective role after cerebral ischemia (Hua et al. 2008; Lo et al. 1996; Murphy 2000). Huang et al. have reported that mice lacking expression of the eNOS developed larger infarcts after MCA occlusion (Huang et al. 1996). Cilostazol induces NO production by eNOS activation (Hashimoto et al. 2006), and it is posible that hyperoxia could augment eNOS activity in ischemic area (Shin et al. 2007). From these points of view, we have evaluated eNOS activity and rCBF before and after the ischemia. The eNOS is activated by phosphorylation at Ser^{1177} (Chen et al. 1999). The present study demonstrated that the amount of phospho-Ser^{1177} eNOS in ischemic area after reperfusion was significantly elevated in the combination therapy, and this

result may correlate with the result that combination therapy was not only improving rCBF after reperfusion, but also protecting the ischemic brain with functional and morphological outcome. And eNOS activity may be partly mechanism under combination therapy against neuroprotective effect.

Recent studies suggest that the benefits of NBO may exceed the risk of enhanced oxidative stress (Kim et al. 2005; Singhal et al. 2002a), which reportedly plays a major role in the pathogenesis of the cerebral infarction occurring as a reperfusion injury after focal ischemia (Yang et al. 1994). However, a theoretical concern remains about reperfusion injury being caused by an increase in oxygen free radicals due to NBO. Indeed, a previous study indicated that free radicals increased in the blood of humans undergoing hyperbaric oxygen therapy (Narkowicz et al. 1993). However, the generation of superoxide and hydrogen peroxide can be suppressed by increased cyclic AMP (Takei et al. 1998), and previous reports suggested that cilostazol suppresses superoxide production via an increase in cyclic AMP (Choi et al. 2002; Park et al. 2006). We have reported that cilostazol provided neuroprotection against brain ischemia-mediated increases in metallothionein-1 and -2, having a potential of anti-oxidative stress (Wakida et al. 2006). Hence, it is possible that cilostazol suppresses any superoxide production that might be induced by NBO.

Both of NBO and cilostazol therapy have been reported to have side effects. In

humans, NBO may occur lung damage (Jenkinson 1993), and prolonged high oxygen exposure is reported to induce cough, shortness of breath, decrease vital capacity and increase alvelocapillary permeability (Capellier et al. 1999). For these reasons, prolonged exposure to high oxygen concentration should be avoidance. Clinically, despite cilostazol being generally well tolerated (Huang et al. 2008), untoward side effects (including headache and palpitation) limit its use, even though they are generally mild to moderate in intensity (Chapman and Goa 2003). From these points of view, it is desirable to gain enough effect by two or more therapies used even insufficient dose or time. This study demonstrated that concurrent treatment with insufficient time of NBO and a suboptimal dose of cilostazol had neuroprotective effects no less than sufficient single therapies. And this study may bring a hope for the patients who cannot receive sufficient therapy because of their complication.

In conclusion, the present study demonstrated that combination therapy involving NBO and cilostazol (a) protected against not only acute, but also subacute cerebral injury in mice that had undergone focal cerebral ischemia, and (b) inhibited of apoptotic cell death. These effects may be, at least in part, due to improvement of rCBF just after reperfusion in association with eNOS activity.

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Legends for Figures

Figure 1 A and B; the protocol of experiment 1. C and D; the protocol of experiment 2. A-C; the length and timing of normobaric hyperoxia (NBO) exposure, and the dose and timing of cilostazol administration are mentioned. D. experimental course of the experiment 2. The day 0 refers to the day of surgery.

Figure 2 Infarct area and volumes in Experiment 1. A and B; exposing time-dependent effect of normobaric hyperoxia (NBO) on the infarct between normoxia- and NBO-treated mice subjected to 2 h ischemia/22 h reperfusion. 2-h NBO and 3-h NBO groups received NBO during ischemia, and 3-h NBO group received additional 1 h NBO treatments after reperfusion. Infarct size significantly decreased by treatment with 3-h, but not 2-h, NBO. *P<0.05 versus normoxia (a Student-Newman-Keuls test, n = 10). C and D; dose-dependent effect of cilostazol on the infarct between vehicle- and cilostazol-treated mice. Cilostazol (3 or 10 mg/kg, i.p.) was injected just after the induction of ischemia. Infarct size significantly decreased by treatment with 10 mg/kg, but not 3 mg/kg, cilostazol. *P<0.05; **P<0.01 versus vehicle (a Dunnett's test, n = 12 to 18). Data are expressed as means ± S.E.M.

Figure 3 Regional cerebral blood flow (rCBF). A, laser Doppler measurements in the left MCA territory. Area under the rCBF curve during ischemia (B) and from immediately after to 30 min after reperfusion (total 30 min) (C). $^{\dagger}P<0.01$, $^{*}P<0.05$ combination versus vehicle. $^{\#}P<0.05$ sham versus vehicle (a Student-Newman-Keuls test, n = 5 to 7). Data are expressed as mean ± S.E.M.

Figure 4 Acute brain injuries in Experiment 2. A, 2,3,5-Triphenyltetrazolium chloride (TTC)-stained coronal sections show infarct tissues (pale unstained region). Brain sections (from extreme left) were obtained from vehicle, 2-h NBO, cilostazol 3, and combination mice, respectively. B, total infarct area, C, total infarct volume, and E, infarct volumes in cortex and subcortex. $^{*}P<0.05$, $^{**}P<0.01$ versus vehicle (a Student-Newman-Keuls test, n = 11 to 17). Data are expressed as mean ± S.E.M. D, neurological deficit scores 22 h after reperfusion. $^{*}P<0.05$ versus vehicle (Mann-Whitney *U*-test with Bonferroni correction, n = 27 to 36 adding the mice that were assessed subacute brain injury).

F-I, TUNEL staining. F and G show representative photomicrographs of TUNEL staining in the ischemic boundary zones of the cerebral cortex (F, upper row) and subcortex (G, lower row). Bar = 50 μm. H, Schematic drawing showing the brain regions at a level of 0.4 to 1.0 mm anterior to bregma (through the anterior commissure).

1, cortical; 2, subcortical ischemic boundary zones. I-J, quantitative representation of TUNEL-positive cells in ischemic brains from each group. *P<0.05, **P<0.01 versus vehicle, $^{\#}P$<0.05 and $^{\#\#}P$<0.01 between indicated groups (a Student-Newman-Keuls test, n = 5). Data are expressed as mean ± S.E.M.

Figure 5 Western blot analysis of phospholyrated-eNOS (P-eNOS) and eNOS. A, P-eNOS was increased after ischemia. It was more prominent in all therapeutic groups than in control. eNOS was not different between all groups. B, Quantitative analysis of Western blotting of P-eNOS: P-eNOS was increased at NBO, cilostazol and combination groups. Moreover, P-eNOS was significantly greater in combination group than in vehicle group. *P<0.05, **P<0.01 versus control (Student's t-test); $^{\#}P$<0.05 versus vehicle (Student's t-test, n = 9 or 10). C, Quantitative analysis of Western blotting of eNOS: eNOS was not different between all groups. D, The ratio of P-eNOS/eNOS: There was significant difference between control and combination group. But there were no significant differences between any other pair. *P<0.05 versus control (Student's t-test). Data are expressed as mean ± S.E.M.

Figure 6 Neurobehavioral assessment in experiment 2. A, neurological deficit scores up to Day 7 after ischemia. B, rotarod performance on Day 5. Percentage of

mice that spent more than 600 sec on the rod. C, locomotor activity on Day 7. D, total number of beam-breaks in the first 60 min of measurement. $^{\#}P<0.05$ versus sham, $^{*}P<0.05$ between indicated groups [a Mann-Whitney *U*-test with Bonferroni correction (A, B), a Student-Newman-Keuls test (C, D), n = 12 to 15]. Data are expressed as mean ± S.E.M.

Figure 7 Subacute histological assessment in experiment 2. A, photographs of whole brains 7 days after ischemia. Arrows indicate lesion area and brain atrophy. Bar = 5 mm. B, photographs of brain slices stained with cresyl violet. The hypo-cellular or neutrophil infiltration areas were judged the lesion area. The red dotted line encloses the lesion area. Bar = 5 mm. C, Higher magnification of demarcated a square area in the vehicle of (B), and showing the border between lesion and intact area. Bar = 1 mm. Combination therapy attenuated the brain atrophy (D), and reduced the lesion area (E) and lesion volume (F). $^{*}P<0.05$, $^{**}P<0.01$ versus vehicle (a Student-Newman-Keuls test, n = 12 to 15). Data are expressed as mean ± S.E.M.

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Figure 1

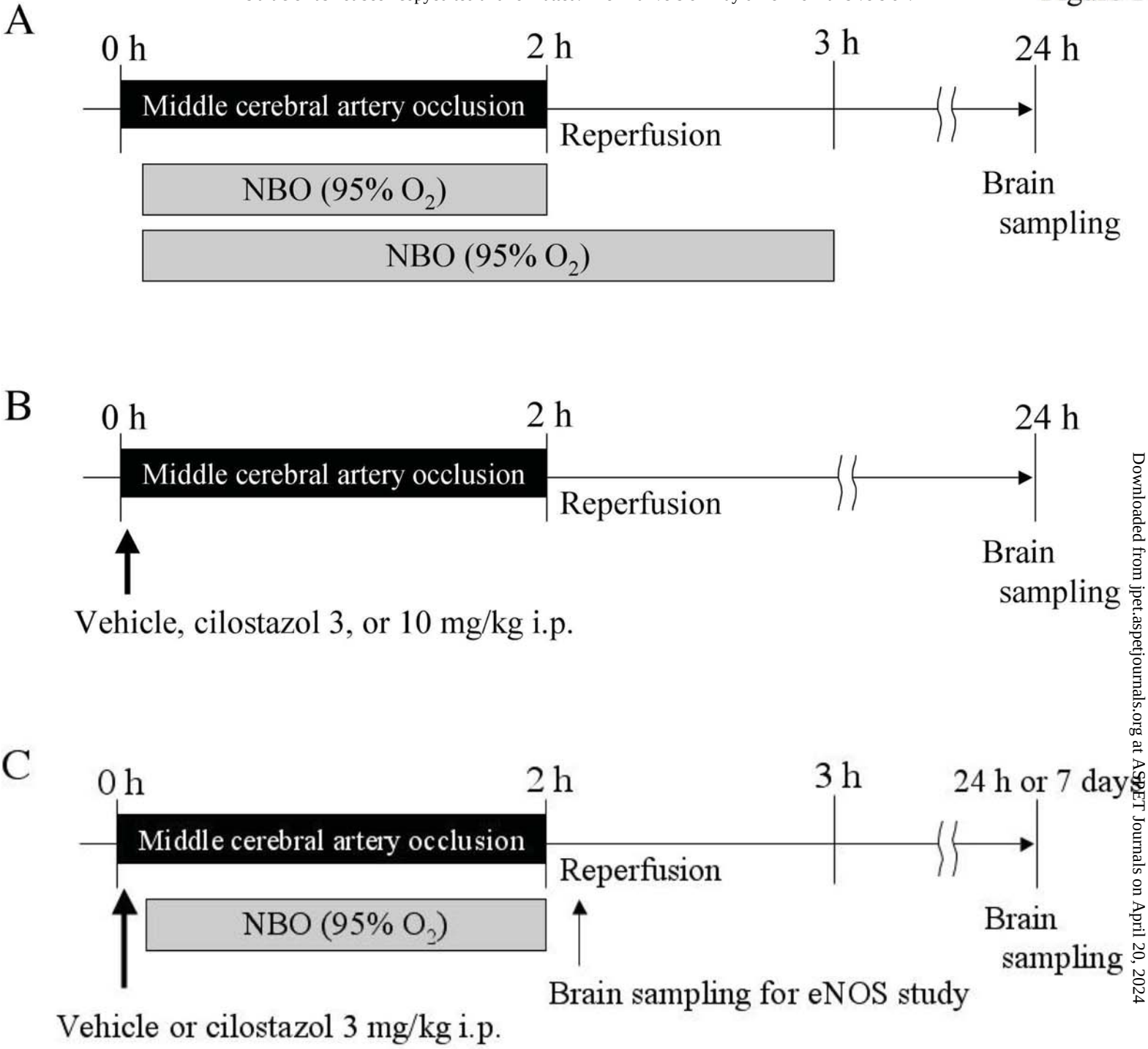

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Figure 2

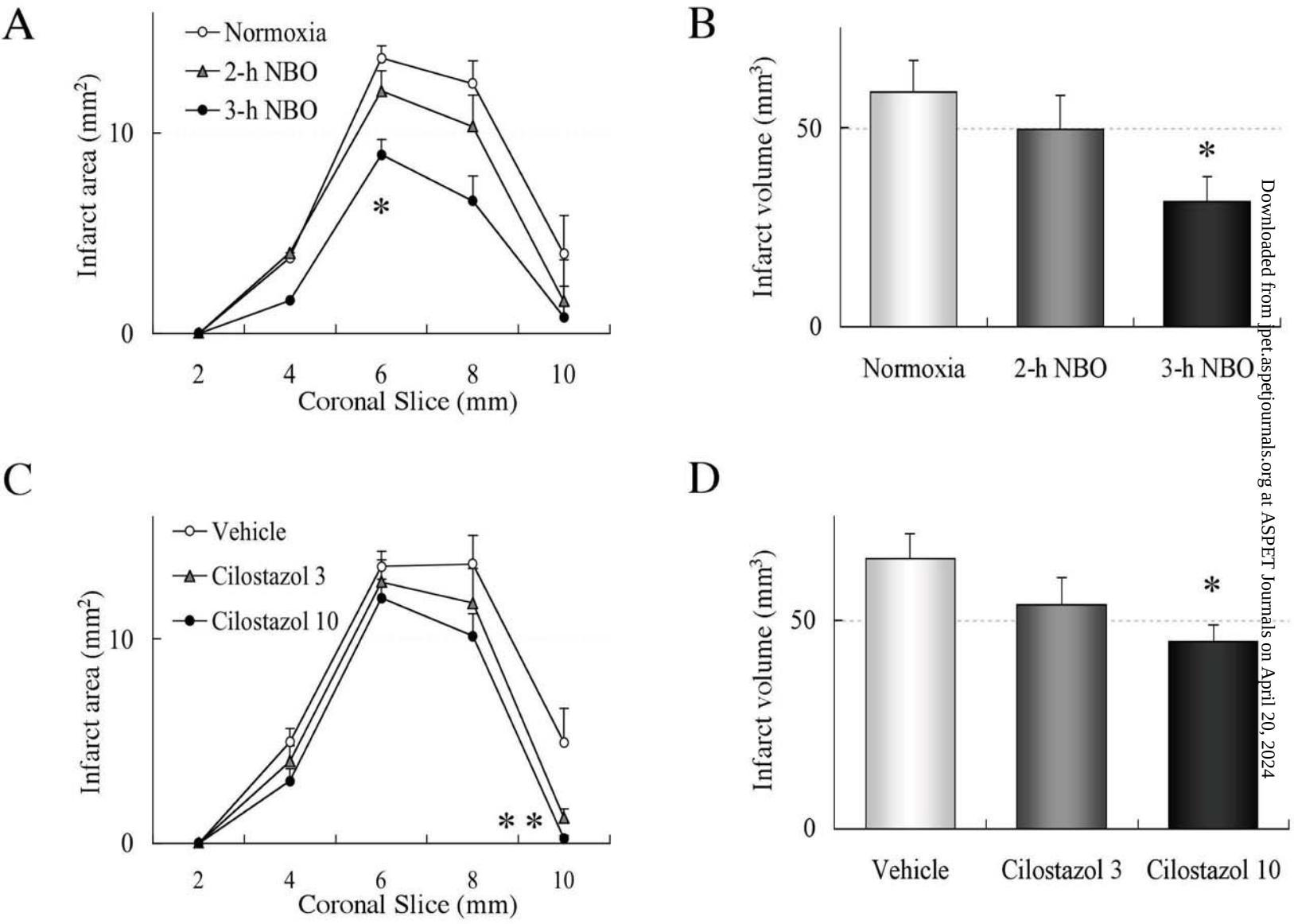

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Figure 3

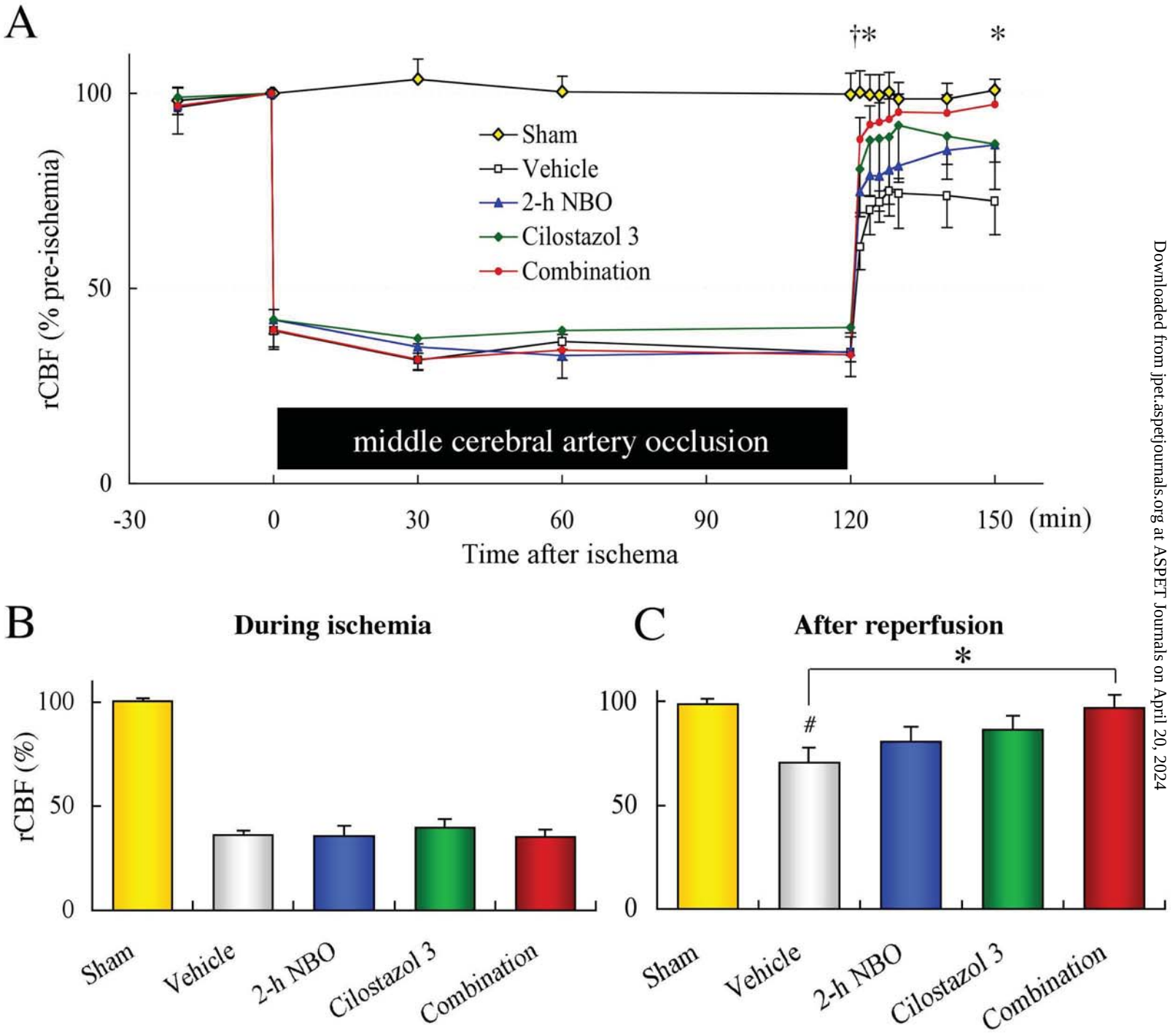

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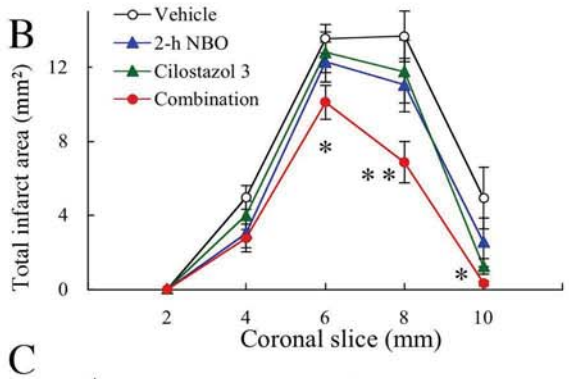
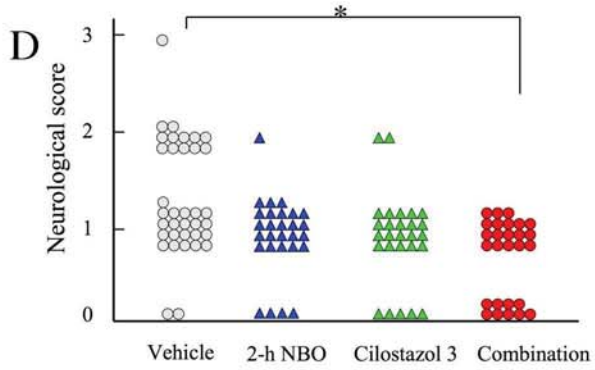
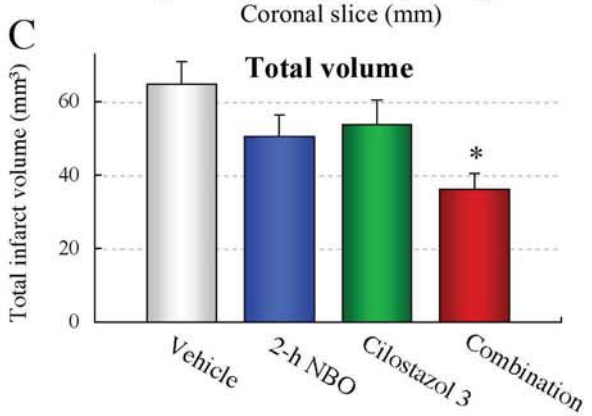
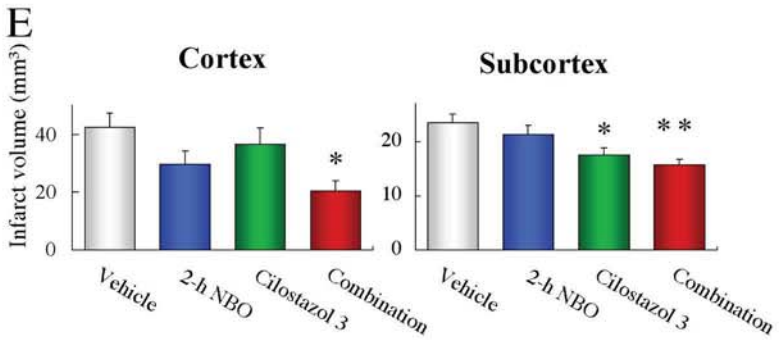
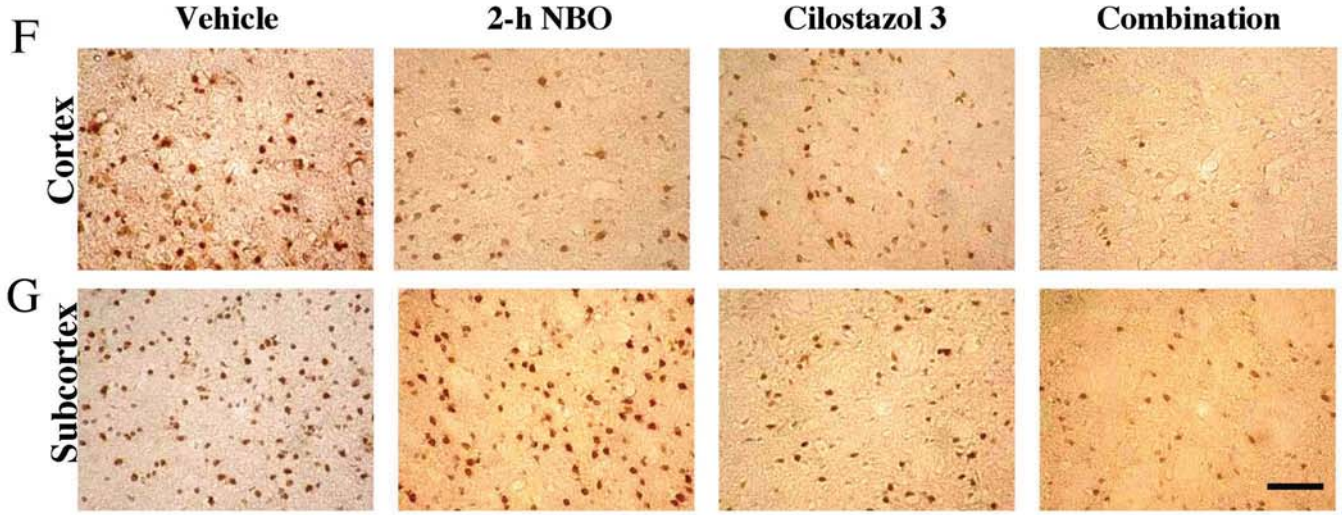
Figure 4











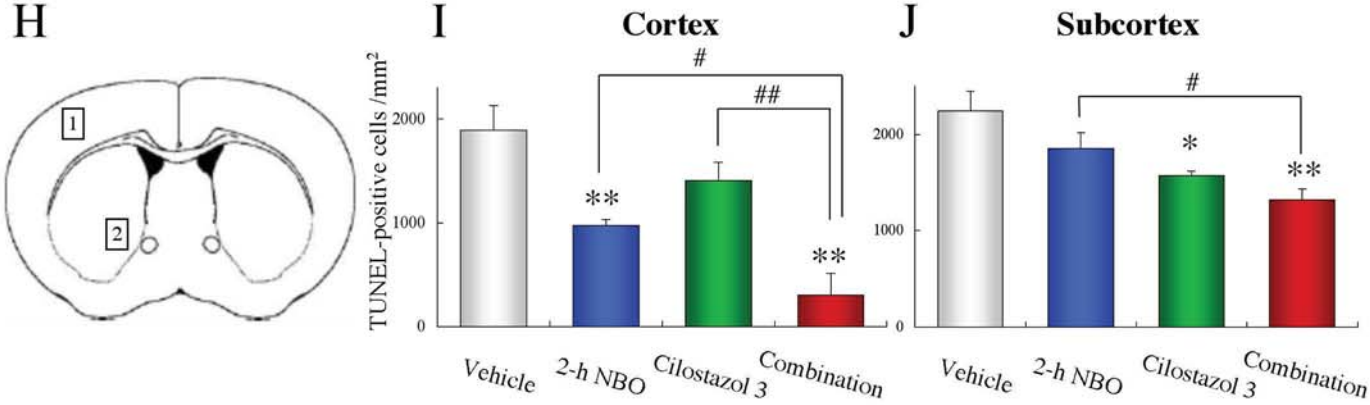


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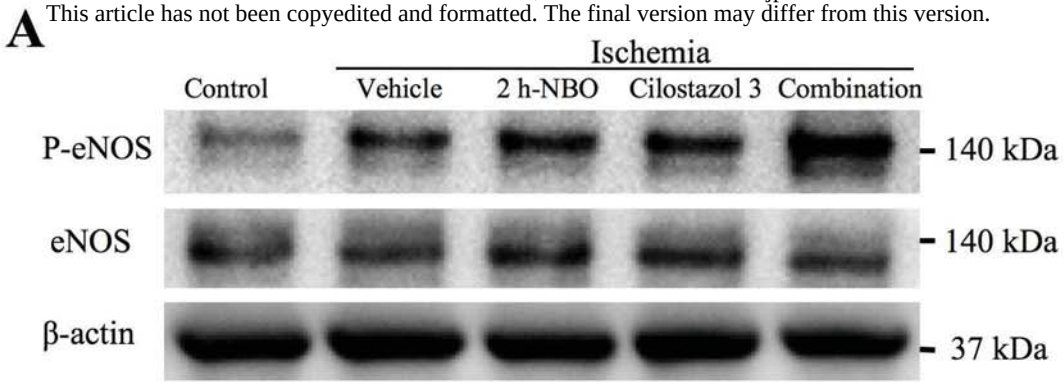
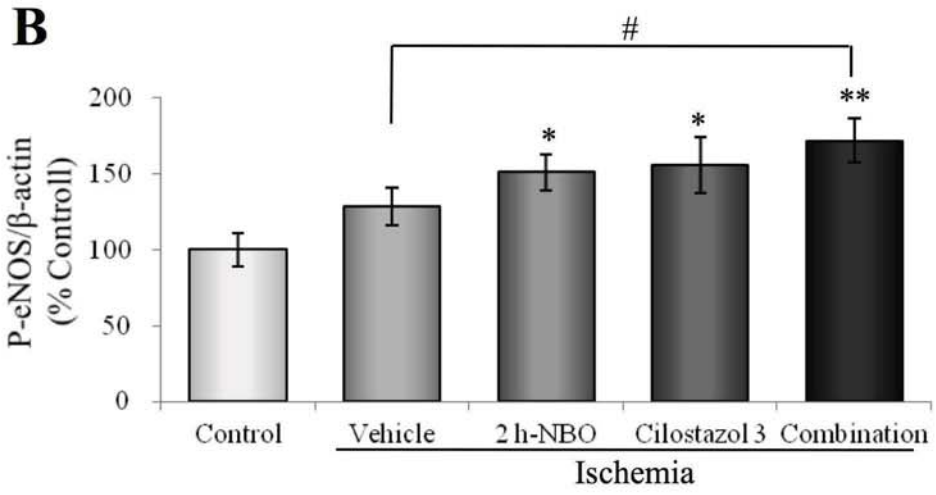
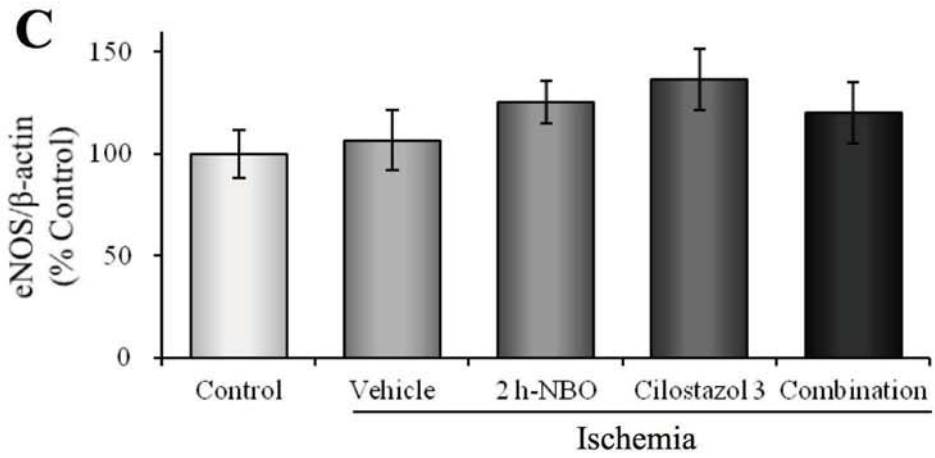
Figure 5







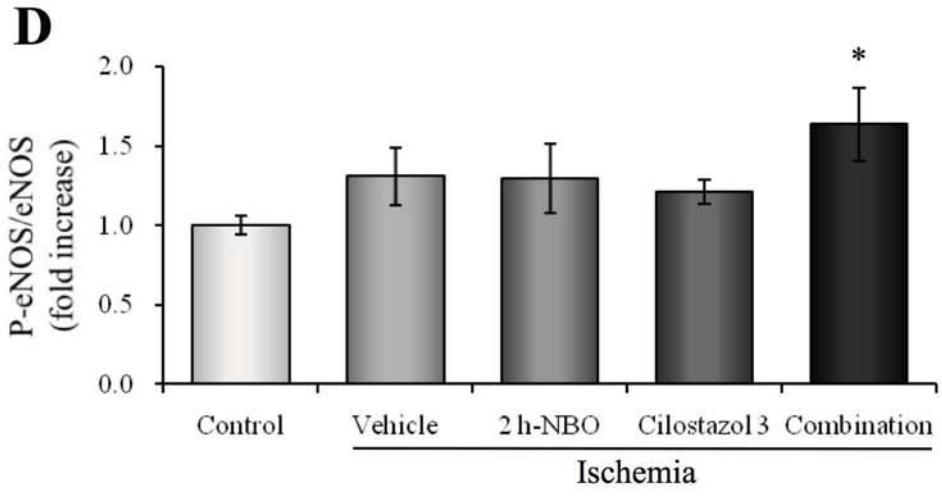


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Figure 6

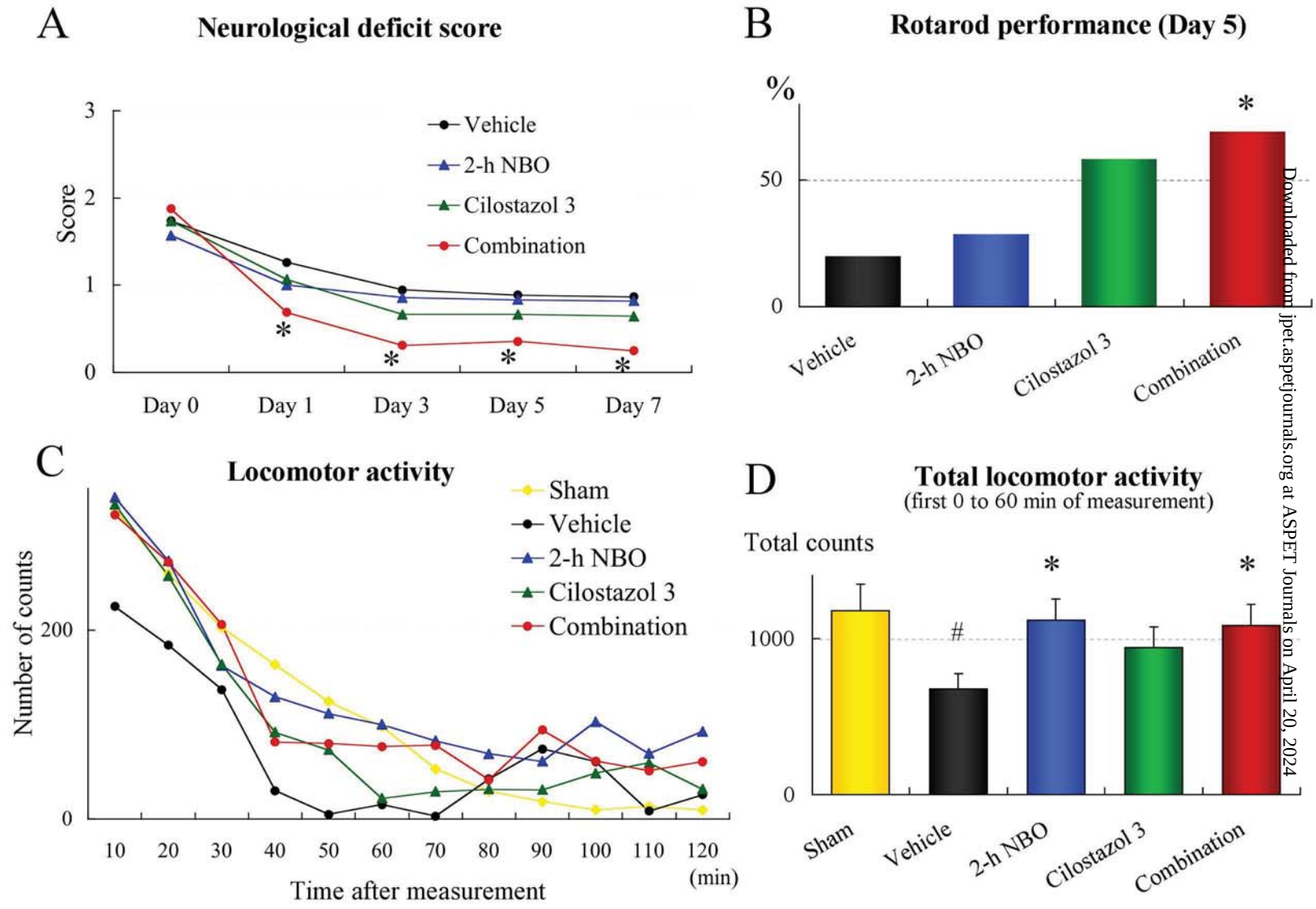

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Figure 7