Auraptene/Naringin-Rich Fruit Juice of *Citrus kawachiensis* (Kawachi Bankan) Prevents Ischemia-Induced Neuronal Cell Death in Mouse Brain through Anti-Inflammatory Responses

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(Received July 23, 2018)

Summary Cerebral ischemia/reperfusion leads to delayed neuronal cell death, resulting in brain damage. Auraptene (AUR) and naringin (NGIN), which exert neuroprotective effects in ischemic brain, are abundant in the peel of *Citrus kawachiensis*. Although parts of AUR/NGIN are transited from the peel to the juice during the squeezing of this fruit, these amounts in juice might be too low to exert effects. We thus prepared the AUR/NGIN-rich fruit juice of *C. kawachiensis* by addition of peel paste to the raw juice. The present study revealed that orally administration of the dried powder of this AUR/NGIN-rich fruit juice (2.5 g/kg/d) for 7 d to ischemic mice significantly suppressed the ischemia-induced neuronal cell death in the hippocampus, which was coincidentally with the reduction of hyper-activation of microglia and astrocytes. These results suggest that AUR/NGIN-rich juice of *C. kawachiensis* may possess therapeutic potential for the prevention of neurodegenerative diseases via inhibition of inflammatory processes.

Key Words ischemic brain injury, anti-inflammation, auraptene, naringin, fruit juice

Our studies have previously shown that the peel of *Citrus kawachiensis* (Kawachi Bankan) is exceptionally abundant in auraptene (AUR) compared with that of other citrus (1). The amount of AUR in *C. kawachiensis* was about four times more than that in *Citrus hassaku* (Hassaku) which was known until then to be rich in AUR among citrus fruits. AUR, a citrus coumarin derivative, was reported to have potent anti-cancer and anti-inflammatory activities in peripheral tissues (2). Our studies also revealed that it exerts anti-inflammatory effects not only in peripheral tissues but also in the central nervous system (CNS) (3–6). This peel is also a rich source of 3,5,6,7,8,3′,4′-heptamethoxyflavone (HMF) and naringin (NGIN). HMF, a citrus polymethoxyflavone, was reported to have both anti-inflammatory (7) and immune-modulatory (8) effects in peripheral tissues. We also showed that it has the neuroprotective effects in the CNS which result from its ability to suppress inflammation (9) and to increase the expression of brain-derived neurotrophic factor (BDNF) in the hippocampus (10–13). As for NGIN, which is a biolflavonoid commonly found in citrus fruit, it exerts its neuroprotective effects against various brain injuries by reducing oxidation- and inflammation-mediated alterations (14–17).

On the basis of these findings, we prepared dried peel powder of *C. kawachiensis* (the content of AUR, 4.07±0.033 mg/g; of HMF, 0.27±0.0039 mg/g; and of NGIN, 44.02±0.491 mg/g) and orally (p.o.) administered it at the dose of 1.2 or 2.4 g/kg/d to lipopolysaccharide (LPS)-induced systemic inflammatory model mice (4) and a transient global cerebral ischemia mouse model (18). As a result, we succeeded in revealing that p.o. administration of the dried peel powder of *C. kawachiensis* exerts potent neuroprotective activities in the brain of these model mice (4,18). *C. kawachiensis* is a citrus fruit that is mainly eaten as raw fruit and drunk as a juice. During the squeezing of this fruit to obtain the juice, parts of some bioactive constituents including AUR and NGIN are transited from the peel to the juice. When we prepared the dried juice powder of *C. kawachiensis*, the amounts of AUR and NGIN in the dried juice powder were 0.32±0.033 mg/g; of HMF, 0.27±0.0039 mg/g; and of NGIN, 44.02±0.491 mg/g respectively, and the HMF content was below the detection limit (4). We thus prepared test juice enriched in AUR/NGIN by adding peel paste to the raw juice. The aim of this study was to assess whether this AUR/NGIN-rich test juice would have a neuroprotective effect against the damage caused to the mouse brain by transient cerebral global ischemia.

As a model animal of transient cerebral global ischemia, C57BL/6 strain mice were subjected to bilateral common carotid occlusion (2-vessel occlusion: 2VO), because this strain is most susceptible to cerebral ischemia following 2VO among seven common mouse strains.
strains (C57BL/6, ICR, BALB/c, C3H, CBA, ddY and DBA/2) (19).

MATERIALS AND METHODS

Preparation of AUR/NGIN-rich fruit juice. Fruits of C. kawachiensis were harvested in Yawatahama (Ehime, Japan) and squeezed to obtain the juice, to which was added the pasty peel residue (1/5 volume of juice) in order to increase the AUR/NGIN content. This fruit juice enriched in AUR/NGIN was then freeze-dried. This dried test sample was suspended in distilled water for p.o. administration.

Animals. Nine-week-old male C57BL/6 strain mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan). All groups of mice were kept at 23±1°C under a 12-h light/dark cycle (light on 8:00–20:00). During the experimental period, the mice were given free access to tap water and food until 08:30 and then deprived of food until the time of administration of sample or vehicle (16:00). All animal experiments were carried out in accordance with the Guidelines for Animal Experimentation specified by the Animal Care and Use Committee of Matsuyama University, and the experiments were implemented by the approved protocol (No. 9002; 2, September 2009).

Procedures for ischemic surgery. On Day 5 (4 d after the start of administration of citrus sample), mice were subjected to 2VO as previously described (3, 10). Throughout surgery, body temperature and brain surface temperature were maintained at 37±0.5°C and 36.5±0.2°C, respectively. After surgery, all animals were placed in a recovery cage under a heat lamp and had free access to drinking water.

Administration of citrus samples. For experiments, mice weighing about 25 g were randomized into 3 groups: sham-control group (Sham; n=11), 2VO-control group (2VO; n=14), and 2VO surgery and sample-treatment group (2VO+Juice; n=14). In the test-sample group (2VO+Juice), mice were p.o. administered sample solution (0.75 mL) once a day for 7 d (from Day 1 to Day 7). Mice in the other 2 groups (Sham and 2VO) were administered vehicle (distilled water) in the same way. The dose of citrus sample was set at 2.5 g/kg/d. In this powder, the amounts of AUR, HMF, and NGIN were 0.946±0.0096 mg/g, 0.071±0.0006 mg/g, and 3.17±0.0449 mg/g, respectively.

Immunohistochemistry. At Day 8 (1 d after the last administration of test sample or vehicle), the mice were anesthetized and transcardially perfused with ice-cold phosphate-buffered saline (PBS). Their brains were then harvested in Yawatahama (Ehime, Japan) and squeezed to obtain the juice, to which was added the pasty peel residue (1/5 volume of juice) in order to increase the AUR/NGIN content. This fruit juice enriched in AUR/NGIN was then freeze-dried. This dried test sample was suspended in distilled water for p.o. administration.

Effects of the dried AUR/NGIN-rich juice of C. kawachiensis on 2VO-induced neuronal cell death in the hippocampus

The hippocampal region is vulnerable to transient global cerebral ischemia, and the neurons in it easily undergo delayed neuronal cell death within a few days after the start of ischemia (21). We thus estimated the effect of the test sample on the neuronal cell death in CA1, CA2, and CA3 regions of the hippocampus at Day 8 (namely 3 d after 2VO) by using immunohistochemical methods (MAP-2/Nissl staining). Figure 1A shows representative photographs of each region of the hippocampus. In the Sham group, the pyramidal cells with dendrites gave positive staining with both cresyl violet (blue signal) and the anti-MAP-2 antibody (brown signal). In the 2VO group, the number of intact vivid neurons with defined cell membranes and nuclei was counterstained with 0.1% cresyl violet (Nissl staining). The stained sections (more than two sections per each experimental mice) were examined under an optical microscope (CX21; Olympus, Tokyo, Japan). For microscopic staining for optical microscopy, the primary antibody was a rabbit polyclonal antibody against ionized calcium-binding adaptor molecule 1 (iba1, 1:1,000; Wako Pure Chemical Industries, Ltd., Osaka, Japan) and the secondary antibody was EnVision-plus system HRP-labeled polymer (anti-rabbit; Dako). Images captured with a microscope (more than two sections per each experimental mice) were analysed by using Image J software (NIH, Bethesda, MD, USA).

In order to stain glial fibrillary acidic protein (GFAP), an intermediate filament protein (50 kDa) of mature astrocytes, for confocal fluorescence microscopy, mouse anti-GFAP antibody (1:200; Sigma-Aldrich) and Alexa Fluor 568-labeled goat anti-mouse IgG (H+L) was used, as the primary antibody and the secondary antibody, respectively. The mounting medium used was VECTASHIELD® (Vector Laboratories). Images of the hippocampus were captured with a confocal fluorescence microscopy system (LSM510; Zeiss, Oberkochen, Germany). More than two sections were taken from each experimental mice to analyze their intensities of immunoreactivity.

Statistical analysis. Data were expressed as the mean±SE. Significant differences in values obtained in experiments involving 2 groups were analyzed by Student’s t-test. Significance was defined as p<0.05.

RESULTS

Effect of the dried AUR/NGIN-rich juice of C. kawachiensis on 2VO-induced neuronal cell death in the hippocampus

The hippocampal region is vulnerable to transient global cerebral ischemia, and the neurons in it easily undergo delayed neuronal cell death within a few days after the start of ischemia (21). We thus estimated the effect of the test sample on the neuronal cell death in CA1, CA2, and CA3 regions of the hippocampus at Day 8 (namely 3 d after 2VO) by using immunohistochemical methods (MAP-2/Nissl staining). Figure 1A shows representative photographs of each region of the hippocampus. In the Sham group, the pyramidal cells with dendrites gave positive staining with both cresyl violet (blue signal) and the anti-MAP-2 antibody (brown signal). In the 2VO group, the number of intact vivid neurons with defined cell membranes and nuclei was apparently reduced. In the 2VO+fortified Juice group, this neuronal loss was effectively suppressed. Figure 1B shows the results of a quantitative analysis of the cell number of Nissl staining-positive intact cells, indicating that 1) the number of viable cells in the CA1, CA2, and CA3 regions of the 2VO group was significantly (**p<0.001) lower than that of the Sham group; 2) the most severe neuronal cell death occurs in the CA2 region as previously reported (3, 21, 22); 3) the administration of the dried powder of AUR/NGIN-rich C. kawachiensis juice markedly and significantly (###p<0.001) suppressed 2VO-induced neuronal loss in the CA1, CA2 and
CA3 region.

**Effect of the dried AUR/NGIN-rich juice of C. kawachiensis on 2VO-induced inflammation**

It is well known that cerebral ischemia induces a robust neuroinflammatory response and oxidative stress that include marked changes in the gene-expression profile and phenotype of a variety of endogenous CNS cell types (microglia, astrocytes, and neurons) (23, 24). Regarding the estimation of the anti-inflammatory action of this dried juice enriched in AUR/NGIN on the 2VO-injured brain, at first we stained microglia in the hippocampal region of each group of mice with antibody against Iba1 (a microglial marker). Figure 2A shows representative photographs of the stratum lacunosum-moleculare/stratum radiatum in the hippocampal region. In the Sham group, only a few Iba1-positive cells were observed as being in the ramified form (inactivated form). In the 2VO group, the shape of Iba1-positive cells changed to the hypertrophied form (an activated form called ameboid microglia). In the 2VO+Juice group, the shape of most Iba1-positive cells indicated the inactive ramified form. Figure 2B shows the density of Iba1-positive cells. That of the 2VO group (5.22±0.163/mm²) was about twice that of the Sham group (2.78±0.0646/mm²), and there was a significant difference (**p<0.001) between them. That of 2VO+Juice group (4.03±0.336/mm²) was significantly (###p<0.001) lower than that of 2VO group, suggesting that the 2VO-induced activation of microglial cells was suppressed by the dried AUR/NGIN-rich juice of *C. kawachiensis*.

We then stained astrocytes in the hippocampal region of each group of mice with antibody against GFAP (an astrocytic marker). Representative photographs of the stratum lacunosum-moleculare/stratum radiatum in the hippocampal region (Fig. 3A) showed that the number and size of GFAP-positive cells in the 2VO group was obviously larger than those for the Sham group or 2VO+Juice group. As shown in Fig. 3B, the density of GFAP-positive cells in the 2VO group (42.1×10⁵±6.84×10⁵/mm²) was significantly (**p<0.01) higher than that in the Sham group.
Neuroprotective Effect of Fruit Juice of *Citrus kawachiensis*

(13.6x10^5 ± 2.06x10^5/mm²); and this 2VO-induced increase in GFAP-positive cell density was significantly (### p<0.001) suppressed in the 2VO+Juice group (8.37x10^5 ± 1.49x10^5/mm²), suggesting that the 2VO-induced activation of astrocytes was suppressed by the dried AUR/NGIN-rich juice of *C. kawachiensis*. The above results indicated that the dried AUR/NGIN-fortified juice had the ability to suppress the ischemia-induced inflammation in the CNS.

**DISCUSSION**

The data presented here suggested that the dried powder of AUR/NGIN-rich *C. kawachiensis* juice had a neuro-protective effect against ischemic brain via its anti-inflammatory action. As for the mechanism of anti-inflammatory effects of AUR, we previously showed that AUR suppressed the LPS-induced expression of the mRNA of pro-inflammatory enzyme (COX-2) and pro-inflammatory cytokines (IL-1β and TNF-α) in cultured astrocytes (5). As for the mechanism of anti-inflammatory effects of NGIN, it was reported to reduce IL-8, MCP-1, and MIP-1α secretion and mRNA expression, possibly by blocking the activation of the NF-κB and MAPK signaling pathways in LPS-induced RAW 264.7 macrophages (25). When AUR alone was subcutaneously (s.c.) injected into the same model of ischemic mice, the effective dose of AUR was 25 mg/kg/d (3, 5). The dose of AUR in the present study is 2.37 mg/kg/d, which revealed that about one-tenth dose of AUR was sufficient to exert similar effects, when the dried juice powder was p.o. administered. When NGIN alone was orally p.o. injected into the same model of ischemic mice, the effective dose of NGIN was 106 mg/kg/d (18). The dose of NGIN in the present study is 7.93 mg/kg/d, indicating that the intake of juice enriched in AUR/NGIN might be more effective than that the p.o. administration of sole NGIN. These results indicated that the intake of juice enriched in AUR/NGIN might be more effective than the administration of sole AUR or sole NGIN. We will investigate in near future why the peel of *C. kawachiensis* has more potent neuroprotective ability than AUR or NGIN alone does.

Anyway, we are convinced that the peel of *C.
**kawachiensis** has neuroprotective ability. Because our recent studies revealed that the experimental diet containing the peel of *Citrus kawachiensis* has the ability 1) to ameliorate microglial activation, tau hyperphosphorylation, and suppression of neurogenesis in the hippocampus of senescence-accelerated mice (26); and 2) to suppress astrogial activation, tau phosphorylation, and inhibition of neurogenesis in the hippocampus of type 2 diabetic db/db mice (27), in addition to the aforementioned papers (4, 18). The accumulation of evidence has confirmed that inflammation, a physiological response to infection/injury, is the key contributor to the pathophysiological process of chronic diseases including asthma and various intracerebral diseases such as age-related neurological disorders, Alzheimer’s disease, multiple sclerosis, and brain tumors (28). The present findings taken together with the above findings and the results of a clinical trial (29) indicate that this Auraptene/NGIN-rich *C. kawachiensis* juice might be a functional food.

**Acknowledgments**

We would like to thank Ehime Beverage Inc. (Ehime, Japan) for supplying juice of *C. kawachiensis* and its peel residue for this study.

**Author contributions**

S.O. and Y.F. conceived and designed the experiments. S.O., M.K., T.K., Y.K., A.S., and M.N. performed the experiments. Y.A. and M.Y. contributed to the analysis and interpretation of the data. S.O. and Y.F. wrote the paper. All authors read and approved the final manuscript.

**Funding**

This work was supported by the Strategic Research and Developmental Project of Ehime Prefecture.

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