Preventive Effect of *Morinda citrifolia* Fruit Juice on Neuronal Damage Induced by Focal Ischemia

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It is known that the fruit juice of *Morinda citrifolia* (*M. citrifolia*, Noni, Rubiaceae) has various pharmacological effects such as antioxidant or anti-inflammatory activities, which may help the inhibition of ischemic neuronal damage. Here, we examined the effect of the fruit juice of *M. citrifolia* (Noni juice) on the brain damage caused by ischemic stress in mice. Noni juice was obtained from the mature fruit grown in Okinawa (about 1.5 l/4 kg of fruit; 100% Okinawa Noni juice (ONJ). Male ddY mice were supplied with 3% or 10% juice in the drinking water for 7 d, and compared to the control group. On the 7th day, mice were subjected to 2 h of middle cerebral artery occlusion (MCAO). Interestingly, the intake of juice reduced the infarct volume as analyzed by 2,3,5-triphenyltetrazolium chloride (TTC) staining on the 3rd day of MCAO when compared to the control group. Furthermore, we found that the neurological deficit scores (NDS) were decreased after the reperfusion in the juice-supplied mice. On the other hand, the intake of juice did not affect the expression levels of antioxidant such as Cu/Zn superoxide dismutase. The present study suggests that Noni juice may have a preventive effect against cerebral ischemic stress, while further studies are needed to explain the detailed mechanism.

Key words middle cerebral artery occlusion; ischemia; *Morinda citrifolia*; infarction; self-medication

Morinda citrifolia, also called Noni, has been extensively used in folk medicine in Polynesia, Tahiti, Southeast Asia, Australia and Hawaii.[1)] The fruits, roots, leaves, stems and bark are all used and it has been shown that they are effective against minimizing the symptoms of lifestyle-related diseases such as diabetes and hypertension, cancer and atherosclerosis.[1)] Furthermore, it has been demonstrated that Noni juice contains some antioxidative or anti-inflammatory ingredients.[1—3)]

It is known that oxidative stresses such as generation of damaging reactive oxygen species will lead to cell death under ischemic condition. In addition, it is reported that ischemic neuronal damage could be restored by vitamins and polyphenols which both have antioxidative properties.[4—6)] Furthermore, oxidative stress involves redox signaling to molecular mediators of inflammation pathways, which induce further cell damage. Thus, it is proposed that the antioxidative and anti-inflammatory properties of Noni juice may provide protective effect against the neuronal damage caused by ischemic stress.

In the past decade, several significant breakthroughs have occurred in the management of cerebral ischemia and consequently several new therapeutic strategies have been reported.[7—9)] However, still in Japan, cerebral ischemia is a major cause of morbidity and mortality, the third most common cause of death and the main cause for neurological disability among adults. Recently, from the point of view of "self-medication" or "preventive medicine," several dietary supplements are used in the prevention of life-style related diseases including cerebral ischemia. Here, we determined the protective effect of daily supplementation of Noni juice on ischemic neuronal damage.

MATERIALS AND METHODS

Animals Male ddY mice (5—6 weeks old) were obtained from SLC (Osaka, Japan). The animals were housed at a temperature of 23—24 °C with a 12 h light–dark cycle (lights on 8:00 a.m. to 8:00 p.m.). Food and water were available *ad libitum*. The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, adopted by the Japanese Pharmacological Society. In addition, all experiments were approved by ethical committee for animals in Kobe Gakuin University (approved number: A060601-10).

Morinda citrifolia The fruit juice of *M. citrifolia* was obtained from the mature fruit grown in Okinawa (100% Okinawa Noni juice (ONJ); about 1.5 l/4 kg of fruit), and stored at −20 °C until use. For an experiment, 100% ONJ was diluted by adding water to make 3% or 10% ONJ. Male ddY mice were supplied with 3% or 10% juice in their drinking water for 7 d, while the control group was only supplied with drinking water. The sham operation (sham) was also only supplied with drinking water. The percentage of drinking volume and amount of ingestion were calculated using the following formula: drinking volume (% of control)$=100\times$ $(\text{drinking volume/d/mouse})_{\text{each group}}/(\text{drinking volume/d/mouse})_{\text{water group}}$, amount of ingestion (% of control)$=100\times$ $(\text{amount of food ingestion/d/mouse})_{\text{each group}}/(\text{amount of food ingestion/d/mouse})_{\text{water group}}$.

Middle Cerebral Artery Occlusion (MCAO) and Reperfusion Seven days after intake of 3%, 10% ONJ or water, three groups of mice were subjected to 2 h of MCAO using a modification of the intraluminal filament technique.[10)] Mice were anesthetized with 2% isoflurane (Abbott Japan, Osaka, Japan) and maintained with 1% isoflurane. The rectal temperature was maintained at 37±0.5 °C with the

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use of a heating blanket (FH-100, Unique Medical, Osaka, Japan) that was feedback controlled by a rectal temperature probe (PTE-101, Unique Medical, Osaka, Japan) and small animals heat controller (ATC-101B, Unique Medical, Osaka, Japan). Physiological parameters (mean arterial blood pressure (MABP), heart rate (Hr), partial arterial pressure of O_2 (PaO_2), partial arterial pressure of CO_2 ($PaCO_2$), pH, and blood glucose (BG)) were measured before, during, and 30 min after MCAO by using sphygmomanometer (TK-370C; BrainScience idea, Osaka, Japan), i-STAT (300F; FUSO Pharmaceutical Industries, Osaka, Japan) and Glucose Pilot (Aventir Biotech, CA, U.S.A.). The left common carotid artery (CCA) and external carotid artery (ECA) underwent a midline pretracheal incision. The vagus nerves were separated carefully from the artery. CCA and ECA were ligated, and then the internal carotid artery (ICA) was isolated. The bifurcation of CCA was made through a small incision, and then a 8-0 nylon monofilament (Shirakawa, Fukushima, Japan) with a 4 mm tip coated in silicon resin (Xantopren Blue and Activator, Heraeus Kulzer, Germany) was introduced through a small incision and was advanced 9 mm along the ICA beyond the bifurcation of CCA, thus stopping the blood flow to the middle cerebral artery (MCA). After 2 h of ischemia, mice were re-anesthetized with isoflurane, and then the filament was withdrawn for blood reperfusion. The sham-operated mice were subjected to the procedure mentioned above without MCAO. The operative site was sutured and mice were allowed to awake from the anesthesia. We eliminated mice with brain hemorrhage at 3 d after MCAO. Thus, the final number of mice was as follows: sham group $n=5$; control group $n=12$; 3% ONJ group $n=7$; and 10% ONJ group $n=10$.

Measurement of Cerebral Blood Flow The relative cerebral blood flow (rCBF) was measured by laser Doppler flowmetry (LDF; TBF-LN1, Unique Medical, Osaka, Japan) to assess the adequacy of the vascular occlusion and reperfusion.[11—13] A laser Doppler probe (ALP-NC, Unique Medical, Osaka, Japan) inserted into the acrylic sheath was positioned over the left skull 2 mm posterior to the bregma and 6 mm to the left side of the midline. Baseline rCBF values measured before the occlusion were defined as 100%. The MCAO was documented by a decrease in rCBF to 40% of control values and rCBF was recovered to about 100% by reperfusion.

Measurement of the Volume of Ischemic Brain Injury Mice were euthanized by cervical dislocation on 3rd day of reperfusion. The brains were cut into 2-mm thick coronal slices (−2, 0, +2 and +4 mm from the bregma) using a brain slicer. The brain slices were then incubated in normal saline containing 2% 2,3,5-triphenyltetrazolium chloride (TTC; SIGMA, MO, U.S.A.) for 10 min at 37 °C. After staining, the brain slices were fixed with 4% paraformaldehyde (SIGMA, MO, U.S.A.) for 2 h and then they were stored in phosphate-buffered saline. Areas not stained red with TTC were considered to be lesions. The brain slices were scanned as pictures into the computer. Unstained areas (infarct areas) were measured using image analysis software (image J and Adobe Photoshop Elments 5.0). The infarct volume was calculated by infarct area and intensity (intensity=intensity of left hemisphere−intensity of right hemisphere).

Neurological Examination Neurological examination was performed using the neurological deficit score (NDS) as described previously with some modification.[14—16] Briefly, consciousness (0, normal; 1, restless; 2, lethargic; 3, stuporous; 4, seizures; and 5, death), walking (0, normal; 1, paw; 2, unbalanced walking; 3, circling; 4, unable to stand; and 5, no movement), limb tone (0, normal; 1, spastic; and 2, flaccid) and pain reflex was scored on 3rd day of reperfusion. In particular, NDS was assessed by the following behavior: restless=hyper locomotion; lethargic=moveless; stuporous=sleepy mode and short periods of responsibility can be achieved by intense stimulation; seizures=twitches and violent shaking of their limbs; paw=adduction of forelimb; unbalanced walking=can not walk straight or walking with a limp; circling=circling towards the paretic side; spastic=cramp in their limbs with violent movements; flaccid=inability to exert force with their muscle. Pain reflex was assessed using the tail flick test (MK-330B, Muromachi kikai, Tokyo, Japan) (Pain reflex=latency of 3rd day after MCAO−pre latency). A cut-off time of 10 s was used to prevent any injury to the tail.

Western Blot Analysis for Cu/Zn Superoxide Dismutase (SOD-1) Expression in Ipsilateral Cortex One day after reperfusion, mice were decapitated and brains were removed. Ipsilateral cortex was dissected, and homogenized with homogenize buffer (20 mM Tris–HCl, pH 7.5, 120 mM NaCl, 4% Tween-20, 2 mM β-mercaptoethanol, 1 mM Na_3VO_4, 5 mM benzamidine, 20 mM NaF, 1 mM *p*-nitrophenyl phosphate, 5 mM imidazole, 50 μg/ml trypsin inhibitor, 50 μg/ml leupeptin, 50 μg/ml aprotinin, 5 μg/ml pepstatin, 1 mM PMSF). After centrifugation at 15000 ***g***, the supernatant was collected and immediately lysed in SDS-sample buffer (50 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol), boiled and reduced with β-mercaptoethanol. Samples (50 μg) and molecular weight standards (BioRad, Hercules, CA, U.S.A.) were electrophoresed in 15% SDS-PAGE acrylamide gels and transferred onto nitrocellulose membranes (BioRad). The membranes were blocked for 2 h at 25 °C with 5% blocking agent (GE Healthcare, Tokyo, Japan) in sodium phosphate buffer containing 0.1% Tween 20, incubated with a rabbit anti-SOD-1 antibody (1 : 1000; Santa Cruz Biotechnology, CA, U.S.A.), and mouse anti-GAPDH antibody (1 : 1000; Chemicon, Temecula, CA, U.S.A.) overnight at 4 °C, and then with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (1 : 1000; KPL, Guildford, U.K.) or HRP-conjugated anti-mouse IgG (1 : 10000; KPL) for 1 h at 25 °C. All visualization of immunoreactive bands was performed using Light-Capture (AE-6981; ATTO, Tokyo, Japan) with an enhanced chemiluminescent substrate for the detection of horseradish peroxidase, ECL™ Western Blotting Analysis System (GE Healthcare). The signal intensity of immunoreactive bands was analyzed by use of Cs-Analyzer (Ver. 3.0; ATTO, Tokyo, Japan).

Statistical Analysis The infarct volume was analyzed using one-way ANOVA followed by the Sheffe test. Data are presented as mean±S.E. The data of NDS were analyzed using a Steel–Dwass' test of post hoc nonparametric multiple comparison tests. The differences were regarded as statistically significant when the p value was less than 0.05.

RESULTS

Change of Cerebral Blood Flow after Ischemia-Reperfusion The rCBF was measured by LDF. When the levels of rCBF before ischemia was 100%, it decreased to 60% during ischemia and recovered to about 100% by reperfusion (Fig. 1).

Physiological Variable at Baseline (before), during and at 30 min after MCAO Hr, MABP, BG, pH, $PaCO_2$ and PaO_2 were within normal physiological ranges in all animals at baseline, during MCAO, and during early reperfusion (Table 1).

Effect of ONJ on Water Intake, Ingestion and Body Weight The free intake of 3% or 10% ONJ for 7 d did not affect the water intake, ingestion, or body weight in comparison to the control group (Figs. 2A—C).

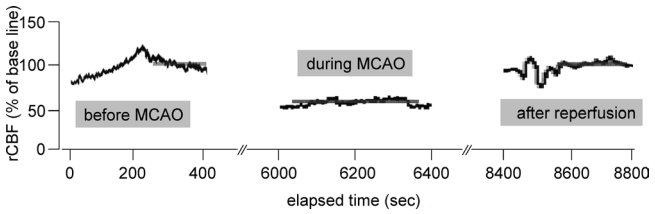

Fig. 1. Change of Cerebral Blood Flow after Ischemia-Reperfusion

The rCBF was measured using LDF to confirm adequate decreases in blood flow during the MCAO. The horizontal grey lines indicate the mean value of rCBF during the indicated periods.

Effect of ONJ on the Development of Infarction after Cerebral Ischemic Stress In the histological observation using the TTC stain, the control group showed clear infarction on 3rd day after cerebral ischemic stress (Fig. 3). On the other hand, the ONJ intake group definitely suppressed the development of infarction in every brain region as compared with the control group in a dose-dependent manner (Fig. 3). The infarct volume in the control group was 1200879±164985, in the 3% ONJ intake group it was 801833±230014 and in the 10% ONJ intake group it was 593784±186770. It is evident that the 10% ONJ intake group significantly suppressed the development of infarction as compared with the control group (Fig. 4).

Effect of ONJ on the Development of Behavioral Abnormalities after Cerebral Ischemic Stress On 3rd day after the cerebral ischemic stress, the measurement of NDS (median NDS (25th—75th percentile)) was performed in all groups. The NDS for the sham group was 0.7 (0.6—0.9), the control group was 4.2 (2.3—7.3), the 3% ONJ intake group was 1.2 (0.8—5.2) and the 10% ONJ intake group was 2.0 (1.0—2.5). It is clear that the behavioral abnormality was significantly developed in the control group as compared with the sham (Fig. 5). In contrast, the 10% ONJ intake group significantly suppressed the development of behavioral abnormality as compared with the control group (Fig. 5).

Effect of ONJ on SOD-1 Expression after Cerebral Ischemic Stress On 1st day after cerebral ischemic stress, SOD-1 expression levels in ipsilateral cortex were estimated

Table 1. Physiological Parameters for Mice with MCAO Treated with Water or 10% ONJ

Group	Before MCAO			During MCAO			30 min after MCAO		
	Water +sham	Water +MCAO	10% ONJ +MCAO	Water +sham	Water +MCAO	10% ONJ +MCAO	Water +sham	Water +MCAO	10% ONJ +MCAO
Hr (beat/min)	630.3 ±23.2	654.0 ±6.1	663.0 ±20.2	667.0 ±10.7	644.0 ±24.7	635.0 ±18.4	620.6 ±16.9	613.5 ±20.7	630.1 ±19.6
MABP (mm Hg)	63.6 ±1.7	65.8 ±2.5	70.2 ±3.0	71.6 ±2.2	73.4 ±4.9	68.1 ±2.3	71.0 ±2.0	70.4 ±4.9	68.2 ±2.4
BG (mg/dl)	207.6 ±12.9	205.6 ±9.8	200.4 ±12.4	207.9 ±6.4	213.3 ±14.8	193.6 ±14.5	215.9 ±12.6	260.4 ±13.2	233.1 ±14.3
pH	7.3 ±0.02	7.3 ±0.03	7.4 ±0.02	7.3 ±0.04	7.3 ±0.02	7.3 ±0.02	7.3 ±0.02	7.3 ±0.04	7.4 ±0.03
$PaCO_2$ (mm Hg)	42.0 ±0.9	38.4 ±0.9	39.1 ±1.7	33.9 ±1.2	42.5 ±1.8	39.4 ±2.4	36.0 ±0.9	38.7 ±1.7	35.8 ±1.5
PaO_2 (mm Hg)	29.8 ±2.1	32.3 ±1.2	32.6 ±1.8	32.7 ±2.2	35.1 ±3.4	28.4 ±1.8	31.6 ±2.0	37.4 ±2.0	37.5 ±1.1

Hr=heart rate; MABP=mean arterial blood pressure; BG=blood glucose, n=7—8.

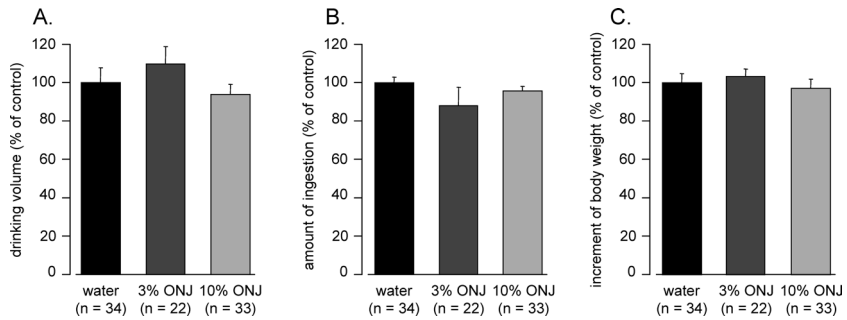

Fig. 2. Effect of ONJ on the Water Intake, Ingestion and Body Weight

The data were obtained from a daily dose (A, B) or the weight gain during 7 d (C). Control is the water group.

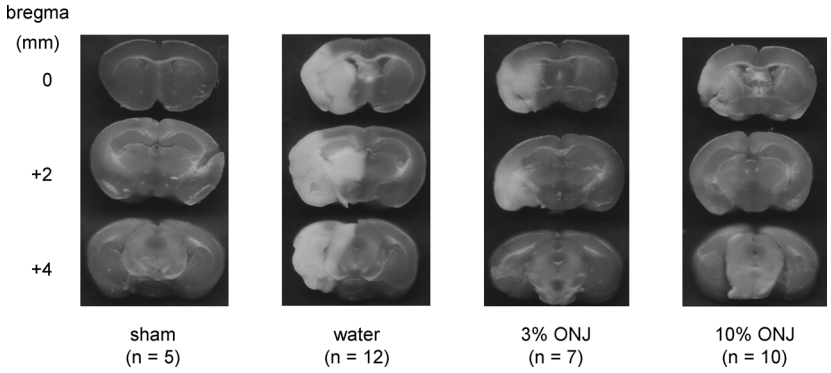

Fig. 3. Effect of ONJ on the Development of Infarction after Cerebral Ischemic Stress

Representative photographs of infarct volume at 0, +2, +4 mm from the bregma in the coronal section with 2,3,5-triphenyltetrazolium at 3 d after 2 h of MCAO.

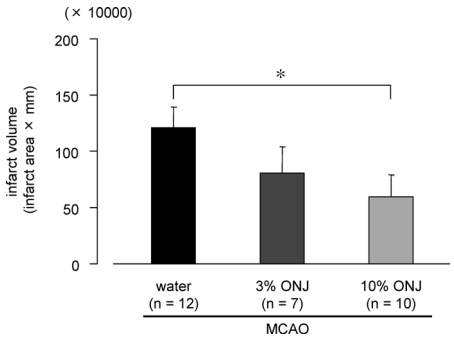

Fig. 4. Quantitative Analysis of the Infarct Volume after Cerebral Ischemic Stress

Results are expressed as the mean±S.E. $*p<0.05$.

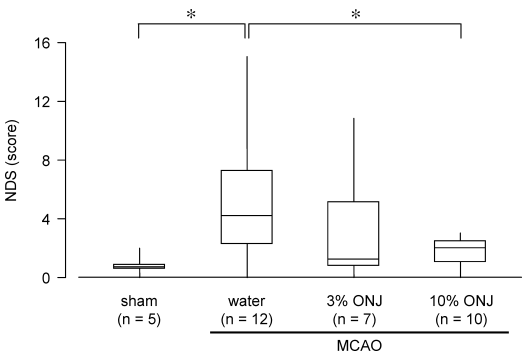

Fig. 5. Effect of ONJ on the Development of Behavioral Abnormalities after Cerebral Ischemic Stress

The neurological examination was performed using the NDS. Results are expressed as median (25% to 75%). $*p<0.05$.

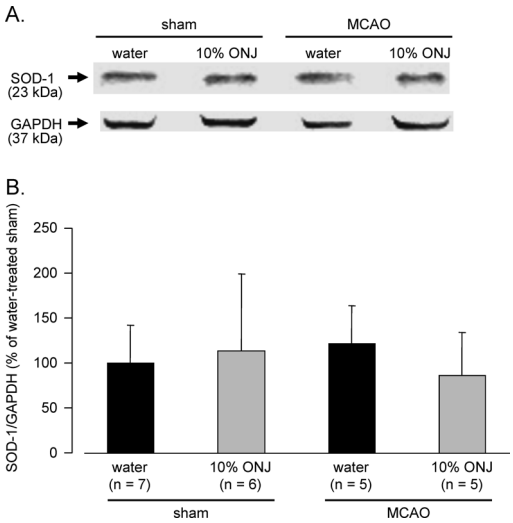

Fig. 6. Effect of ONJ on SOD-1 Expression after Cerebral Ischemic Stress

Representative photographs of Western blot analysis (A) and the relative SOD-1 expression levels (B) analyzed by determining the ratio of SOD-1/GAPDH (the endogenous standard protein) at 1 d after 2 h of MCAO.

by use of Western blot analysis. The ischemic stress did not alter SOD-1 expression levels at all (Figs. 6A, B). In addition, 10% ONJ also did not affect SOD-1 expression levels (Figs. 6A, B).

DISCUSSION

In this study, the cerebroprotective effect of intake of Noni juice on ischemic neuronal damage was clearly demonstrated using focal ischemia model mice. In the present focal ischemia, infarction was observed not only in cortex and striatum, which are involved in MCA territory, but also hippocampal region, which is not MCA territory. The development of infarction in hippocampus might be due to the secondary deleterious phenomena, such as spreading depolarization or expanding post-ischemic inflammation, following the anoxic depolarization- or breakdown of ion homeostasis-induced cell death in core region.[17] As shown in Figs. 3 and 4, the development of infarction was suppressed by ONJ in a dose-dependent manner. Furthermore, Fig. 5 indicates that the increment of NDS after MCAO was also significantly decreased by the daily intake of ONJ for 7 d prior to MCAO. In addition, we found that the intake of ONJ only for 3 d also could suppress the development of infarction significantly (data not shown). As shown in Fig. 2, the daily intake of drinking water or of feeding stuff and the weight change were not affected by the free intake ONJ at all, let us to believe that the neuroprotective effect of Noni juice was not merely due to dietary factors. These results suggest that Noni juice contains some neuroprotective ingredients. Surprisingly, ONJ completely suppressed the development of ischemic infarction in every brain region (*i.e.*, including ischemic core and penumbra region). It is possible that ONJ provides the resistance against "ischemic stress" *per se*, such as generation of free radicals, decrement of ATP or increment of intracellular calcium concentration.[18,19] In this study,

we believe that ONJ treatment showed the "preventive effect" against the development of ischemic core itself, but not "recovery effect" from the cell death in ischemic core.

It is well known that oxidative stress such as generation of free radical participates in the development of neuronal damage after ischemic stress.[20] Antioxidants such as edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one), a strong free radical scavenger, is known to have neuroprotective effects on ischemic stroke and is used for treatment of patients with acute brain infarction.[8,9] In fact, the ingredients contained in Noni juice which demonstrate an antioxidative effect have been identified.[2,3] The prior intake of ONJ might be providing an antioxidative effect and thus thought to be able to inhibit ischemic neuronal damage. However, since ONJ was provided prior to the onset of ischemic stress, any ingredients in ONJ were seemed not to scavenge oxidants such as free radicals after ischemic stress directly, suggest that some indirect effect such as the increment of expression levels of preventive antioxidants (*e.g.* SOD or glutathione-peroxidase) or neurotrophic factors (*e.g.* brain-derived neurotrophic factor (BDNF) or nerve growth factor) might be involved in the neuroprotective effect of ONJ. Our results, indicating that SOD-1, a well-known antioxidant, was not affected by ONJ (Fig. 6), reduce the possibility of antioxidant-mediated neuroprotective effect of ONJ. Further study will be needed to explain the effect of ONJ.

Previously, Noni juice has been reported to have a brought range of nutritional value. Wang *et al.* reported that intake of 10% of Tahitian Noni juice for 12 d inhibited the lipid hyperoxidation in the liver.[1] They also reported several effects of Noni juice including antitumor, anti-inflammatory and antibacterial effects.[1] We believe that this is the first report to show the neuroprotective effect of Noni juice against focal ischemia as there is no reported regarding neuroprotective effect of Noni juice.

In conclusion, we have shown that the ONJ significantly prevents the development of ischemic neuronal damage in a dose-dependent manner when provided in the drinking water before the ischemic stress. Recently, self-medication with dietary supplements has become increasingly popular for health-maintenance and prevention of life-style related diseases including cerebral ischemia. Our results may help the development of preventive medicine against the cerebrovascular diseases.

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