Evaluation of Neuronal Protective Effects of Xanthine Oxidoreductase Inhibitors on Severe Whole-brain Ischemia in Mouse Model and Analysis of Xanthine Oxidoreductase Activity in the Mouse Brain

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Abstract

Global cerebral ischemia and reperfusion (I/R) often result in high mortality. Free radicals play an important role in global cerebral I/R. Xanthine oxidoreductase (XOR) inhibitors, such as allopurinol, have been reported to protect tissues from damage caused by reactive oxygen species (ROS) by inhibiting its production through XOR inhibition. The recently introduced XOR inhibitor febuxostat, which is a more potent inhibitor than allopurinol, is expected to decrease free radical production more effectively. Here, we analyzed the effects of allopurinol and febuxostat in decreasing global severe cerebral I/R damage in mice. Mice were divided into three groups: a placebo group, an allopurinol group, and a febuxostat group. Pathological examinations, which were performed in each group in the CA1 and CA2 regions of the hippocampus 4 days after I/R surgery, revealed that there was a decrease in the number of neuronal cells in the 14-min occlusion model in both regions and that drugs that were administered to prevent this damage were not effective. The enzymatic activity was extremely low in the mouse brain, and XOR could not be detected in the nonischemic and ischemic mice brains with western blot analyses. Thus, one of the reasons for the decreased effectiveness of XOR inhibitors in controlling severe whole-brain ischemia in a mouse model was the low levels of expression of XOR in the mouse brain.

Key words: whole-brain ischemia, xanthine oxidoreductase, three-vessel occlusion model, allopurinol, febuxostat

Introduction

Resuscitation from global cerebral ischemia that is associated with cardiac arrest often results in poor neurological outcomes.1,2) Postcardiac arrest treatment is important to minimize brain injury3); however, even with good treatment, outcomes need to be improved. Further development of treatments is necessary for patients with poor neurological prognoses.

Several rat models of global cerebral ischemia have been established.4–6) However, taking into consideration the use of gene-modified animals, there is merit to developing a mouse model, and some models have been reported. Yonekura et al. have presented a three-vessel occlusion model involving temporary occlusion of the basilar artery.7) This model is reproducible; however, the technique is extremely challenging. In contrast, Thal et al. have reported a two-step approach for disconnecting cerebral circulation from the vertebral collateral blood flow by coagulating the basilar artery.8) These models are useful for studying events, such as cardiopulmonary arrest states, that involve whole-brain ischemia.

Some enzymes are activated during global and focal cerebral ischemia and reperfusion (I/R), leading to the production of excessive reactive oxygen species (ROS).9–11) Among them, xanthine oxidoreductase (XOR) is one of the best studied ROS-generating enzymes.12) This enzyme catalyzes the oxidation of hypoxanthine to xanthine and xanthine to uric acid with the concomitant reduction of NAD+ or molecular oxygen. Mammalian XOR exists as xanthine dehydrogenase (XDH), which prefers NAD+ with a miniature clip.7) This model is reproducible; however, the technique is extremely challenging. In contrast, Thal et al. have reported a two-step approach for disconnecting cerebral circulation from the vertebral collateral blood flow by coagulating the basilar artery.8) These models are useful for studying events, such as cardiopulmonary arrest states, that involve whole-brain ischemia.
as an electron donor. However, XDH is converted to xanthine oxidase (XO) in some situations, and XO prefers O2. McCord has proposed that the conversion of XDH to XO during tissue reperfusion is the basis of the I/R mechanism. Several experimental and clinical studies have been conducted on cerebral ischemia in order to study the role of XOR and the protective effects of XOR inhibitors, such as allopurinol (GlaxoSmithKline Pharma Ltd., Research Triangle Park, North Carolina, USA). Recently, some XOR tight-binding inhibitors have been developed for anti-gout drug. Febuxostat (Teijin Pharma Ltd., Tokyo) is a novel potent XOR inhibitor and that was recently approved by the Food and drug administration for clinical use.

Here, we adopted a three-vessel occlusion mouse model as a global severe cerebral I/R model and analyzed the effects of allopurinol and febuxostat in the model.

Materials and Methods

I. Animals and materials

Six- to nine-week-old male C57BL/6 mice (CLEA Japan Inc., Tokyo), weighing 19–22 g, were allowed free access to food and water before the experiments. All experimental procedures in this study were approved by the institutional animal care committee of Nippon Medical School. Extreme care was taken throughout the study to minimize the pain and discomfort to the animals.

Febuxostat, 2-[3-cyano-4-(2-methylpropoxy)phenyl]-4-methyl-5-thiazolecarboxylic acid, was obtained from Carbosynth Ltd. (Berkshire, UK). Allopurinol [4-hydroxypyrazolo(3,4-d)pyrimidine] and oxypurinol [4,6-dihydroxypyrazolo(3,4-d)pyrimidine] were obtained from Sigma-Aldrich Co. LLC (St. Louis, Missouri, USA). All other chemicals and reagents were of reagent grade or equivalent and were purchased from Wako Pure Chemical Industries, Ltd., Osaka.

II. Determination of plasma XOR inhibitor concentrations

Blood samples were collected with heparinized syringes from the descending aorta while the mice were deeply anesthetized. The samples were centrifuged as soon as possible after blood withdrawal, and the plasma was recovered. Half of the volume of 10% perchloric acid was added to the allopurinol-treated plasma samples. An equal volume of acetonitrile was added to the febuxostat-treated plasma samples. The mixtures were centrifuged for 10 min at 15,000 × g, and 10 μL of the supernatant was injected into a liquid chromatography (LC) column.

III. Determination of plasma oxypurine concentrations

Mouse plasma samples were prepared as described above. One-twentieth volume of 60% perchloric acid was added to the plasma sample. The mixture was centrifuged for 10 min at 15,000 × g, and the supernatant was added to one-fifth volume of 3 M sodium bicarbonate to remove the perchlorate. After vortex mixing for about 1 min, the solution was centrifuged, and the supernatant was collected into a new tube. The sample was neutralized by adding one-fifth volume of 0.5 M potassium phosphate buffer at pH 5.5.

Ten microliters of the supernatant was injected into the column. An ÄKTA LC system that was equipped with a SUPELCOSIL LC-18 T column (4.6 mm × 25 cm; Sigma-Aldrich Co. LLC) was used to separate the oxypurines. The oxypurines were eluted isocratically with 100 mM potassium phosphate buffer, pH 5.5. The flow rate through the column was 1 mL/min, and the column was maintained at ambient temperature. Hypoxanthine, xanthine, and uric acid were detected at 250 nm, 268 nm, and 289 nm, respectively.

Calibration curves were constructed for hypoxanthine, xanthine, and uric acid on the basis of the peak areas of the chromatograms. The concentration of each oxypurine was calculated with the calibration curves.

IV. Preparation for the surgical procedure

As a preliminary experiment, we monitored the heart rate, blood pressure, and body temperature of ischemic mice with a NP-NIBP monitor (MK-2000ST, Muromachi Kikai Co., Ltd., Tokyo) for mice. We confirmed that the changes in both parameters were...
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V. Surgical procedures

In order to adjust the peak time of the XOR inhibitor concentrations and the start time of reperfusion, the XOR inhibitors (50 mg/kg) were administered orally to the mice 30 min before the start of surgery, and the same volume of 0.5% methylcellulose was administered to the placebo group.

We followed the method employed by Yonekura et al. with minor modifications. All animals were subjected to one surgical intervention. A single surgeon performed all of the surgical operations in a blinded manner. After the cessation of blood flow in the basilar artery was confirmed visually, both common carotid arteries were occluded with 2 Sugita temporary miniclips (Mizuhco Ikakogyo Co., Ltd., Tokyo). The duration from the start of the surgical procedure until application of the final clip was 10–15 min. Ischemic duration was measured from the application of the last clip at the left common carotid artery. Spontaneous respiration was assisted through a face mask during and after the ischemia. After 14 min of ischemia, the three clips were removed, and the restoration of blood flow was confirmed in every case by direct inspection of each artery under a microscope. Halothane exposure was discontinued immediately after skin closure. The animals were returned to their cages and placed in a humidified incubator set to 33.0°C. Rectal temperature was maintained at 36.0–37.0°C with a heating blanket (Animal Blanket Controller, Model ATB-1100, Nihon Kohden Corporation, Tokyo).

VI. Specimen collection and histopathological evaluation

The mice were anesthetized with 2.0% halothane 4 days after surgery and transcardially perfused with 10% neutral buffered formalin. The brains were removed and fixed overnight. The fixed tissues were cut into 2-mm thick coronal sections 2 mm caudal from bregma with a brain slicer (MB-SS1-C, Muromachi Kikai Co., Ltd., Tokyo) and embedded in paraffin using a routine procedure with a Vacuum Rotary VRX-23 (Sakura Finetek Japan Co., Ltd., Tokyo). The paraffin-embedded sections (3.5-μm thickness) were subjected to Nissl staining. Each image was captured with an Olympus DP71 digital camera (Olympus Optical Co., Ltd., Tokyo) that was attached to an Olympus AX80 microscope at 400× magnification. Each area to be analyzed was determined by WinROOF ver. 6.1.3 software (Mitani Co., Ltd., Tokyo), and the number of neurons/μm² was evaluated. All specimens were reviewed by a pathologist who was blinded to the study. We conducted a pilot study during which we determined the appropriate duration of the vascular occlusion and the follow-up period after surgery and the evaluation method.

VII. Enzyme assay

Tissue samples were frozen under liquid nitrogen and stored at −80°C until they were analyzed. At the time of analysis, the tissue samples were homogenized by a hand homogenizer with 50 mM potassium phosphate buffer (pH 7.4), 0.25 M sucrose, 1 mM salicylate, 0.3 mM ethylenediaminetetraacetic acid (EDTA), and a complete protease inhibitor cocktail (Roche Applied Science, Indianapolis, USA), and a 15,000 × g supernatant was produced. The total protein concentration of the supernatant was determined with a Coomassie (Bradford) Protein Assay Reagent (Thermo Fisher Scientific Inc., Rockford, Illinois, USA). Enzyme assays were performed at 25°C in 50 mM potassium phosphate buffer (pH 7.8) containing 0.4-mM EDTA, 0.15 mM xanthine, and 500 μM NAD⁺. XOR activities were determined by the uric acid formation rate and by monitoring the absorbance change at 295 nm. An extinction coefficient of 9.6 mM⁻¹ cm⁻¹ was used for the uric acid. Photometric experiments were performed with a UV-3210 spectrophotometer (Hitachi High-Technologies Co., Ltd., Tokyo) that was equipped with a temperature-control system. High-performance LC (HPLC) was used to separate and detect the accumulated uric acid. The HPLC conditions for separating and detecting uric acid are described above.

VIII. Western blot analysis

The tissue distribution of XOR was determined by western blot analyses. The tissue lysate from brain and liver samples that were prepared for enzyme assay described above were used. Equal amounts of protein were resolved with sodium...
dodecyl sulfate-polyacrylamide gel electrophoresis (Mini-PROTEAN TGX 4–20%, Bio-Rad Laboratories, Inc., Hercules, California, USA), which was followed by electrophoretic transfer onto a polyvinylidene fluoride membrane with the iBlot system (Life Technologies Corporation, Grand Island, New York, USA). After blocking the nonspecific binding sites by incubation in 5% (w/v) skimmed-milk Tris-Buffered Saline-containing 0.1% Tween-20 (TBS-T) for 60 min, the membranes were incubated for 60 min at room temperature with 10,000-fold dilution of anti-XOR polyclonal rabbit antibody that was previously produced by Hattori et al.16) The membranes were further incubated with a horseradish peroxidase-conjugated anti-rabbit immunoglobulin antibody (Dako Denmark A/S, Glostrup, Denmark) for 60 min at room temperature at a 1:5,000 dilution. After washing in TBS-T, the membranes were reacted with ECL-prime (GE Healthcare, Uppsala, Sweden) at room temperature for 5 min. Immunoreactive bands were visualized and analyzed with the Chemidoc™ XRS Plus System with Image Lab™ SVP Software (Bio-Rad Laboratories, Inc., Hercules, California, USA).

IX. Statistical analysis

The average number of residual neurons in both hemispheres of all of the mice was evaluated. A one-way analysis of variance and Dunnett’s test were used to assess the intragroup differences regarding the number of surviving neurons. The data were presented as mean ± standard deviation (SD). Adjusted P values were considered significant at P < 0.05. The statistical analysis was performed with the SigmaStat 20.0 Statistical Software package (Systat Software, Inc., San Jose, California, USA).

Results

I. Effects of XOR inhibitors and mode of administration

In order to investigate the pharmacological effect and pharmacokinetics of both XOR inhibitors on mice, the plasma samples from XOR inhibitor-administered mice were assayed.

Mice were randomly divided into 6 groups (5, 20, 50 mg/kg allopurinol-administered groups, and 5 mg/kg, 20 mg/kg, 50 mg/kg febuxostat-administered groups, n = 5 per group). Blood samples were taken from one animal per group at 1 h, 2 h, 4 h, 8 h, and 16 h after drug administration. Allopurinol was hardly detected in the mouse plasma at any dose, while oxypurinol, a hydroxylated form of allopurinol, was detected in a dose-dependent manner. Similarly, plasma febuxostat levels increased with increases in the administered dose (Fig. 1A). A dose

Fig. 1 Blood levels of the xanthine oxidoreductase (XOR) inhibitors and oxypurines. A: Allopurinol and febuxostat were administered orally to mice at 5 mg/kg, 20 mg/kg, and 50 mg/kg (n = 1). Blood concentrations of the XOR inhibitors were analyzed at the indicated times. B: Allopurinol and febuxostat were administered orally to mice at 50 mg/kg. Blood concentrations of the XOR inhibitors were analyzed 1 h, 2 h, and 4 h after administration (n = 5). C: Allopurinol and febuxostat were administered to mice at 50 mg/kg, and the same volume of 0.5% methylcellulose was administered to the placebo group. Blood oxypurine concentrations 1h after the administration of the XOR inhibitors were analyzed (n = 5).
greater than 50 mg/kg was not tested because the mouse urine after the administration of 50 mg/kg became remarkably turbid, and we were afraid of acute renal failure with a higher dose. Therefore, the doses of the inhibitors were maintained at 50 mg/kg for the subsequent experiments.

For the determination of XOR inhibitors or oxypurines concentration in blood, 30 mice were divided into 50 mg/kg allopurinol-administered group, and 50 mg/kg febuxostat-administered group, and blood samples were taken from 5 mice in each group 1 h, 2 h, and 4 h after drug administration. The maximum plasma concentrations of both XOR inhibitors were observed 1 h after administration (Fig. 1B). Furthermore, we measured the plasma levels of oxypurines, such as hypoxanthine, xanthine, and uric acid, in order to confirm that both drugs effectively inhibited XOR activity. Plasma samples that were prepared 1h after administering 50 mg/kg of each drug were assayed. Methylcellulose was administered in the same manner as the placebo control. Plasma hypoxanthine and xanthine levels increased remarkably in the XOR inhibitor-administered groups compared to that in the placebo control group (Fig. 1C). In contrast, the plasma levels of uric acid decreased remarkably in the XOR inhibitor-administered group compared to that in the placebo control, indicating that the drugs effectively inhibited XOR activity in vivo. Therefore, the oral administration of 50 mg/kg of the XOR inhibitors 1h before surgery was considered appropriate for our experiment.

II. Neuronal cell count and histopathological evaluation

Although various methods to evaluate I/R injury have been reported, we chose to ascertain the extent of neuronal injury by assessing the number of surviving cerebral neurons in this study. As most significant changes were observed in the CA1 and CA2 regions of the hippocampus with 14 min occlusion (Fig. 2A), these regions in both hemispheres were evaluated for the number of viable neurons. We also conducted a three-vessel occlusion study with occlusion times of 6 min and 10 min (data not shown). However, the damage varied between the samples with 6 min and 10 min of occlusion,
and the obtained data were inadequate for objective evaluation. We also tried ischemia for over 14 min; however, the survival 4 days after the procedure was too low for an evaluation. As we needed a model that simulated a severe whole-brain ischemic event, such as cardiopulmonary arrest, we conducted the study using the 14-min occlusion model.

The 14-min occlusion induced a decrease in the number of viable neurons in the CA1 region. However, I/R did not induce any differences in the loss of neuronal cells in the CA1 region among the three groups (Fig. 2B). Similar to the CA1 region, treatment-related changes in the CA2 region were not detected between the placebo and drug-administered groups in the 0-min-occlusion mice and the 14-min-occlusion mice (Fig. 2C). The cerebral I/R-induced necrotic changes in the neurons included swollen cytoplasm as well as pyknosis and karyorrhexis of the nuclei. The numbers of neurons/μm² of 10 mice/group were determined to be adequate for an objective evaluation of the CA1 and CA2 regions (Fig. 2D, E). However, no significant differences in the number of neuronal cells were observed between the groups.

### III. XOR distribution in the brain

We investigated XOR activities and expression in the ischemic and nonischemic mouse brains and compared them with those in the liver in order to determine the distribution of XOR in brain tissue.

The whole brain of surgically treated mice (n = 5) was excised 4 days after the occlusion surgery. The liver and brain were excised from nonsurgically treated mice (n = 6) and used as a control. XOR activity was difficult to detect in the nonischemic mouse brain and ischemic mouse brain by a photometric assay. Therefore, HPLC was employed to detect uric acid as the final XOR product. The nonischemic mouse brain and ischemic mouse brain XOR activities were extremely lower than that of the liver (Table 1). Consistent with the XOR activity assay, XOR protein was not detected in the cytosolic fraction of the nonischemic mouse brain and ischemic mouse brain both in the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and in the western blot analysis, while XOR was clearly detected in the liver cytosolic fraction (Fig. 3). We concluded that XOR expression was particularly low in the nonischemic mouse brain and ischemic mouse brain.

### Discussion

Two whole-brain ischemia models have been described according to the methods reported by Thal et al.⁸ and Yonekura et al.⁷ to date. In this study, we employed the method of Yonekura et al. because the method has the advantage that the time needed for manipulation is markedly short and can be completed with

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Table 1 Xanthine oxidoreductase (XOR) activities in mouse brain and liver

<table>
<thead>
<tr>
<th>Protein (nmol/min/mg)</th>
<th>Tissue weight (nmol/min/g)</th>
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<tbody>
<tr>
<td>Non-ischemic brain</td>
<td>0.0047 (±0.0031)</td>
</tr>
<tr>
<td>Ischemic brain</td>
<td>0.0053 (±0.00478)</td>
</tr>
<tr>
<td>Liver</td>
<td>2.217 (±1.22)</td>
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The whole brain of surgically treated mice (n = 5) was excised 4 days after the occlusion surgery. The liver and brain were excised from nonsurgically treated mice (n = 6) and used as a control. XOR activities in mouse brain and liver lysate were detected by measuring uric acid formation rates. Numbers in parenthesis represent standard deviation.

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Fig. 3 Detection of the xanthine oxidoreductase (XOR) protein by western blotting analysis. Whole brains of a surgically treated mouse and a sham operated mouse were excised 4 days after the occlusion surgery. The liver and brain were excised from a nonsurgically treated mouse (n = 6) and used as a control. XOR activities in mouse brain and liver lysate were detected by measuring uric acid formation rates. Numbers in parenthesis represent standard deviation.

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Fig. 3 Detection of the xanthine oxidoreductase (XOR) protein by western blotting analysis. Whole brains of a surgically treated mouse and a sham operated mouse were excised 4 days after the occlusion surgery. The liver and brain were excised from a nonsurgically treated mouse and ischemic mouse brain and used as a control. A: Cytosol fractions of mouse tissue samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and in the western blot analysis, while XOR protein was visualized with the Oriole™ fluorescent gel stain (BioRad Laboratories, Inc., Hercules, California, USA) on the duplicate gel. B: A western blot analysis was performed with an anti-rat XOR antibody. Two µg or 10 µg of protein was applied to a polyacrylamide gel for total protein stain or a western blot analysis, respectively. Lane M, Precision Plus Protein™ Standards (Bio-Rad Laboratories, Inc.); Lane 1: control mouse (not surgically treated) brain cytosol, lane 2: sham mouse brain cytosol, lane 3: ischemic mouse brain cytosol, lane 4: mouse liver cytosol, which was used as a positive control for XOR. The position of XOR is indicated by the arrow.
one surgical operation. The observed brain damage was similar to that reported by Yonekura et al.71

Our mouse experiment showed that both allopurinol and febuxostat reached the maximum blood level concentrations (Fig. 1A, B). These results were consistent with the pharmacokinetics of these inhibitors in other animals.17 The plasma levels of hypoxanthine and xanthine were sufficiently high after the administration of the inhibitors, indicating that the dose was sufficient to inhibit XOR (Fig. 1C). The plasma uric acid levels in the XOR-inhibitor-administered mice were decreased compared to that in mice receiving methylcellulose; however, the uric acid levels could not be evaluated precisely because an uricase inhibitor was not used in our study.

The CA1 and CA2 regions were segmented according to the report of Lein et al.18 Transient global cerebral ischemia causes selective neuronal injury in vulnerable regions of the brain, such as the CA1 sector of the hippocampus.19–21 Furthermore, Yonekura et al. have used a global ischemia model in C57BL/6 mice and showed high reproducibility in addition to neuronal injury in the CA1 region.71 Li et al. have evaluated an ischemia model for nerve cell damage in other parts of the hippocampus and found that the most severe damage was observed in the CA1 region, followed by the CA2 region. No significant neuronal damage was observed in the CA3 and CA4 regions.22 Therefore, we decided to add the CA2 region to our evaluation, and similar results were noted of a decrease in the number of neurons in the CA2 region. These results were similar to those reported by Li et al.

The two XOR inhibitors that were administered in our study are used clinically as hypouremic agents for treating gout; however, their biochemical and pharmacological properties are quite different. Allopurinol is oxidized to oxypurinol by XOR, and oxypurinol binds tightly to the enzyme.23 Febuxostat is significantly more potent than allopurinol.24 Febuxostat inhibits the enzyme in vivo for long periods of time because the enzyme inhibitor complex is very stable and is not influenced by changes in cofactor redox status.15

Many previous reports have indicated a neuroprotective effect of allopurinol during focal cerebral ischemia.25–30 However, no obvious neuroprotective effects were observed in our experiments with both inhibitors in contrast to our expectation. One possibility is that the occlusion in our study was so severe that the observed tissue damages were not caused by reperfusion but by ischemia. However, increases in some biochemical markers due to oxidative damage were detected in the I/R brain (the detailed report will be described elsewhere), suggesting that it was likely that the tissue suffered I/R damage. Analysis of neuroprotective effects with other radical scavengers would be useful for further study of the whole brain ischemic model. Another possible reason involves the expression levels of XOR in the brain tissue. The distribution of XOR in the brain is quite low;31 however, some in vivo studies have reported that XOR activity increases during hypoxia and reperfusion.28,32 We re-evaluated the activity and expression in the nonischemic mouse brain and ischemic mouse brain. XOR expression levels in brains were extremely low compared to that in the liver even after reperfusion (Fig. 3). In addition, the XOR activities were significantly lower (Table 1). These results were consistent with the finding that the effects of XOR inhibitors were limited in brain ischemia.

We estimated the effects of XOR inhibitors in a mouse model of severe brain ischemia. Allopurinol and febuxostat did not decrease the brain I/R damage in mice. We concluded that when brain ischemia is extremely severe, XOR inhibitors are ineffective for protecting against ischemia in the CA1 and CA2 regions of the hippocampus.

Acknowledgments
The author’s thank Takeshi Nishino, MD, PhD and Zenya Naito, MD, PhD for their helpful discussions and critical inputs after reading the manuscript. In addition, we thank Mr. Takayuki Asakura for technical assistance.

Conflicts of Interest Disclosure
The authors have no personal, financial, or institutional interests in any of the drugs, materials, or devices in the article. All authors who are members of the Japan Neurosurgical Society (JNS) have registered online Self-reported COI Disclosure Statement Forms through the website for JNS members.

References
4) Kameyama M, Suzuki J, Shirane R, Ogawa A: A new


22) Li LX, Campbell K, Zhao S, Knuckey NW, Meloni BP: The effect of blood pressure (37 vs 45 mmHg) and carotid occlusion duration (8 vs 10 min) on CA1-4 neuronal damage when using isoflurane in a global cerebral ischemia rat model. *Brain Res Bull* 86: 390–394, 2011


27) Itoh T, Kawakami M, Yamauchi Y, Shimizu S,


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