Anti-oxidative nutrient rich diet protects against acute ischemic brain damage in rats
Taijun Yunoki, Kentaro Deguchi, Yoshio Omote, Ning Liu, Wentao Liu, Nozomi Hishikawa, Toru Yamashita, and Koji Abe

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Abbreviations used:
AGES, advanced end glycation products; ANOVA, analysis of variance; AO, anti-oxidative; BP, blood pressure; CV, cresyl violet; DBP, diastolic blood pressure; h, hour; Iba-1, ionized calcium-binding adapter molecule 1; IR, ischemic-reperfusion; MCA, middle cerebral artery; MC, methylcellulose; MCP-1, monocyte chemotactic protein-1; min, minute; OCT, optimal cutting temperature; PBS, phosphate buffered saline; rCBF, regional cerebral blood flow; ROS, reactive oxygen species; SBP, systolic blood pressure; Sirt-1, sirtuin-1; tMCAO, transient middle cerebral artery occlusion; TNFα, tumor necrosis factor α; 4-HNE, 4-hydroxynonenal; 8-OHdG, 8-hydroxy-2-deoxyguanosine; W, week.

Abstract
We evaluated the neuroprotective effects of an anti-oxidative nutrient rich enteral diet (AO diet) that contained rich polyphenols (catechins and proanthocyanidins) and many other anti-oxidative ingredients. Wistar rats were treated with either vehicle, normal AO diet (containing 100 kcal/100 ml, catechin 38.75 mg/100 ml and proanthocyanidin 19 mg/100 ml, 1 ml/day), or high AO diet (containing 10 times the polyphenols of the normal AO diet) for 14 days, and were subjected to 90 minutes of transient middle cerebral artery occlusion. The AO diet improved motor function, reduced cerebral infarction volume, and decreased both peroxidative markers such as 4-hydroxynonenal, advanced glycation end products, 8-hydroxy-2-deoxyguanosine and inflammatory markers such as monocyte chemotactic protein-1, ionized calcium-binding adapter molecule-1, and tumor necrosis factor-α. Our study has shown that an AO diet has neuroprotective effects through both anti-oxidative and anti-inflammatory mechanisms, indicating that nutritional control with polyphenols could be useful for patients with acute ischemic stroke.
1. Introduction

Because ischemic stroke is a major cause of neurological disorders and death in the world (Truelsen et al., 2007), effective therapies for preventing cell death by cerebral blood flow restoration and neuroprotection are urged in the acute stage. Oxidative stress is one important factor that can aggravate ischemic brain damage during such an acute stage (Abe et al., 1995; Hayashi et al., 1999). After reperfusion of acute ischemic stroke, reactive oxygen species (ROS) are excessively generated, which promote apoptotic cell death through protein, lipid, and DNA peroxidation (Schaller and Graf, 2004; Zhang et al., 2004). Inflammation also plays an important role in acute ischemic stroke through activating macrophage and tumor necrosis factor-α (TNF-α) (Dirmagl et al., 1999). Thus, anti-oxidative and anti-inflammatory actions could be an indispensable strategy for ameliorating ischemic brain damage (Sun et al., 2002; Villegas et al., 2004).

We have previously reported that a free radical scavenger, edaravone, strongly reduced brain edema after cerebral ischemia in rats (Abe et al., 1988), which is not only in clinical use for stroke patients in Japan, but also currently showing good clinical effects in Europe (Kaste et al., 2013). Meanwhile, polyphenols are contained in many foods such as green tea (catechins), grape seed (proanthocyanidins), and red wine (resveratrol), which also show both anti-oxidative and anti-inflammatory effects (Landete, 2012). Polyphenols have been shown partly to prevent coronary heart disease, dementia, cancer, and arteriosclerosis (Ghosh and Scheepsens, 2009). In the present study, therefore, we investigated whether an anti-oxidative nutrient rich enteral diet (AO diet) therapy could reduce ischemic rat brain damage through anti-oxidative and anti-inflammatory mechanisms after transient middle cerebral artery occlusion (tMCAO) in rats.

2. Results

2.1. Physiological parameters

Body weights were not significantly different among the three diet groups (vehicle, 270.5 ± 12.2 g; normal AO diet, 280.5 ± 4.6 g; high AO diet, 273.7 ± 14.7 g). The time-dependent changes in systolic blood pressure (SBP), diastolic blood pressure (DBP), and regional cerebral blood flow (rCBF) are shown in Table 1. SBP (vehicle, 124.4 ± 7.6 mmHg; normal AO diet, 121.5 ± 7.8; high AO diet, 123.0 ± 3.2) and DBP (vehicle, 95.7 ± 4.4 mmHg; normal AO diet, 91.9 ± 11.2; high AO diet, 91.8 ± 6.8) before tMCAO showed no significant difference between the three diet groups. SBP 24 h after reperfusion also showed no significant difference between the three diet groups (vehicle,
151.2 ± 6.5 mmHg; normal AO diet, 120.5 ± 12.3; high AO diet, 135.4 ± 4.2). However, DBP 24 h after reperfusion was significantly lower in the high AO diet group than the vehicle group (vehicle, 111.2 ± 11.2 mmHg; normal AO diet, 99.5 ± 6.3; high AO diet, 88.4 ± 9.5, \( p < 0.05 \) versus vehicle).

Although rCBF rates among the three diet groups were not significantly different, rCBF seemed to be higher in the two AO diet groups (vehicle, 79.5 ± 36.1%; normal AO diet, 82.6 ± 20.9; high AO diet, 91.2 ± 27.3; Figure 2).

2.2. Motor function and infarct volume

As compared to the vehicle group (2.4 ± 0.6), Bederson scores showed an improvement in normal and high AO diet groups (normal AO diet, 1.7 ± 0.7; \( p < 0.05 \); high AO diet, 1.6 ± 0.7, \( p < 0.01 \); Figure 3A).

Compared with the vehicle group (75.9 ± 12.1 mm\(^3\)), the AO diet groups showed a reduction in the infarct volume (normal AO, 67.5 ± 14.6 mm\(^3\); \( p = \text{n.s.} \) versus vehicle) with significant reductions in the high AO diet group (54.6 ± 10.3 mm\(^3\); \( p < 0.05 \) versus vehicle; Figure 3B). The examples of CV sections are shown in Figure 3C.

2.3. Oxidative stress markers

Typical immunohistochemical stains for 4-hydroxynonenal (4-HNE), advanced end glycation products (AGEs), and 8-hydroxy-2-deoxyguanosine (8-OHdG) at the peri-ischemic areas are shown in Figure 4. Compared with the vehicle group (5.3 ± 32.5/mm\(^2\); AGEs, 243.2 ± 30.1; 8-OHdG, 147.5 ± 24.7), the normal AO diet group significantly reduced the number of positive cells for each of the three antibodies (4-HNE, 148.2 ± 31.7/mm\(^2\); AGEs, 157.3 ± 21.1; 8-OHdG, 97.2 ± 27.8; \( p < 0.01 \) versus vehicle). The high AO diet group showed further reductions in the number of positive cells in 4-HNE (114.3 ± 26.2/mm\(^2\); \( p < 0.01 \) versus vehicle), AGEs (121.8 ± 32.7 mm\(^2\); \( p < 0.01 \) versus vehicle), and 8-OHdG (74.4 ± 12.0/mm\(^2\); \( p < 0.01 \) versus vehicle).

2.4. Inflammatory markers

Typical immunohistochemical stains for monocyte chemotactic protein-1 (MCP-1), ionized calcium-binding adapter molecule 1 (Iba-1), and TNFα at the peri-ischemic areas are shown in Figure 4. Compared with the vehicle group (MCP-1, 186.3 ± 35.4/mm\(^2\); Iba-1, 127.1 ± 20.4; TNFα, 121.7 ± 28.4), the normal AO diet group showed a slight reduction in the number of positive cells for MCP-1 (177.1 ± 34.2/mm\(^2\)), Iba-1 (116.4 ± 14.3), and TNFα (101.8 ± 19.2). On the other hand, the high AO diet group showed significant reductions in the number of positive cells for all three inflammatory markers (MCP-1, 131.1 ± 39.2/mm\(^2\); Iba-1, 77.3 ± 21.4; TNFα, 79.9 ±
30.0; \( p < 0.05 \) versus vehicle).

Staining for Sirt-1 also is shown in Figure 4 (bottom). Although there was a tendency toward an increase in Sirt-1 staining, the change was not significant among the three diet groups (vehicle, 157.0 ± 33.3/ mm²; normal AO diet, 161.2 ± 52.4; high AO diet, 189.0 ± 37.4).

3. Discussion

It appeared that an AO diet improved the motor function (Figure 3A), reduced the infarct volume (Figure 3B and C), and reduced oxidative (Figure 4) and inflammatory (Figure 5) markers, except for Sirt-1 (Figure 5, bottom). Oxidative stress plays an important role in ischemic stroke (Abe et al., 1995). ROS were generated after tMCAO, which increased brain injury by direct damage of cellular lipids, proteins, and DNA or indirect damage by affecting cellular signaling and gene regulation (Schaller and Graf, 2004; Zhang et al., 2004). Normal and high AO diets contained incremental doses of anti-oxidants such as catechins, proanthocyanidins, vitamin C, vitamin E, zinc, copper, selenium, and chromium, and thus ameliorated the oxidative stress (Ghosh and Scheepens, 2009).

In the present study, 4-HNE, AGEs, and 8-OHdG were selected as oxidative stress markers. 4-HNE is one lipid peroxidative product that was produced by ischemic-reperfusion (IR) injury in brain (Eaton et al., 1999). AGEs are end products of protein peroxidation related to ischemic stroke (Zimmerman et al., 1995). 8-OHdG is a well-known oxidative DNA marker as previously described (Lukic-Panin et al., 2007; Zhang et al., 2004). A reduction of peroxidative damages to lipids, proteins, and DNA following ischemic stroke (Fig. 3).

In the pathogenesis of ischemic stroke, the inflammatory response is another important factor in the deterioration and primary ischemic damage during the later stage (Dirnagl et al., 1999). Post ischemic inflammation disrupts neurovasculature and allows inflammatory cells to enter into the brain, which contributes to neuronal injury (del Zoppo, 2006). Polyphenols also have been shown to have anti-inflammatory effects (Ghosh and Scheepens, 2009), which were confirmed in the present study with staining for MCP-1, Iba-1, and TNF-\( \alpha \) (Figure 5). MCP-1 activates microglia to be Iba-1 positive, and increases infiltration of macrophages to promote inflammation through TNF-\( \alpha \) production. It seemed that an AO diet inhibited such inflammatory changes in the rat brain following tMCAO (Fig. 4).

Sirt-1 is known to be a longevity gene with various effects including anti-oxidation, anti-inflammation, glycolytic metabolism, lipid metabolism, and protection
against brain damage from ischemia (Hernandez-Jimenez et al., 2013; Potente and Dimmeler, 2008). Resveratrol is a polyphenol that activates Sirt-1 (Villalba and Alcain, 2012). It makes sense to find that the present study did not show a significant Sirt-1 activation (Figure 5, bottom) because the preset AO diet contained only catechins and proanthocyanidins but not resveratrol. The present study also showed that catechins and proanthocyanidins improved ischemic brain damage without activating the Sirt-1 pathway (Figure 5).

In conclusion, our present study demonstrated that an AO diet showed an advantage in reducing oxidative and inflammatory brain damage following tMCAO, while improving both clinical symptoms and infarct volume. Increased improvement of cerebral infarction in high AO groups compared with the normal AO groups suggests a main anti-oxidative effect greater than vitamins or minerals. The present study suggests that an AO diet has potential to improve ischemic stroke damage through the anti-oxidative and anti-inflammatory effects of polyphenols.

4. Experimental Procedure
4.1. Diet

All rats had free access to the diet (MF, Oriental Yeast, Tokyo, Japan). The vehicle diet contained 359 kcal, 23.1 g protein, 5.1 g fat, vitamins (C, 4 mg; E, 9.1 mg; A, 1283 IU; B1, 2.05 mg; B2, 1.10 mg; B6, 0.87 mg; B12, 5.5 μg; D, 137 IU; K, 8 μg; niacin, 0.04 mg; pantothenic acid, 2.45 mg; and folic acid, 170 μg), and minerals (Na, 190 mg; K, 900 mg; Ca, 1070 mg; Mg, 240 mg; P, 830 mg; Fe, 10.6 mg; Mn, 4.84 mg; Zn, 4.89 mg; Cu, 0.78 mg) per 100 g. We produced the following experimental diets: vehicle diet; a normal dose anti-oxidative nutrient rich enteral diet (normal AO diet); and a high dose AO diet (high AO diet). The vehicle diet consisted of a daily dose of 0.5% methylcellulose (MC) plus glucose (100 kcal/100 mL) without any electrolytes or polyphenols. The normal AO diet was supplemented with a daily dose of 0.5% MC and a small amount of polyphenol (catechin 38.75 mg/100 mL, Taiyo Kagaku, Yokkaichi, Japan; proanthocyanidin 19 mg/100 mL, Kikkoman, Noda, Japan), and the high AO diet was supplemented with a daily dose of 0.5% MC and 10 times the polyphenols (catechin 387.5 mg/100 mL, proanthocyanidin 190 mg/100 mL) of the normal AO diet. The normal and high AO diets shared common components such as glucose (100 kcal), protein (5 g), fat (2.8 g), carbohydrate (14.0 g), vitamins (C, 100 mg; E, 5.0 mg; A, 233 IU; B1, 0.18 mg; B2, 0.2 mg; B6, 0.30 mg; B12, 0.32 μg; D, 40 IU; K, 8 μg; niacin, 1.6 mg; pantothenic acid, 0.96 mg; and folic acid, 38 μg), and minerals (Na, 130 mg; Cl, 80 mg; K, 136 mg; Ca, 63 mg; Mg, 31 mg; P, 88 mg; Fe, 0.88 mg; I, 13 μg; Mn, 0.335 mg; Zn,
In addition to the above diet delivered by oral gavage (1 kcal/day), rats in all three groups consumed a pellet diet (about 20 g/day = 72 kcal/day) with sufficient energy and basic nutrients (protein, fat, carbohydrate).

4.2. Animals and focal cerebral ischemia

Nine-week-old male Wistar rats were obtained from Japan SLC (Hamamatsu, Japan). Rats were maintained for a week in a temperature-regulated room (21–23 °C) with a 12-h light/dark cycle. All experimental procedures were approved by the Animal Committee of the Okayama University Graduate School of Medicine (OKU-2013378). At age 10 weeks, the animals were divided into three groups: vehicle diet (1 mL/day), normal AO diet (1 mL/day), and high AO diet (1 mL/day) groups (n = 6 in each groups). A small burr hole (1.5 mm in diameter) was drilled at 2 mm posterior and 5 mm lateral to the bregma for the measurement of regional cerebral blood flow (rCBF). Body weight, blood pressure (BP), and pulse rate were measured before tMCAO, and 24 h after reperfusion; measurements for BP and pulse rate were performed using a tail-cuff (Softron BP98A, Tokyo, Japan).

From age 10 weeks, drugs were administered by oral gavage every day for 14 days. At age 12 W, animals were anesthetized with a nitrous oxide/oxygen/isoflurane mixture (69%/30%/1%) during surgery with an inhalation mask. The right middle cerebral artery (MCA) was occluded by insertion of a 4-0 surgical nylon thread with silicon coating through the common carotid artery as described previously (Deguchi et al., 2012). Body temperature was maintained at 37 ± 0.3°C using a heating pad during the surgical treatment. After 90 min of tMCAO, the filament was gently removed. rCBF was measured using a laser-Doppler flowmeter (FLO-C1; Omegawave, Tokyo, Japan) before and during tMCAO, just after reperfusion, and 24 h after reperfusion.

Twenty-four hours after reperfusion, clinical scores were measured using Bederson scores: 0, no observable deficit; 1, forelimb flexion; 2, decreased resistance to lateral push; 3, same behavior as grade 2 with circling (Bederson et al., 1986). Rats were sacrificed under deep anesthesia with pentobarbital (10 mg/250 g rat, i.p.). Rats were then transcardially perfused with heparinized saline, followed by 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.2). The whole brain was removed and immersed in the same fixation for 1 d at 4°C and washed with PBS. The tissues were transferred into graded sucrose solution of 10%, 20%, and 30%, sequentially, then embedded for optimal cutting temperature (OCT) in powdered dry ice, and stored at −80°C until use. Twenty micrometer-thick coronal brain sections were cut.
in a cryostat at −18°C and mounted on a silane-coated glass slide.

4.3. Histology and immunohistochemistry

For evaluation of the infarct volume, brain sections were stained with cresyl violet (CV) and were then examined by microscopy (SZX-12; Olympus Optical, Tokyo, Japan). The sections were made at 2, 0, −2, −4, −6 mm from the bregma. Infarct volumes were measured in five sections by pixel counting using a computer program for Photoshop CS5, and the volume was calculated.

We performed immunohistochemistry for 4-HNE, AGEs, 8-OHdG, MCP-1, Iba-1, TNF-α, and Sirt-1. After fixation with 4% formaldehyde, sections were incubated in 0.3% hydrogen peroxidase/methanol for 10 min to block endogenous peroxidase activity and incubated with bovine serum albumin for 1 h. Sections were then incubated at 4°C overnight with primary antibody for 4-HNE (1:50; MHN-100P; JalCA, Shizuoka, Japan), AGEs (1:200; Transgenic, Kobe Japan), 8-OHdG (1:50; MOG-020; JalCA, Shizuoka, Japan), MCP-1 (1:50; ab7202; Abcam Cambridge, UK), Iba-1 (1:100; ab5076; Abcam Cambridge, UK), TNF-α (1:40; AF-510-NA; R&D Systems; Abingdon, UK), and Sirt-1 (1:500; sc-15404; Santa Cruz, Santa Cruz, CA, USA). The next day, the sections were incubated with the following biotinylated secondary antibodies for 2 h at room temperature: biotinylated anti-mouse monoclonal antibody (1:500; Vector Laboratories) for 4-HNE, AGEs, and 8-OHdG; biotinylated anti-goat monoclonal antibody (1:500; Vector Laboratories) for TNF-α and Iba-1; and biotinylated anti-rabbit monoclonal antibody (1:500; Vector Laboratories) for MCP-1 and Sirt-1. The sections were then incubated with avidin-biotin-peroxidase complex (Vectastain ABC kit; Vector Laboratories) for 30 min and visualized with diaminobenzidine tetrahydrochloride.

4.4. Statistical analysis

All results are presented as mean ± SD. Statistical analyses were performed using analysis of variance (ANOVA) and Tukey–Kramer post-comparison. Differences with a probability value of \( p < 0.05 \) were considered statistically significant.

Acknowledgments

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References


Zhang, W., Sato, K., Hayashi, T., Omori, N., Nagano, I., Kato, S., Horiuchi, S., Abe, K.,

Figure legends

Figure 1. The three experimental rat groups showing vehicle group (0.5% methylcellulose plus glucose 250 mg/1 mL/day, \( n = 6 \)), normal AO diet group (AO diet 1 mL/day, \( n = 6 \)), and high AO diet group (\( n = 6 \)). All groups were sacrificed 24 h after 90 min of transient middle cerebral artery occlusion (tMCAO).

Figure 2. Effect of normal and high AO diets on regional cerebral blood flow before, during, just after, and 24 hours after 90 min of transient MCAO. There were no significant differences among vehicle, normal, and high AO groups (\( n = 6 \)).

Figure 3. Bederson scores 24 h after reperfusion of the vehicle, normal AO and high AO groups (A), infarct volumes (B), and cresyl violet stains of coronal sections (C). Note that Bederson scores were significantly improved in the normal and high AO diet groups (*\( p < 0.05 \), **\( p < 0.05 \) versus vehicle) and infarct volumes were significantly reduced in the high AO diet group (*\( p < 0.05 \) versus vehicle).

Figure 4. Immunohistochemistry for 4-HNE, AGEs and 8-OHdG at the peri-infarcted area in the vehicle, normal and high AO diet groups (left), and quantitative analyses of positive cells (right). The number of positive cells was significantly smaller in the normal and high AO diet groups (**\( p < 0.01 \) versus vehicle, scale bar 200 μm).

Figure 5. Immunohistochemistry for MCP-1, Iba-1, TNFα and Sirt-1 at the peri-infarcted area in the vehicle, normal and high AO diet groups (left), and quantitative analyses of positive cells (right). The number of positive cells for MCP-1, Iba-1, and TNFα was significantly smaller than vehicle (*\( p < 0.05 \), **\( p < 0.01 \), scale bar 200 μm).
### Table 1

time-dependent changes in systolic blood pressure (SBP), diastolic blood pressure (DBP), and rCBF

<table>
<thead>
<tr>
<th></th>
<th>before tMCAO</th>
<th>during tMCAO</th>
<th>just after reperfusion</th>
<th>24h after repurfusion</th>
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<td>Vehicle</td>
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<td><strong>Diastolic blood pressure (mmHg)</strong></td>
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<tr>
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<td><strong>Regional cerebral blood flow(%)</strong></td>
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<td>89.0±14.5</td>
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</table>

Time dependent change in systolic blood pressure, diastolic blood pressure and rCBF.
Diastolic blood pressure of high AO at 24h after the reperfusion was significantly decreased (* p < 0.05 vs vehicle).
Figure 1

Vehicle 9w

10w

Vehicle 1ml/day

12w

90 min
tMCAO

24h

†

Normal AO

Normal AO diet 1ml/day
tMCAO

†

High AO

High AO diet 1ml/day
tMCAO

†

Burr hole
Figure 2

The graph shows the relative cerebral blood flow (rCBF) as a percentage of base control at different time points:
- **before tMCAO**
- **during tMCAO**
- **just after reperfusion**
- **24h after reperrfusion**

The x-axis represents the time points, and the y-axis represents the rCBF (% of base control). The graph compares different treatments:
- **Vehicle**
- **Normal AO**
- **High AO**

The data points indicate a significant decrease in rCBF during tMCAO, followed by an increase in rCBF after reperfusion.
Figure 3

A

B

C

Vehicle Normal AO High AO

Vehicle Normal AO High AO

Vehicle Normal AO High AO
Figure 4

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>Normal AO</th>
<th>High AO</th>
<th>Number of positive cells (/1mm$^2$)</th>
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<td>8-OHdG</td>
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<td><img src="8-OHdG_bar_graph.png" alt="Bar graph" /></td>
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**Note:** The bar graphs indicate statistical significance with **asterisks** indicating a p-value of less than 0.01.
Figure 5

<table>
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<th>Protein</th>
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<th>High AO</th>
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</tbody>
</table>

Number of positive cells (/1mm²)

- MCP-1: Vehicle, Normal AO, High AO
- Iba-1: Vehicle, Normal AO, High AO
- TNFα: Vehicle, Normal AO, High AO
- Sirt-1: Vehicle, Normal AO, High AO

* p < 0.05
** p < 0.01