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The synergetic effect of edaravone and borneol in the rat model of ischemic stroke

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ABSTRACT

Free radical production contributes to the early ischemic response and the neuroinflammatory response to injury initiates the second wave of cell death following ischemic stroke. Edaravone is a free radical scavenger, and borneol has shown anti-inflammatory effect. We investigated the synergistic effect of these two drugs in the rat model of transient cerebral ischemia. Edaravone scavenged •OH, NO• and $ONOO^-$ concentration-dependently, and borneol inhibited ischemia/reperfusion-induced tumor necrosis factor-α (TNF-α), inducible nitric oxide synthase (iNOS), interleukin-1β (IL-1β) and cyclooxygenase-2 (COX-2) expressions. In the rat model of transient cerebral ischemia and reperfusion, the combination of edaravone and borneol significantly ameliorated ischemic damage with an optimal proportion of 4:1. Emax (% inhibition) of edaravone, borneol and two drugs in combination was 55.7%, 65.8% and 74.3% respectively. ED_{50} of edaravone and borneol was 7.17 and 0.36 mg/kg respectively. When two drugs in combination, ED_{50} was 0.484 mg/kg, in which edaravone was 0.387 mg/kg (ineffective dose) and borneol was 0.097 mg/kg (ineffective dose). Combination index (CI) < 1 among effects observed in experiments, suggesting a significant synergistic effect. Reduced levels of pro-inflammatory mediators and free radicals were probably associated with the synergistic effect of edaravone and borneol. The combination exhibited a therapeutic time window of 6 h in ischemia/reperfusion model, and significantly ameliorated damages in permanent ischemia model. Moreover, two drugs in combination promoted long-term effect, including improved elemental vital signs, sensorimotor functions and spatial cognition. Our results suggest that the combination of edaravone and borneol have a synergistic effect for treating ischemic stroke.

1. Introduction

Stroke is a major cause of death and acquired disability in adults (Donnan et al., 2008). To date, the only approved treatment for ischemic stroke is intravenous tissue plasminogen activator (Donnan et al., 2008). However, its efficacy is limited by a very narrow therapeutic time window of 4.5 h and by reperfusion injury and potential hemorrhagic complications because the drug is aimed at restoring cerebral blood flow rather than preventing the actual mechanisms associated with neuronal cell death (Amaro and Chamorro, 2011; Bivard et al., 2013). Thus, the successful treatment of acute ischemic stroke remains one of the major challenges in clinical medicine.

Edaravone prevents neuronal death and brain edema by scavenging hydroxyl radical and inhibiting lipid peroxidation (Yagi et al., 2009; Kikuchi et al., 2010) and has been used to treat ischemic stroke in Japan (Kikuchi et al., 2010; The Edaravone Acute Brain Infarction Study Group, 2003; Adams et al., 2007). Borneol, a terpene and bicyclic organic compound found in several species of *Artemisia* and *Dipterocarpaceae*, has shown anti-inflammatory activities and protective effects against cerebral ischemia/reperfusion injury (Liu et al., 2011).

The development of brain damage after ischemic stroke occurs over time, evolving within hours or days. Overproduction of free radicals including reactive oxygen species (ROS) and reactive nitrogen species (RNS) contributes to the early ischemic response (Lo et al., 2005) and causes neuronal apoptosis (Broughton et al., 2009). ROS include superoxide anion ($O_2•^-$), hydrogen peroxide

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(H_2O_2) and hydroxyl radical ($\cdot OH$), whereas RNS include nitric oxide radical ($NO\bullet$) and the highly toxic peroxynitrite ($ONOO^-$) (Ikonomidou and Kaindl, 2011). The neuroinflammation after ischemic injury initiates the second wave of cell death (Hossmann, 2006). Increasing evidences demonstrate that pro-inflammatory mediators, including inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), play a pivotal role in postischemic brain damage (Amantea et al., 2009). We thus hypothesize that the combination of edaravone (scavenging free radicals) and borneol (inhibiting inflammation) may have synergetic efficacy for ischemic stroke.

2. Materials and methods

2.1. Regents and drugs

5,5-Dimethyl-1-pyrroline N-oxide (DMPO), carboxy-PTIO (C-PTIO), xanthine, xanthine oxidase and 2,3,5-tripenyltetrazolium chloride (TTC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 5,5-Dimethyl-1,3,2-dioxaphosphorinan-2-one (DEPMPO), 1-Hydroxy-2-oxo-3-(N-3-methyl-aminopropyl)-3-methyl-1-triazene (NOC-7) was purchased from Enzo Alexis Biochemicals (Farmingdale, NY, USA). All cell culture medium and supplements were purchased from Gibco (Grand Island, NY, USA). Edaravone and (+)-borneol were supplied by Simcere Pharmaceutical Group.

2.2. Animals

The experimental protocol was approved by the Institutional Animal Care and Use Committee of Nanjing Medical University. We made every effort to minimize the number of mice used and their suffering. In this study, we used Sprague-Dawley rats (9–10 weeks old, 250–270 g) which were purchased from Shanghai Slac Laboratory Animal Co. Ltd. We maintained animals at controlled temperature (22 ± 2 °C) and group housed them (12-h light-dark cycle) with access to food and water ad libitum. Animals were randomly allocated to experimental groups by computer-generated randomization schedules. An experimenter (Y.H.) labeled all animals according to the randomization schedule before allocation. Experiments were performed by investigators who were unaware of the experimental group to which an animal belonged.

2.3. Transient cerebral ischemia model

Focal cerebral ischemia was induced by intraluminal middle cerebral artery occlusion (MCAO) as described previously (Zhou et al., 2010). In brief, under chloral hydrate anesthesia (350 mg/kg, i.p.), a 4/0 surgical nylon monofilament with rounded tip was introduced into the left internal carotid artery through the external carotid stump, advanced 20–21 mm past the carotid bifurcation until a slight resistance was felt. At this point, the intraluminal filament blocked the origin of the middle cerebral artery and occluded all sources of blood flow from the internal carotid artery, anterior cerebral artery, and posterior cerebral artery. Throughout the procedure, body temperature was maintained at 37 ± 0.5 °C. Regional cerebral blood flow was measured by means of a flexible probe and laser Doppler flowmetry (Moor Instruments, Axminster, Devon, UK) (inclusion criteria: regional cerebral blood decreased by 85–95%). The filament was left in place for 120 min and then withdrawn for reperfusion. In the sham-operated animals, the occluding filament was inserted only 7 mm above the carotid bifurcation.

2.4. Assessment of outcome

Infarct volume, neuroscore, modified neurological severity score (mNSS) and spatial cognitive performance were measured by investigators who were unaware of the experimental group to which an animal belongs. The neuroscore assessment and infarct volume measure were performed 24 and 24.5 h after the MCAO respectively as described previously (Zhou et al., 2010). In brief, brains were removed rapidly and frozen at −20 °C for 5 min. Coronal slices were made at 1–2 mm from the frontal tips, and sections were immersed in 2% TTC at 37 °C for 20 min. Infarct volume was expressed as a percentage area of the coronal section in the infracted hemisphere. Neuroscore assessment was performed by an experimenter blinded to the experimental groups (rating scale: 0=no deficit, 1=failure to extent left forepaw, 2=decreased grip strength of left forepaw, 3=circling to left by pulling the tail, and 4=spontaneous circling). mNSS is a composite of motor, sensory, reflex, and balance tests (Chen et al., 2001). This score is derived by evaluating animals for hemiparesis (response to raising rats by the tail or placing rats on a flat surface), abnormal movements (immobility, tremor, seizures), sensory deficits (placing, proprioception), and absent reflexes (pinna, corneal, startle). Neurological function was graded on a scale of 0–18 (normal score 0; mild injury 1–6; moderate injury 7–12; severe injury 13–18). The spatial cognitive performance of rats was evaluated by Morris water maze.

2.5. Western blot analysis

Western bolt analysis was performed as described previously (Zhou et al., 2010). The primary antibodies were as follows: anti-iNOS (1:1000, Alexis), anti-TNF-α (1:500, Abcam, Cambridge, UK), anti-IL-1β (1:1000, Abcam), anti-COX-2 (1:2000, Abcam), anti-β-actin (1:1000, Sigma-Aldrich). Appropriate horseradish peroxidase-linked secondary antibodies were used for detection by enhanced chemiluminescence (Pierce, Rocdford, IL, USA).

2.6. Coculture of neurons and glia cells and OGD/R model

Primary cortical neurons were isolated from embryonic day 16 rats and cultured in neurobasal medium containing 2% B27 supplement as our previously reported (Luo et al., 2010). For subsequent co-culturing with glia cells, primary neurons were seeded in membrane inserts (1 μm pore size). Cultured neurons were identified at 10 days in vitro. For primary glia cells culture, neonatal rats were decapitated and their brains were quickly removed, and glia cells were prepared as described previously (Kim et al., 2002). Dispersed cells were diluted to a concentration of 10^6 cells/ml with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 1% streptomycin/penicillin. Ten days later, cortical neurons cultured in inserts for 10∼12 d were placed into the wells containing glia cells to generate co-culture. After 24 h, the co-culture was washed with sugar-free DMEM twice, and then exposed to oxygen and glucose deprivation (OGD) for 6 h. During the OGD, the co-culture was treated with edaravone or/and borneol or vehicle. After that, the co-culture was incubated with neurobasal/B27 medium instead of sugar-free DMEM for 24 h, and then the inserts with neurons were transferred to new wells and the viability of neurons was detected by MTT assay.

2.7. Morris water maze task

The spatial cognitive performance of rats was evaluated by Morris water maze. The protocol was similar to that for mice, which has been detailedly described in our previous reports