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Anti-oxidative effects of D-allose, a rare sugar, on ischemia-reperfusion damage following focal cerebral ischemia in rat

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ABSTRACT

The present study investigates the anti-oxidative effects of D-allose on ischemic damage. Rats were subjected to transient middle cerebral artery occlusion (MCAO) for 1 h under pentobarbital anesthesia. D-allose was intravenously infused during occlusion and a further 1 h after reperfusion (400 mg/kg). The effects of D-allose on focal cerebral ischemia were examined by measuring brain damage (infarction and atrophy volume) and behavioral deficits 7 days after MCAO. In another set of rats, apurnic/apyrimidic abasic sites (AP-sites) and 8-hydroxy-2′-deoxyguanosine (8-OHdG), oxidative stress markers, were investigated 24 h after MCAO to examine the anti-oxidative effects of D-allose. Brain damage and behavioral deficits were significantly decreased by D-allose administration compared to vehicle. The number of AP-sites and 8-OHdG levels were also reduced by D-allose. Thus, the present study suggests that D-allose has anti-oxidative effects and induces neuroprotection in focal cerebral ischemia.

Rare sugars were defined as monosaccharides and their derivatives that are rare in nature by the 1st International Symposium of International Society of Rare Sugar, Takamatsu, Kagawa, Japan, 2002. In general, rare sugars are known as low-calorie sweeteners [9]. However, their biological functions and pharmacological activities are not well known. A research group at Kagawa University has examined many aspects of rare sugars [1,17]. Bhuiyan et al. [2] reported a simple method to produce D-allose from fructose using microorganisms and their enzymes. Another group in our university demonstrated that D-allose can attenuate the production of reactive oxygen species (ROS) during ischemia-reperfusion of rat liver, leading to protective effects [10].

Oxidative DNA damage results from direct attacks by ROS. DNA damage may significantly contribute to secondary brain damage following permanent or transient ischemia [7,11]. Our preliminary experiments suggested that D-allose was neuroprotective in a model of global brain ischemia [17]. We hypothesized that D-allose might also be protective in a model of focal cerebral ischemia, transient middle cerebral artery occlusion (MCAO). The purpose of the present study was, therefore, to investigate the effectiveness of D-allose in inhibiting brain damage, behavioral deficits and oxidative DNA damage in that model. The latter was assessed using apurinic/apyrimidinic abasic sites (AP-sites) and 8-hydroxy-2′-deoxyguanosine (8-OHdG), hallmarks of oxidative DNA damage [13].

Animal protocols were approved by the Animal Committee of Kagawa University Faculty of Medicine. Male Sprague–Dawley rats (CLEA, Tokyo, Japan), each weighing 250–350 g, were used for all experiments. Rats were allowed free access to food and water. Animals were anesthetized with pentobarbital (40 mg/kg, i.p.) before surgery during which time body temperature was maintained at 37.5 °C. Left femoral artery was catheterized to monitor arterial blood pressure and to measure blood glucose, pH, pCO_2, and pO_2.

Transient (60 min) occlusion of the MCA was achieved using the suture method of Zea Longa and Weinstein [20]. Briefly, the bifurcation of left common carotid artery was exposed, the external carotid

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artery was ligated distally. A 3–0 monofilament nylon suture, its tip rounded by heating, was introduced into the internal carotid artery lumen through the stump of the external carotid artery (ECA) and gently advanced into the internal carotid artery 19–20 mm past the common carotid artery bifurcation to block the origin of MCA. After 60 min of MCAO, reperfusion was achieved by withdrawal of the suture from the ECA and the ECA was tied permanently. D-allose (100 mg/kg or 400 mg/kg, Kagawa University, 98% purity) or D-fructose (400 mg/kg) dissolved in saline, or saline as vehicle was administrated continuous infusion via tail vain during ischemia and reperfusion (for 2 h total). For sham-operated rats, the carotid arteries were exposed but no suture was inserted. After closing the skin incision, rats were allowed to recover from anesthesia, returned to their cages, and allowed to move and eat freely.

This study was performed in three parts. In the first part, we evaluated the effect of D-allose on ischemic damage by histological analysis. Twenty four rats underwent MCAO. These were treated with saline as vehicle, 100 mg/kg D-allose, 400 mg/kg D-allose or 400 mg/kg D-fructose ($n = 6$ per group). Animals were reanesthetized and sacrificed one week after MCAO for examination. The second part investigated the effect of D-allose on behavioral deficits 7 days after MCAO. Ten rats were treated with either saline as vehicle or 400 mg/kg D-allose. Another five rats underwent a sham-operation. In the third part, apurinic/apyrimidic abasic sites (AP-sites) and 8-hydroxy-2′-deoxyguanosine (8-OHdG), oxidative stress markers, were investigated 24 h after MCAO to examine the anti-oxidative effects of D-allose. Eighteen rats were treated with either saline or 400 mg/kg D-allose for examination of AP-sites, ELISA of 8-OHdG, or immunohistochemistry of 8-OHdG (three rats in each). Another nine rats underwent a sham-operation. Animals were reanesthetized and sacrificed 24 h after MCAO for examination.

For histological examination, rats were reanesthetized with pentobarbital (40 mg/kg, i.p.) and underwent intracardiac perfusion with 4% paraformaldehyde in 0.1 mol/l phosphate-buffered saline (PBS; pH 7.4). The brain was removed, paraffinized, and serially sectioned (8 μm thick) at 500 μm intervals with a sliding microtome, from a rostral limit of bregma +2.2 mm to bregma −6.8 mm caudally. The sections were stained with hematoxylin–eosin, and morphometric analysis was performed by computer-assisted hand delineation of the lesion area using NIH image software (Version 1.61, Bethesda, MD, USA) [12]. Because significant atrophy was also present in the ischemic hemisphere, total tissue loss was calculated by summing the areas of cortical necrosis, striatal necrosis, and atrophy (difference between the hemispheres). Volumes of hemispheric tissue loss were determined by integrating the areas of damage in the anteroposterior dimension, from the rostral to the caudal section. Interindividual variation in brain size was controlled for by dividing the volume of tissue loss by the contralateral hemispheric volume, expressing damage as a percent rather than as raw data in mm^3. Then brain damage calculated as:

$$\text{Brain damage}\,(\%) = (\text{Cv} + \text{Sv} + \text{Av}) \times \frac{100}{(\text{contralteral hemisphere volume})}$$

where Cv = cortex lesion volume, Sv = striatum lesion volume and Av = atrophy volume.

To investigate function outcome, forelimb placing [14] and locomotor activity [15] tests were used in this study. Forelimb placing was scored using the vibrissae-elicited forelimb placing test. Independent testing of each forelimb was induced by brushing the respective vibrissae against the corner of a table top once per trial for 10 trials. A score of one was given each time the animal placed its forelimb onto the edge of the table in response to the vibrissae stimulation. The percentage of successful placing responses was determined for both the impaired forelimb and the nonimpaired forelimb. Locomotor activity of the animal in a transparent cage was measured in every 1 h period for 24 h (starting at 6:00 AM) with photobeam interruption sensors (LOCOMO LS-8, Melquest, Toyama, Japan). The number of beam breaks was evaluated as locomotor activity.

To examine oxidative DNA damage, the rats were reanesthetized before undergoing intracardiac perfusion with saline and then the brain were removed. DNA was first extracted using a DNA isolation kit (Dojindo Molecular Technologies, Kumamoto, Japan). In this method, purified DNA (ratio of OD260/280 > 1.8) is isolated from brain tissues by a guanidine method with RNase A and proteinase K. This method avoids the use of phenol and heating which may induce high background [13]. Aldehyde-reactive probe (ARP) labeling and quantification of AP-sites were performed by AP-sites assay kit (Dojindo Molecular Technologies). Purified DNA was dissolved at 100 μg/ml in TE buffer, and 10 μl of the DNA solution was incubated with 10 μl of 5 mM ARP solution at 37 °C for 1 h. The ARP-labeled DNA was quantified using 96 well microplates as in an ELISA study. The ARP-labeled DNA and DNA binding solution were added to each well and incubated overnight in the dark at 37 °C. Each well was washed five times with PBS with Tween 20 (PBST). HRP-streptavidine solution was added and incubated at 37 °C for 1 h. After wells were washed five times with PBST, sensitive substrate solution was added and incubated at 37 °C for 1 h. Optical density (OD) was then measured at 630 nm. ARP assays were performed in triplicate and mean values calculated. The data, expressed as the number of AP sites per 100,000 nucleotides, were calculated based on the linear calibration curve generated for each experiment using ARP-DNA standard solutions.

8-OHdG levels in samples were determined with an ELISA kit (Japan Institute for the Control of Aging, Shizuoka, Japan). This kit can measure extremely low levels of 8-OHdG, and the specificity of the monoclonal antibody has been established [16]. Optical density was measured at 450 nm. 8-OHdG measurements were performed in triplicate and means values calculated. The data, expressed as pg of 8-OHdG per μg of DNA [8,14], were calculated on the basis of a linear calibration curve generated with 8-OHdG standard solutions. We also performed the immunohistochemistry of 8-OHdG [13]. Using the avidin–biotin complex technique, section was incubated in a 1:10 dilution of goat serum for 30 min, rinsed, and incubated overnight with the primary antibody, which was mouse anti-8-OHdG monoclonal antibody (10 μg/ml). Normal mouse immunoglobulin G was used as a negative control.

All data in this study are presented as mean ± SD. Data on brain damage (infarction and atrophy volume), behavioral test (forelimb placing scores and activity counts) and oxidative stress markers (the number of AP-sites and 8-OHdG levels) were analyzed with Student's *t*-test or one-way analysis of variance (ANOVA), followed by Bonferroni's post hoc test. Significance levels were measured at $P < 0.05$.

All physiological variables were measured immediately before 30 and 70 min after ischemia (Table 1). Blood glucose, pH, pCO_2, pO_2, and mean arterial blood pressure were within the normal range (80–120 mg/dL, 7.34–7.44, 34–49 mmHg, 80–100 mmHg, and 80–130 mmHg, respectively). These variables were not influenced by D-allose administration.

Hemotoxylin and eosin staining was performed to examine brain histologic changes 7 days after MCAO in rats (Fig. 1A). The effect of D-allose (100 mg/kg or 400 mg/kg) or D-fructose (400 mg/kg) treatment was assessed 7 days after induction of MCAO. Brain damage (infarction and atrophy volume) was significantly decreased by administration of 400 mg/kg D-allose compared to the vehicle (saline injection) group ($P < 0.05$, Fig. 1B).