Figure legends

Fig. 1. Time-dependent profiles of microRNA expression in gerbil hippocampus

Hierarchical clustering of miRNAs detected in the hippocampal CA1 region showed four distinct clusters for neuroprotection and protein expression. Each column represents a single sample, and each row represents a single miRNA. Black squares represent median level of miRNA, and green or red squares represent lower or higher levels than the median level of miRNA expression, respectively (A). Clusters B, C, D, and E contained microRNAs related to neurogenesis, 43kDa transactivation response DNA-binding protein.(TDP43), fused in sarcoma/translocated in liposarcoma (FUS/TLS), and heat shock protein 70 (HSP70), respectively. tCCAO: transient common carotid artery occlusion. Mmu-miR: *Mus musculus* (mouse) micro RNA; oan-let-7b: *Ornithorhynchus anatinus* (platypus) let-7d microRNA; sha-miR-24: *Sarcophilus harrisii* (Tasmanian devil) microRNA 24.

Fig. 2. Mmu-miR-15a-5p expression during neuroprotection

From the signal strength of 820 ± 22 in sham control (SC) brains (gray circle), the signal strength slowly but progressively increased in the CA1 region after 2 min-tCCAO for one dose (open diamonds) and three doses (filled squares). Note the higher strength after three doses versus one dose (*p<0.05 vs SC, **p<0.01 vs SC)

Fig. 3. MicroRNA changes for FUS/TLS and TDP43

MicroRNA changes from baseline SC brains (gray circles) for FUS/TLS (A, C) and TDP43 (B, D). The signal strength of mmu-miR-125b-5p and mmu-miR-132-5p showed a similar increasing

pattern in both groups of 2 min-tCCAO for one dose (open diamonds) and three doses (filled squares) (*p<0.05 vs SC) (A, C). In contrast, the peak time of sha-miR-24 and oan-let-7b-3p accelerated to 1 d from three doses (filled squares) to one dose (open diamonds), then gradually returned until 6 M. (*p<0.05 vs SC, **p<0.01 vs SC) (B, D)

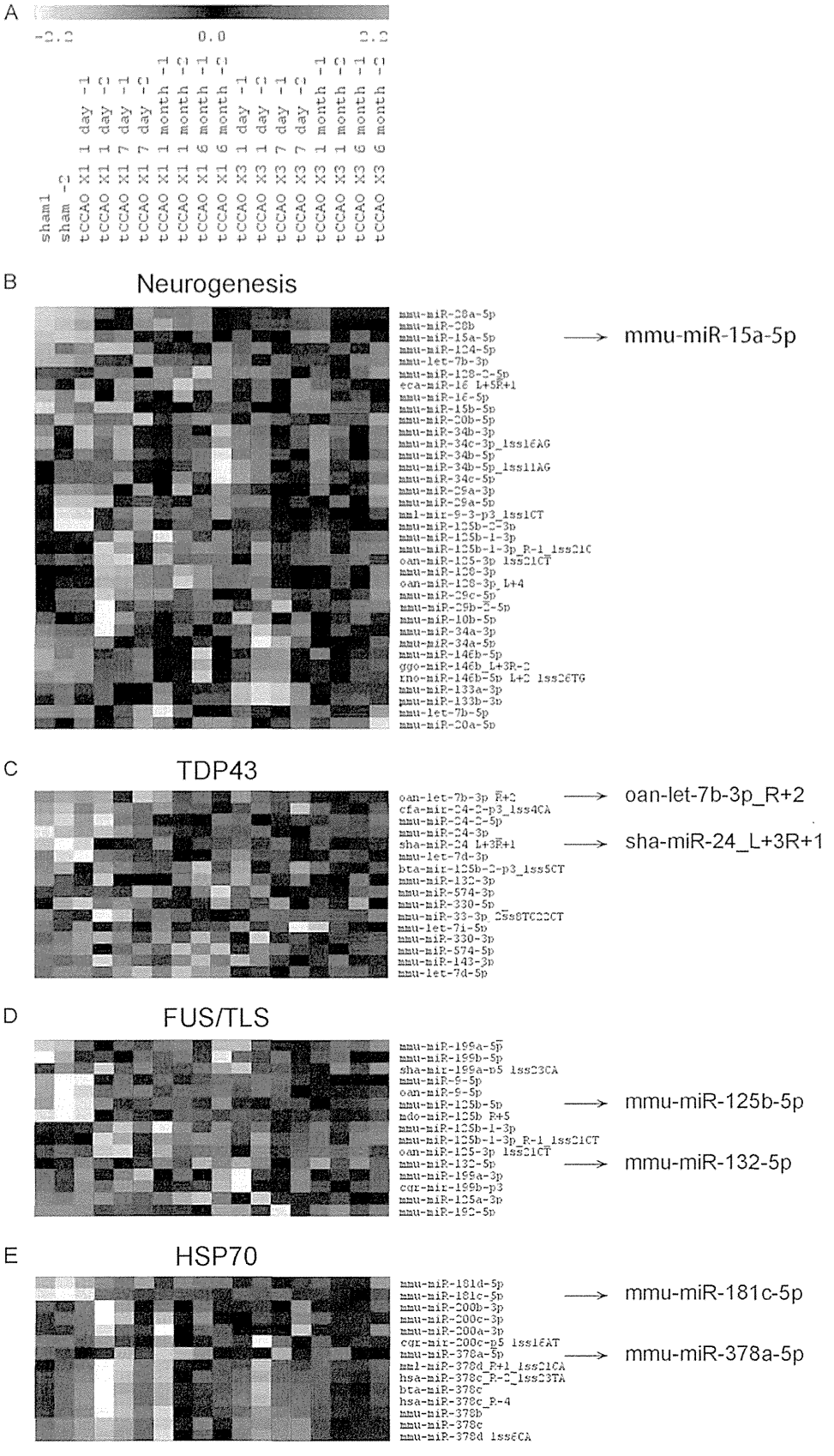
Fig. 4. MicroRNA changes for HSP70

Note the peak time acceleration to 1 d and 7 d from one dose (open diamonds) to three doses (filled squares), with a gradual return to 6 M in mmu-miR-181c-5p and mmu-miR-378a-5p (*p<0.05 vs SC, **p<0.01 vs SC, SC=gray circle)

Table 1 - MicroRNAs targeted by the transient ischemia-induced microRNA

miRNA	*Number of targets*	*Target Sequence (5' to 3')*
mmu-miR-15a-5p	337	UAGCAGCACAUAAUGGUUUGUG
sha-miR-24_L+3R+1	1986	AUCUGGCUCAGUUCAGCAGGAAC
oan-let-7d-3p_R+2	8	CUAUAUAGCCUACUGCCUUCCU
mmu-miR-125b-5p	354	UCCCUGAGACCCUAACUUGUGA
mmu-miR-132-5p	371	AACCGUGGCUUUCGAUUGUUAC
mmu-miR-181c-5p	448	AACAUUCAACCUGUCGGUGAGU
mmu-miR-378a-5p	1074	CUCCUGACUCCAGGUCCUGUGU

Mmu-miRNA: *Mus musculus* (mouse) microRNA; sha-miR: *Sarcophilus harrisii* (Tasmanian devil) microRNA; oan-let-7d: *Ornithorhynchus anatinus* (platypus) let-7d microRNA.



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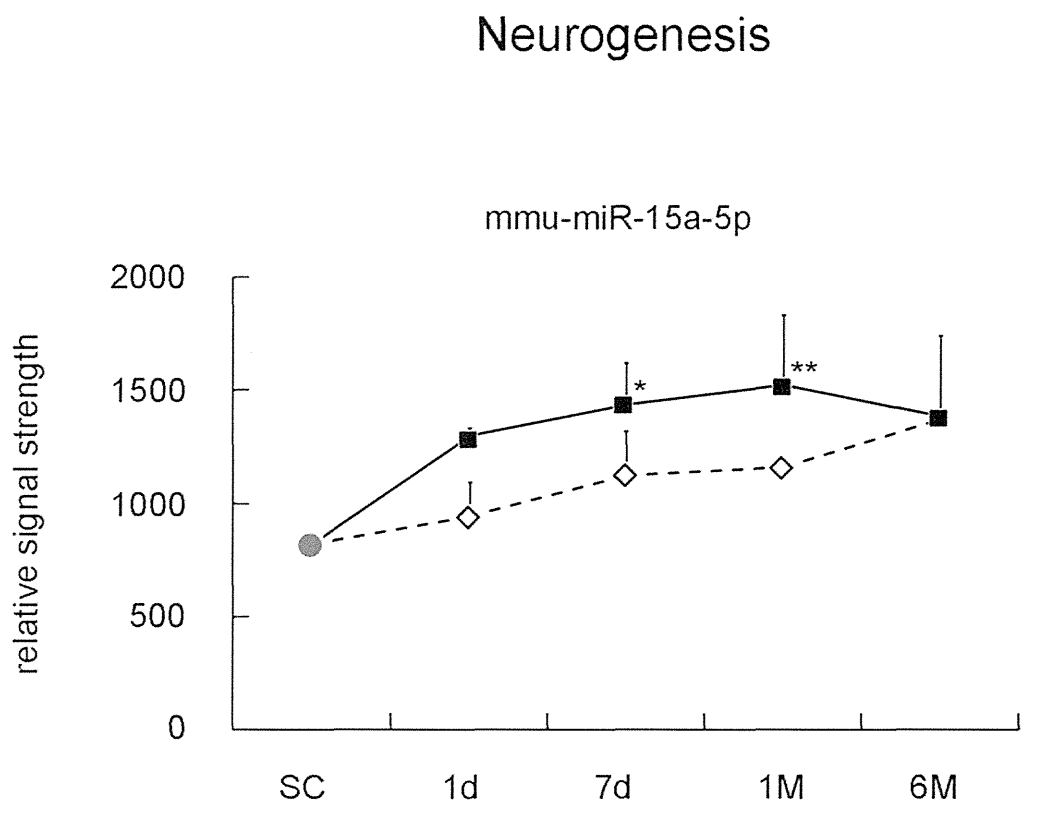

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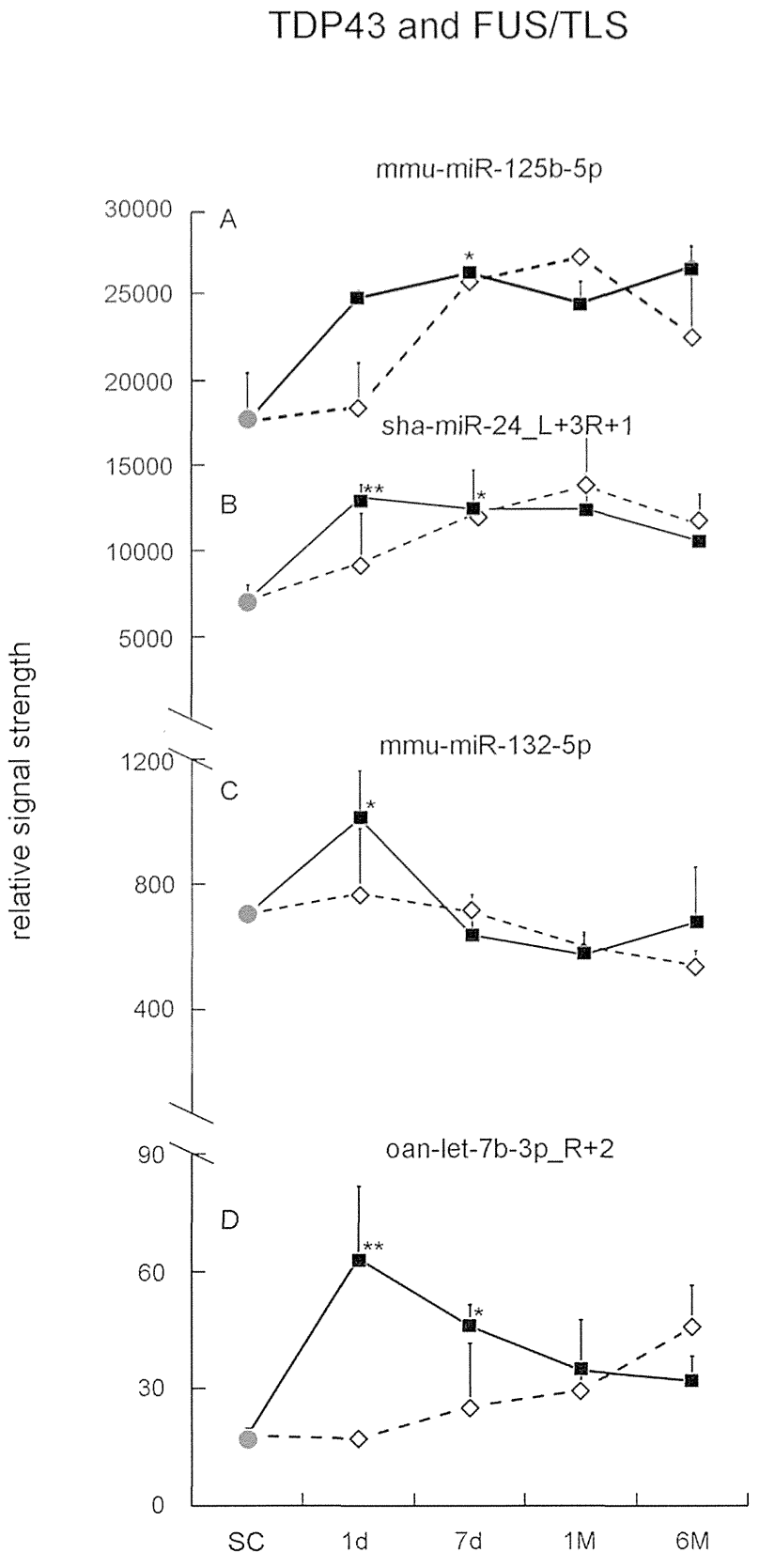

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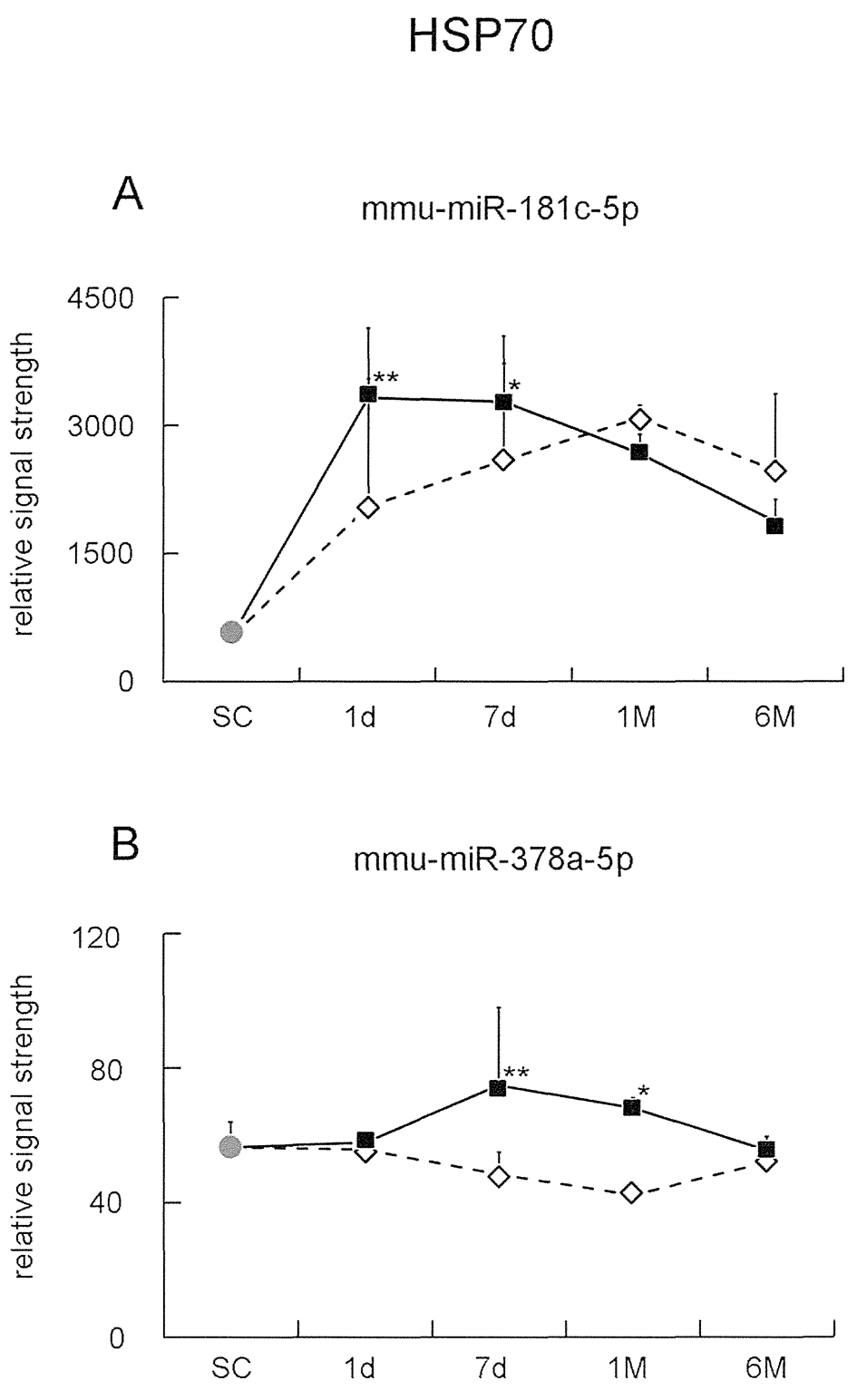

Figure 4:

BRAIN RESEARCH 1587 (2014) 33–39

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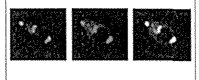

Research Report

Anti-oxidative nutrient rich diet protects against acute ischemic brain damage in rats

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ARTICLE INFO

Article history:
Accepted 19 August 2014
Available online 29 August 2014

Keywords:
Middle cerebral artery occlusion
Rat
Cerebral infarction
Anti-oxidative
Anti-inflammatory

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 min 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

Abbreviations: 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

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http://dx.doi.org/10.1016/j.brainres.2014.08.056
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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) (Dirnagl 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 Scheepens, 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) (Fig. 1).

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; Fig. 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, $p<0.01$; Fig. 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=n.s. versus vehicle) with significant reductions in the high AO diet group (54.6±10. 3 mm^3; $p<0.05$ versus vehicle; Fig. 3B). The examples of CV sections are shown in Fig. 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 Fig. 4. Compared with the vehicle group (4-HNE, 192.7±325/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).

Table 1 – Time-dependent changes in systolic blood pressure (SBP), diastolic blood pressure (DBP), and rCBF.

	Before tMCAO	During tMCAO	Just after reperfusion	24 h After repurfusion
Systolic blood pressure (mmHg)				
Vehicle	124.4±7.6			151.2±6.5
Normal AO	121.5±7.8			120.5±12.3
High AO	123.0±3.2			135.4±4.2
Diastolic blood pressure (mmHg)				
Vehicle	95.7±4.4			111.2±11.2
Normal AO	91.9±11.2			99.5±6.3
High AO	91.8±6.8			88.4±9.5*
Regional cerebral blood flow(%)				
Vehicle	100	55.4±20.3	91.6±36.6	79.5±36.1
Normal AO	100	65.1±21.1	82.8±24.4	82.6±20.9
High AO	100	66.3±29.3	89.0±14.5	91.2±27.3

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

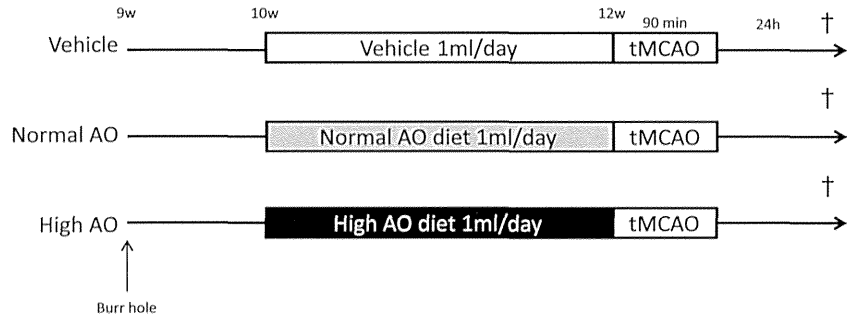


Fig. 1 – The three experimental rat groups showing vehicle group (0.5% methylcellulose plus glucose 250 mg/1 mL/day, $n=6$), normal AO diet group (AO diet 1 mL/day, $n=6$), and high AO diet group ($n=6$). All groups were sacrificed 24 h after 90 min of transient middle cerebral artery occlusion (tMCAO).

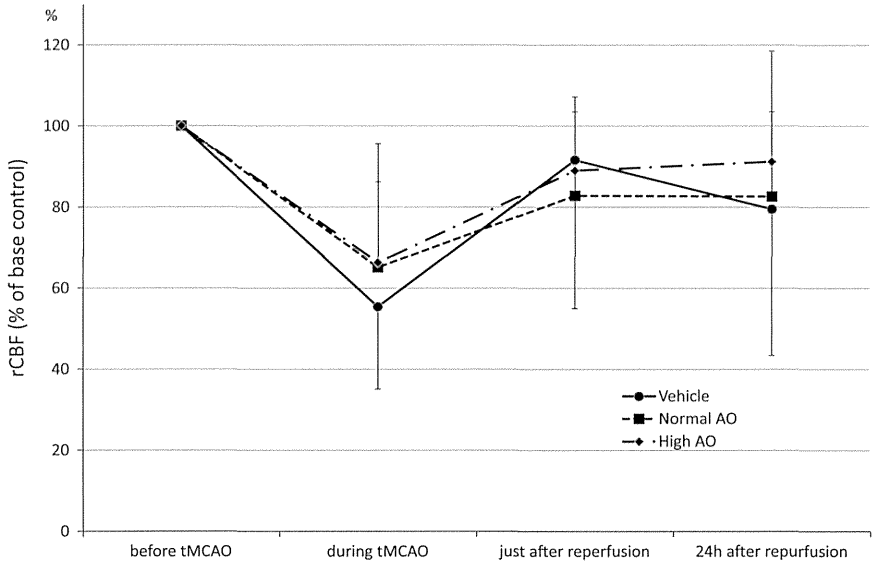


Fig. 2 – Effect of normal and high AO diets on regional cerebral blood flow before, during, just after, and 24 h after 90 min of transient MCAO. There were no significant differences among vehicle, normal, and high AO groups ($n=6$).

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 Fig. 4. Compared with the vehicle group (MCP-1, $186.3\pm35.4/mm^2$; 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 ($177.1\pm34.2/mm^2$), 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, $131.1\pm39.2/mm^2$; Iba-1, 77.3 ± 21.4; TNFα, 79.9 ± 30.0; $p<0.05$ versus vehicle).

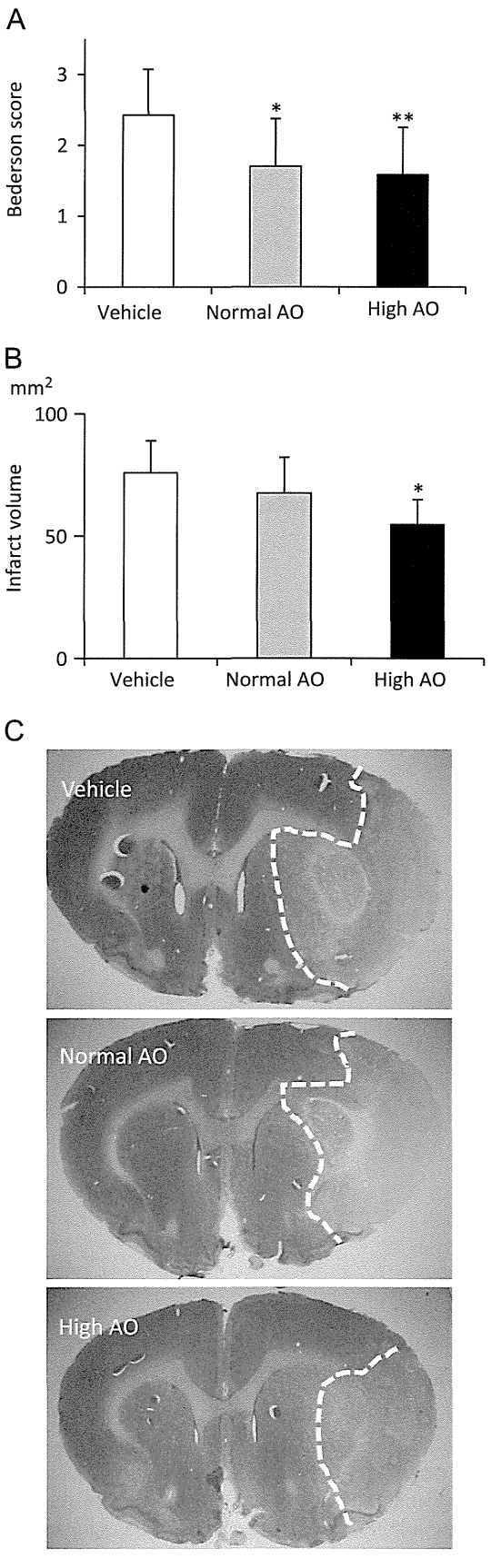
Staining for Sirt-1 also is shown in Fig. 4 (bottom). Although there was a tendency toward an increase in Sirt-1 staining, the change was not significant among the three diet groups (vehicle, $157.0\pm33.3/mm^2$; normal AO diet, 161.2 ± 52.4; high AO diet, 189.0 ± 37.4).

3. Discussion

It appeared that an AO diet improved the motor function (Fig. 3A), reduced the infarct volume (Fig. 3B and C), and reduced oxidative (Fig. 4) and inflammatory (Fig. 5) markers, except for Sirt-1 (Fig. 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 cellar 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 (Dimagl 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-α (Fig. 5). MCP-1 activates microglia to be Iba-1 positive, and increases infiltration of macrophages to promote inflammation through TNF-α 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 (Fig. 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 (Fig. 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 procedures

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

Fig. 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).

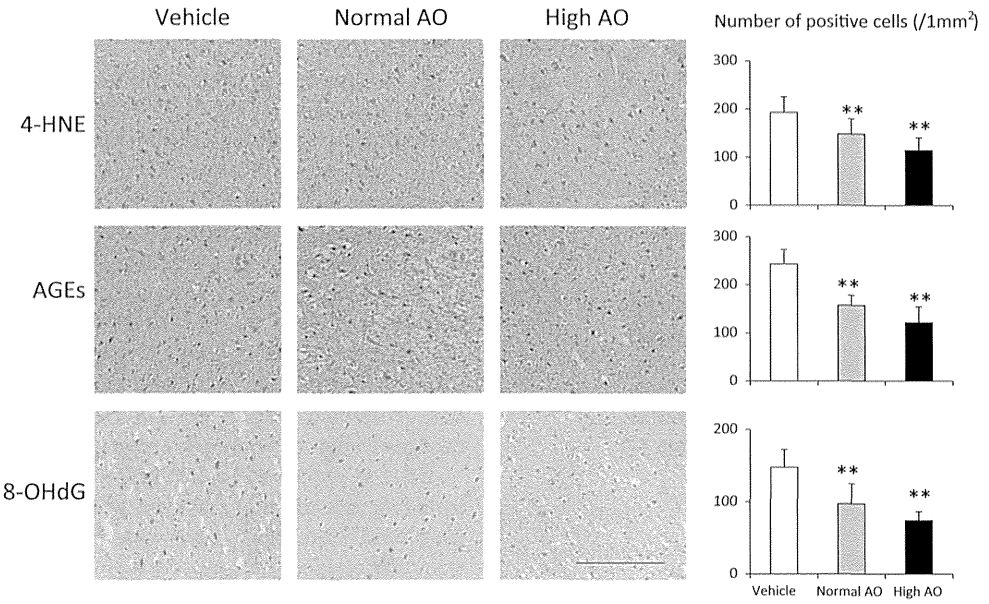


Fig. 4 – Immunohistochemistry for 4-HNE, AGEs and 8-OHdG at the peri-infarcted area in the vehicle, normal and high AO diet groups (left), and quantitative analyses of positive cells (right). The number of positive cells was significantly smaller in the normal and high AO diet groups ($p<0.01$ versus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

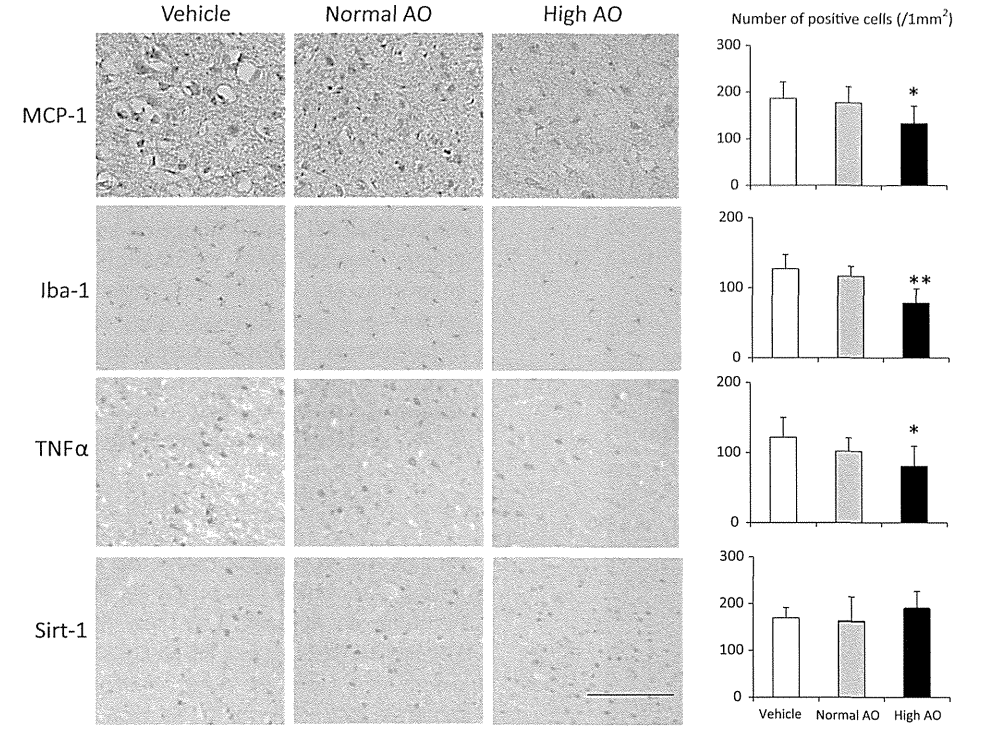


Fig. 5 – Immunohistochemistry for MCP-1, Iba-1, TNFα and Sirt-1 at the peri-infarcted area in the vehicle, normal and high AO diet groups (left), and quantitative analyses of positive cells (right). The number of positive cells for MCP-1, Iba-1, and TNFα was significantly smaller than vehicle (* $p<0.05$,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

(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; B_1, 0.18 mg; B_2, 0.2 mg; B_6, 0.30 mg; B_{12}, 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, 1.5 mg; Cu, 0.15 mg; Se, 5 μg; and Cr, 6 μg) per 100 mL. 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 Benderson 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 day 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; JaICA, Shizuoka, Japan), AGEs (1:200; Transgenic, Kobe Japan), 8-OHdG (1:50; MOG-020; JaICA, 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 TNFα 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

This work was partly supported by a Grant-in-Aid for Scientific Research (B) 25293202 from the Ministry of Education, Culture, Science, Sports and Technology of Japan and by grants (Sobue G, Ishizawa M, Sasaki H, Mizusawa H, and Nakano I) from the Ministry of Health, Labour, and Welfare of Japan.

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Journal of Neuroscience Research

Journal of Neuroscience Research 92:54–63 (2014)

Acceleration of TDP43 and FUS/TLS Protein Expressions in the Preconditioned Hippocampus Following Repeated Transient Ischemia

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The 43-kDa transactivation response DNA binding protein (TDP43), fused in sarcoma/translocated in liposarcoma (FUS/TLS), heat shock protein 70 (HSP70), and β-amyloid (Aβ) are induced and involved in cerebral ischemia, amyotrophic lateral sclerosis (ALS), and Alzheimer's disease (AD), but their relationships in ischemic tolerance have never been examined, although they could be involved in endogenous neuroprotection under ischemic preconditioning. In the present study, Mongolian gerbils were subjected to one or three incidents of basically nonlethal 2-min transient common carotid arteries occlusion (tCCAO). Hippocampal CA1 neurons were lost only in the 2-min three times group at 3 and 7 days, which then gradually recovered from 1 to 6 months. Inductions of TDP43 and FUS/TLS were accelerated from 3 months to 7 days or from 7 days to 1 day, respectively, after 2-min three times ischemia compared with once. The cytoplasmic stainings of TDP43 and FUS/TLS showed a further acceleration of the peaks from 1 months to 3 days or from 1 months to 7 days, respectively, after 2-min three times ischemia compared with once. In contrast, HSP70 was induced only at 7 days after 2-min tCCAO for three times, with no expression for Aβ. These data show that ischemic preconditioning offers a way to induce endogenous neuroprotection and neurogenesis in gerbils, with TDP43, FUS/TLS, and HSP70 involved in this function.

Key words: ischemic preconditioning; TDP43; FUS/TLS; HSP70; neuroprotection

Ischemic stroke remains a leading cause of mortality and long-term disability worldwide in adults (Elkind, 2009; Sunil et al., 2011). Ischemic preconditioning offers a way to induce endogenous neuroprotection by causing the changes of gene expression and alteration of protein synthesis (Kitagawa et al., 1990; Aoki et al., 1993). The proliferating precursor cells show a potential for regeneration and neuronal differentiation at the subependymal zone, dentate gyrus (DG), and cornu ammonis 1 (CA1) pyramidal neurons in the hippocampus after stroke (Nakatomi et al., 2002; Dempsey et al., 2003; Abe et al., 2012).

The 43-kDa transactivation response DNA-binding protein (TDP43) is a predominantly nuclear protein but translocates to the cytosol under a pathological condition, where it is ubiquitnated and/or phosphorylated and cleaved into smaller fragments (Neumann et al., 2006; Mackenzie et al., 2007; Hasegawa et al., 2008). Fused in sarcoma/translocated in liposarcoma (FUS/TLS) can function as an RNA and a DNA binding protein (Crozat et al., 1993; Perrotti et al., 1998) and is involved in diverse cellular processes, including cell proliferation (Bertrand et al., 1999), DNA repair (Baechtold et al., 1999), transcriptional regulation, RNA splicing (Yang et al., 1998), and transport of RNA between intracellular compartments (Zinszner et al., 1997; Neumann et al., 2009). TDP43 expression is altered after acute ischemic stroke (Lee et al., 2008; Kanazawa et al., 2011), and genetic mutations of TDP43 and FUS/TLS cause an inherited amyotrophic lateral sclerosis (ALS; Lagier-Tourenne et al., 2010).

The heat shock protein 70 (HSP70) family is a major molecular chaperone (Abe et al., 1993), which could dramatically protect the brain from ischemic damage (Rajdev et al., 2000; Tsuchiya et al., 2003). The

Contract grant sponsor: Ministry of Health, Labour and Welfare of Japan; Contract grant numbers: (B) 21390267; (C) 24591263; 24659651; Contract grant sponsor: Grants-in-Aid from the Research Committees (H. Mizusawa, I. Nakano, M. Nishizawa, H. Sasaki, and M. Aoki).

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Received 17 June 2013; Revised 28 August 2013; Accepted 29 August 2013

Published online 28 October 2013 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/jnr.23301

deposition of extracellular β-amyloid (Aβ) plaques characterizes the pathology of Alzheimer's disease (AD; Hardy and Selkoe, 2002). Aβ1–42 is induced and accumulated in the brain of AD patients (Skovronsky et al., 1998; Greenfield et al., 1999; Gouras et al., 2000) and in cerebral ischemia (Abe et al., 1991a,b; Kurata et al., 2011). Although these molecules are induced and involved in cerebral ischemia, ALS, and AD, their relationships in ischemic tolerance have never been examined. In the present study, therefore, we evaluated their expressions in the hippocampal CA1 region of gerbils by comparing a short and nonlethal cerebral ischemia (2 min once) and a repeated cerebral ischemia (2 min three times).

MATERIALS AND METHODS

Experimental Model

Experiments were performed on male Mongolian gerbils at 10–12 weeks of age with body weights of about 80 g (Japan SLC, Shizuoka, Japan). Before starting the surgery, animals were maintained in a temperature-regulated room (23–25°C) on a 12-hr light/dark cycle for at least 7 days. The gerbils were fasted but were allowed free access to water overnight before surgery.

Transient global forebrain ischemia was induced in gerbils by occluding bilateral common carotid arteries (CCA) as in our previous method (Abe et al., 1991a,b). Briefly, the gerbils were lightly anesthetized by inhalation of a nitrous oxide/oxygen/isoflurane (69%/30%/1%) mixture. Body temperature was monitored in all animals and was maintained at 37°C ± 0.3°C by using a heat pad (model BMT-100; Bio Research Center, Nagoya, Japan) during the surgery. A midline neck incision was made, and both CCA were exposed. Then, 65 gerbils were randomized into three groups (Fig. 1). When the animals began to regain consciousness after the termination of the anesthesia, both CCA of the second and third groups (n = 30 each) were occluded with surgical microclips (RS-5424; Roboz Surgical Instrument, Gaithersburg, MD) for 2 min of transient CCA occlusion (tCCAO). For the third group (n = 30), secondary occlusion of both CCA for 2 min was induced 2 days later, and then in the same way for the tertiary occlusion, with in total three incidents of tCCAO with 2-day intervals. The sham control (SC) group (n = 5) underwent only one neck incision. The animals recovered for 1, 3, and 7 days and 1, 3, and 6 months (n = 5 at each time point) at the ambient temperature (23–25°C) and then were deeply anesthetized with pentobarbital (i.p., 40 mg/kg) and decapitated. SC animals were sacrificed in the same way just after the operation. All brains were dissected, quickly frozen in powdered dry ice, and stored at −80°C until subsequent preparation. On the dorsal hippocampal level, coronal brain sections with 10 μm thickness were prepared with a cryostat (HM 500M; Microm) at −18°C and mounted on silane-coated glass slides. All the animal experiments were approved by the Animal Committee of the Graduate School of Medicine and Dentistry, Okayama University.

Cresyl Violet Staining

To investigate the morphological changes of the hippocampus after transient forebrain ischemia, cresyl violet staining was performed. In brief, the frozen brain sections (10 μm thickness) were immersed in 0.1% cresyl violet for 10 min at room temperature. Cryosections were dehydrated in graded alcohol, coverslipped with microcoverglass, and analyzed with a light microscope (Olympus BX-51; Olympus Optical, Tokyo, Japan).

Immunohistochemistry

As in our previous report (Liu et al., 2010), the frozen brain sections were first fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4) for 30 min and rinsed three times in phosphate-buffered saline (PBS; pH 7.4). After incubation in 0.3% hydrogen peroxide/methanol for 30 min to block endogenous peroxidase activity, the sections were blocked in 10% bovine serum albumin in PBS for 60 min. Slides were incubated at 4°C overnight with rabbit polyclonal anti-TDP43 antibody (T1705; Sigma-Aldrich Japan, Tokyo, Japan) diluted 1:500, rabbit polyclonal anti-FUS/TLS antibody (ab23439; Abcam, Cambridge, United Kingdom) diluted 1:500, rabbit polyclonal anti-HSP70 (4876; Cell Signaling Technology Japan, Tokyo, Japan) antibody diluted 1:200, or rabbit anti-β-amyloid 1–42 antibody (ab10148; Abcam) diluted 1:400. Slides were then washed with PBS and incubated with biotinylated anti-rabbit IgG secondary antibody (PK-6104; Vector, Burlingame, CA) diluted 1:500 for 2 hr at room temperature. The sections were then incubation with avidin-biotin-peroxidase complex (Vectastatin ABC kit PK-6100; Vector) for 30 min. As a color substrate, diaminobenzidine tetrahydrochloride (DAB) was used. A set of sections was also stained in a similar way but without the primary antibodies, serving as a negative control.

Quantitative Analysis

To analyze cresyl violet staining and immunoreactivity quantitatively, corresponding areas of the hippocampus CA1 region were measured from sections of each animal. Images of all structures were taken from the CA1 region of the hippocampus proper through a light microscope equipped with a digital camera (Olympus DP70; Olympus Optical) connected to a personal computer monitor. The numbers of neurons for cresyl violet staining and DAB staining in the CA1 region were determined by summing in five regions of adjacent 200-μm lengths of pyramidal layer arc dorsal to the dentate gyrus (visualized under ×20 objectives using an eyepiece equipped with a calibration reticle). The counts of neurons in bilateral CA1 regions were averaged for five sections of each hippocampus.

Data from cresyl violet staining were presented as the number of surviving neurons/field (cells/mm^2). To evaluate the neuronal survival in the hippocampal CA1 subfield, neurons defined as large cells of >10 μm with palely stained nuclei, discrete nucleoli, and basophilic cytoplasm were regarded as surviving cells, whereas shrunken neurons with pyknotic nuclei were regarded as damaged cells.

The numbers of positive cells for TDP43, FUS/TLS, and HSP70 in the hippocampal CA1 region were counted within the five regions of pyramidal layer arc dorsal level. Cells positive for TDP43 or FUS/TLS expressed in cytoplasm were

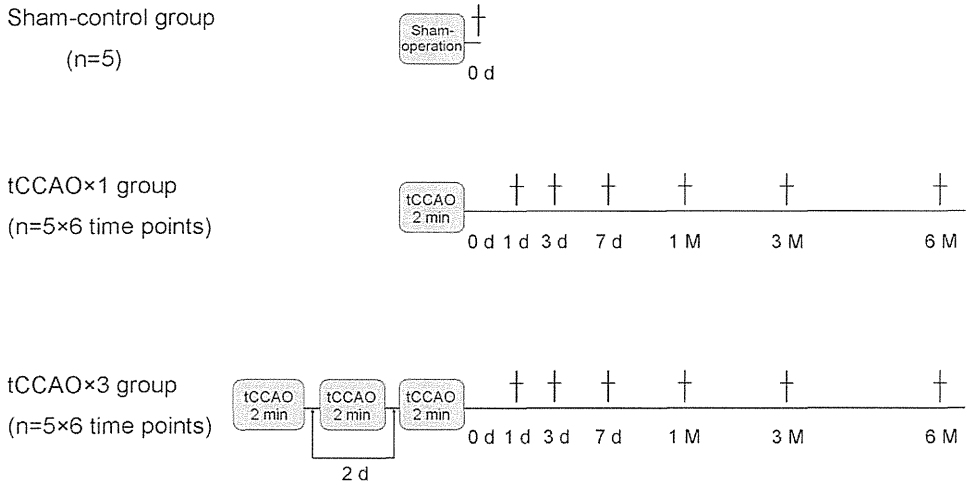

Fig. 1. Three experimental groups for transient common carotid arteries occlusion (tCCAO) with 65 gerbils randomized into three groups. Both CCA of the second and third groups (n = 30 each) were occluded for 2 min of tCCAO, and 3 × 2-min tCCAO were used for the third group, with 2-day intervals. The animals recovered for 1, 3, and 7 days and 1, 3, and 6 months (n = 5 at each time point) and then were decapitated. The sham control (SC) group (n = 5) was treated with only one neck incision and then sacrifice.

quantified by counting stained cells, and the results are shown as the percentage of positive cells over this region. To determine the frequency of positive neurons with cytoplasmic redistribution of TDP43 or FUS/TLS, the five chosen nonoverlapping fields, which were same as those described above at the level of the pyramidal layer arc dorsal of CA1 region, were examined. We counted cytoplasmic TDP43- or FUS/TLS-positive neurons in which diffuse TDP43 or FUS/TLS signal was detected in cytoplasm, in combination with the absence of nuclear immunoreactivity.

Statistical Analysis

Data are expressed as mean ± SD. At first, we tested for normal distribution of samples with SPSS. Then, statistical analysis was performed by using a one-factor ANOVA, followed by a Tukey post hoc comparison. Statistical analyses were performed in SPSS (version 13.0; SPSS Inc., Chicago, IL). All tests were considered statistically significant at $P < 0.05$ (Deguchi et al., 2006).

RESULTS

Histology in the CA1 Region

Typical cresyl violet staining for the hippocampal CA1 region is shown in Figure 2. The density of hippocampal CA1 pyramidal neurons was 4,146.3 ± 350.7 cells/mm^2 in the SC brains (Fig. 2A, top; n = 5). There was no change of the density of the CA1 pyramidal neurons between the SC group and the gerbil group subjected to 2-min tCCAO once from 1 day to 6 months (Fig. 1; n = 5 at each time point; Fig. 2A, left, B, left, open bars). However, in the gerbil group subjected to 2-min tCCAO three times (Fig. 1; n = 5 at each time point), time-dependent damaged neurons with pycnotic nucleus and neuronal loss were observed in the CA1 region (Fig. 2A, right, B, right, solid bars). Although most hippocampal CA1 neurons survived at 1 day after 3 × 2-min tCCAO, large numbers of CA1 neurons were gradually lost from 3 to 7 days (2,693.2 ± 303.1 cells/mm^2 at 3 days, $^{*}P < 0.05$ vs. SC; 1,963.3 ± 483.1 cells/mm^2 at 7 days, $^{**}P < 0.01$ vs. SC). After the extensive loss at 7 days, CA1 cell density gradually and significantly recovered from 1 to 6 months (2,553.0 ± 201.7 cells/mm^2, 3,200.6 ± 323.6 cells/mm^2 and 3,587.3 ± 200.5 cells/mm^2 at 1, 3, and 6 months, respectively; $^{\#}P < 0.05$ at 6 months vs. 7 days). At 6 months, the CA1 cell density had recovered to 86.5% of SC (Fig. 2).

TDP43 Immunohistochemistry and the Intracellular Distribution

TDP43 immunostaining in the CA1 region is shown in Figure 3. In SC gerbils, TDP43 was weakly stained in the nuclei of some CA1 pyramidal neurons (Fig. 3, top). After a single 2-min tCCAO, the TDP43 immunoreactivity was slowly but progressively induced in CA1 pyramidal neurons, with a peak at 3 months, which slightly returned at 6 months (Figs. 3, left, 5A, left, open bars). From the baseline level of SC (1,396.8 ± 130.6 cells/mm^2), the total numbers of TDP43-positive cells increased to 1,514.5 ± 151.2 cells/mm^2 at 1 month ($^{*}P < 0.05$ vs. SC) and 1,728.5 ± 141.2 cells/mm^2 at 3 months ($^{**}P < 0.01$ vs. SC). In the group of 3 × 2-min tCCAO, the TDP43 immunoreactivity showed a pattern similar to that of single-tCCAO group, but the peak time accelerated to 7 days, with much stronger staining (Figs. 3, right, 5A, right, black bars). Subsequently, the numbers gradually returned until 6 months. From the

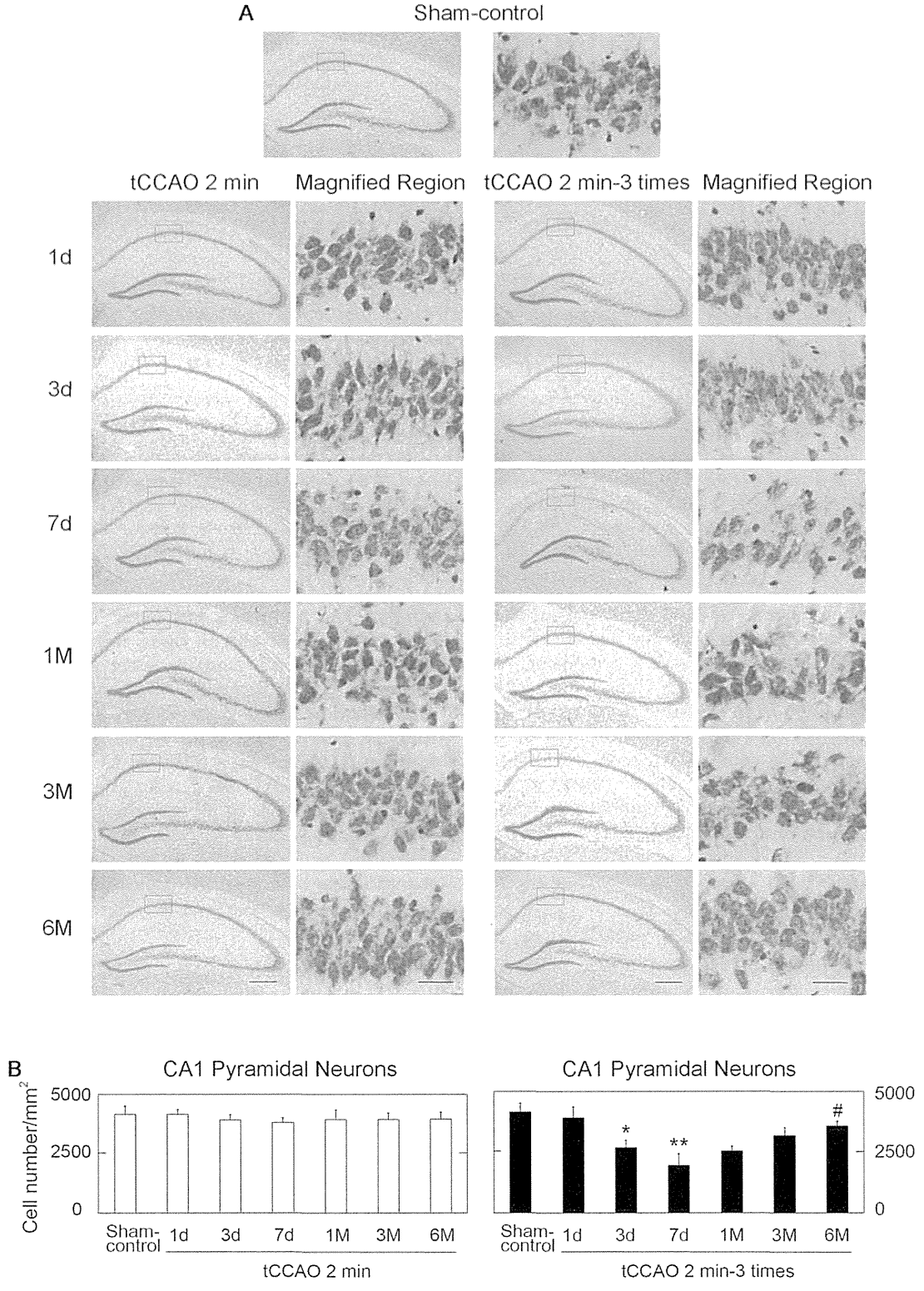

Fig. 2. **A**: Cresyl violet staining of the gerbil dorsal hippocampus and magnification of the CA1 pyramidal layers of the three groups. Note CA1 cell loss only in the 3 × 2-min tCCAO group at 3 and 7 days, which then recovered from 1 to 6 months. **B:** Statistical analysis shows no change in the the CA1 pyramidal neuron density after 1 × 2-min tCCAO (left, white bars), but a large CA1 neuron loss from 3 ($*P < 0.05$ vs. SC) to 7 ($**P < 0.01$ vs. SC) days after 3 × 2-min tCCAO and a gradual and significant recovery from 1 to 6 ($^{\#}P < 0.05$ vs. 7 days) months. Scale bars = 500 μm; 25 μm in magnified regions. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

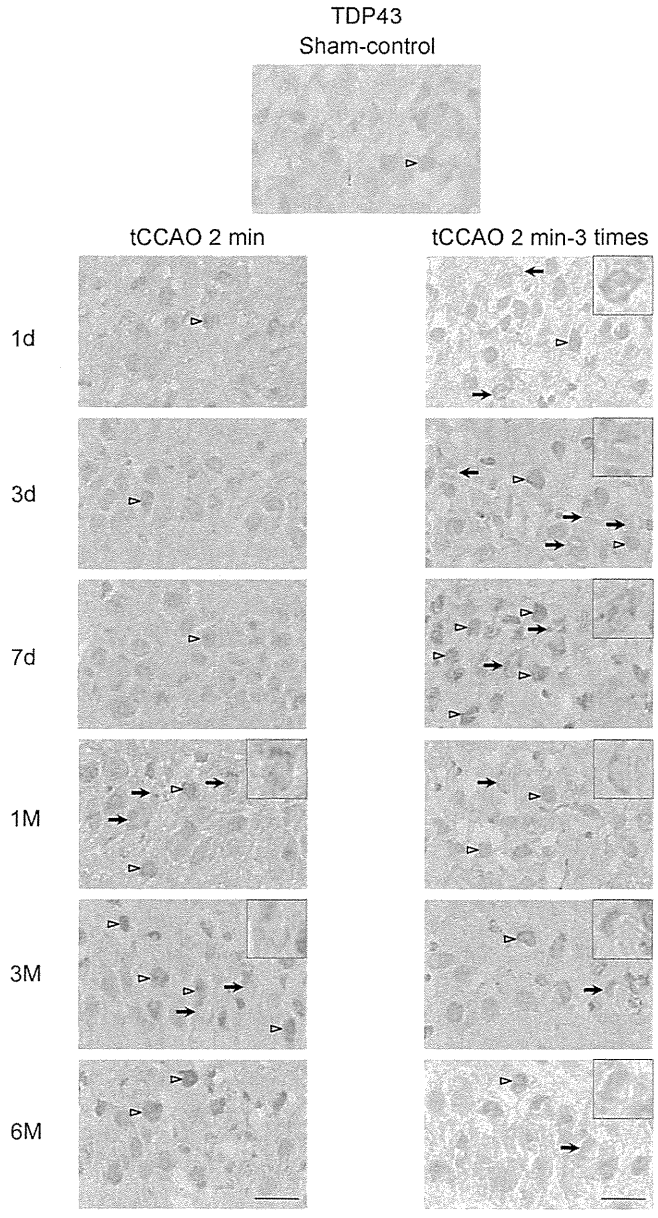

Fig. 3. Immunohistochemistry for TDP43 in the CA1 pyramidal neurons of the three groups. Note the different peaks of the nuclear (arrowheads) and cytplasmic (arrows) expressions. Typical cytoplasmic stainings are shown in magnified **insets**. Scale bars = 23 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

baseline level of SC (1,396.8 ± 130.6 cells/mm^2), the total numbers of TDP43-positive cells had increased to 1,624.6 ± 117.5 cells/mm^2 at 3 days (*$P < 0.05$ vs. SC) and 1,813.1 ± 155.4 cells/mm^2 at 7 days (**$P < 0.01$ vs. SC).

Although nuclear staining (Fig. 3, arrowheads) of TDP43 became stronger (Figs. 3, 5B, left), cytoplasmic staining (Fig. 3, arrows) of TDP43 became evident from 1 day, with a peak at 1 month (Fig. 5B, left, blue bars) after the forebrain ischemia from a single 2-min tCCAO, which then gradually returned until 6 months. Typical cytoplasmic staining is shown in magnified insets in Figure 3. Among total TDP43-positive cells, the ratios of cytoplasmic positive pyramidal neurons were 1.8 ± 1.3%, 2.7 ± 1.2%, 5.2 ± 2.4%, 10.8 ± 3.1%, 7.1 ± 2.5% and 6.6 ± 3.3% at 1 day, 3 days, 7 days, 1 months (§§$P < 0.01$ vs. 1 day), 3 months (§$P < 0.05$ vs. 1 day), and 6 months, respectively. In the group of 3 × 2-min tCCAO group (Figs. 3, 5B right), cytoplasmic staining showed a pattern similar to that of 1 × 2-min tCCAO group, but the peak time accelerated to 3 days. Among total TDP43-positive cells, the ratios of cytoplasmic-positive pyramidal neurons were 15.1 ± 7.5%, 32.6 ± 10.6%, 24.5 ± 10.3%, 21.8 ± 9.0%, 22.7 ± 9.5%, and 16.5 ± 7.2% at 1, 3 (§§$P < 0.01$ vs. 1 day), and 7 days (§$P < 0.05$ vs. 1 day) and 1, 3, and 6 months, respectively.

FUS/TLS Immunohistochemistry and the Intracellular Distribution

FUS/TLS immunostaining in the CA1 region is shown in Figure 4. In SC gerbils, FUS/TLS was weakly stained in the nuclei of some CA1 pyramidal neurons (Fig. 4, top). After 1 × 2-min tCCAO, the FUS/TLS immunoreactivity was slowly but progressively induced in CA1 pyramidal neurons, with the peak at 7 days, then slightly returned toward the SC level at 6 months (Figs. 4, left, 5C, left, open bars). From the baseline level of SC (1,842.22 ± 195.3 cells/mm^2), the total numbers of FUS/TLS-positive cells had increased to 2,247.4 ± 208.0 cells/mm^2 at 7 days (**$P < 0.01$ vs. SC) and 2,113.3 ± 204.0 cells/mm^2 at 1 months (*$P < 0.05$ vs. SC). In the group undergoing 3 × 2-min tCCAO, the FUS/TLS immunoreactivity showed a pattern similar to that of the 1 × 2-min tCCAO group, but the peak time accelerated to 1 day, with much stronger staining, then gradually returned until 6 months (Figs. 4, right, 5C, right, black bars). From the baseline level of SC (1,842.22 ± 195.3 cells/mm^2), the total numbers of FUS/TLS-positive cells had increased to 2,555.2 ± 174.1 cells/mm^2 at 1 day (**$P < 0.01$ vs. SC) and 2,282.9 ± 208.3 cells/mm^2 at 3 days (*$P < 0.05$ vs. SC).

Although nuclear staining (Fig. 4, arrowheads) of FUS/TLS became stronger (Figs. 4, 5D, left), the cytoplasmic staining (Fig. 4, arrows) of FUS/TLS became evident from 1 day, with the peak at 1 months (Figs. 4, 5D, left, blue bars) after the forebrain ischemia of 1 × 2-min tCCAO, which then slightly returned by 6 months. Typical cytoplasmic staining is shown in magnified insets in Figure 4. Among total FUS/TLS-positive cells, the ratios of cytoplasmic-positive pyramidal neurons were 3.3% ± 1.5%, 5.1% ± 2.6%, 7.6% ± 4.1%, 11.4% ± 6.9%, 10.3% ± 2.5%, and 9.7% ± 3.2% at 1, 3, and 7 days and 1 (§§$P < 0.01$ vs. 1 day), 3 (§$P < 0.05$ vs. 1 day), and 6 months, respectively. In the groups undergoing 3 × 2-min tCCAO (Figs. 4, 5D, right), cytoplasmic staining showed a pattern similar to that of the 1 × 2-min tCCAO group, but with the peak time accelerated to 7 days. Among total FUS/TLS-positive cells, the

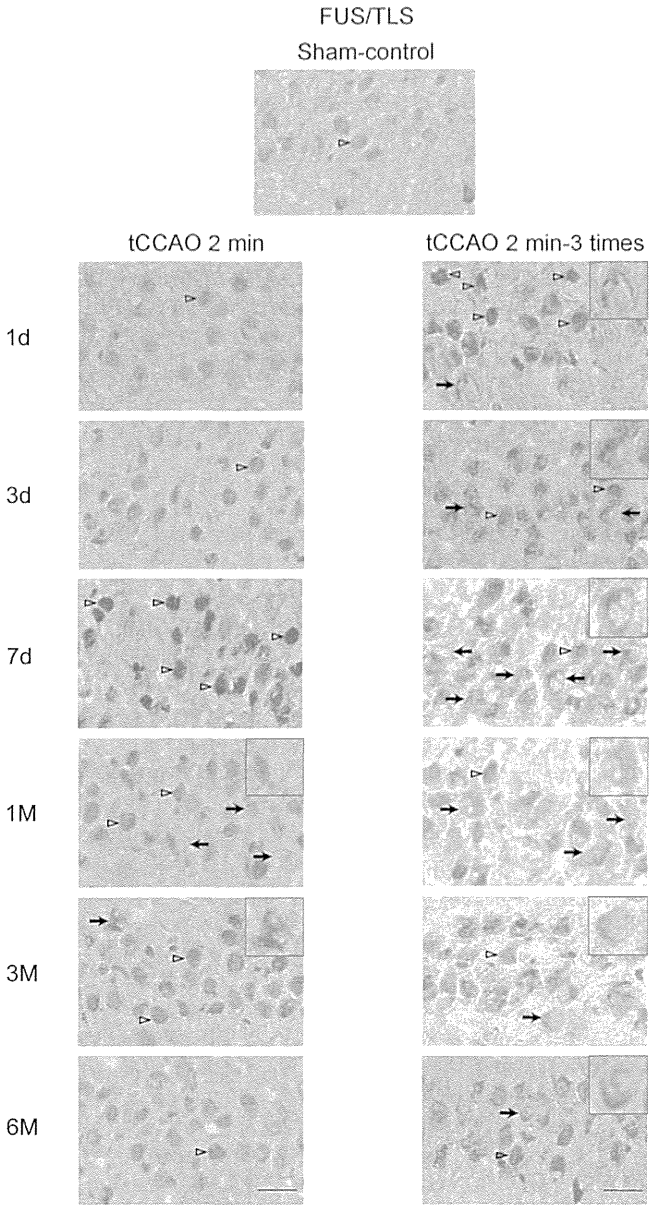

Fig. 4. Immunohistochemistry for FUS/TLS in the CA1 pyramidal neurons of the three groups. Note the different peaks of the nuclear (arrowheads) and cytplasmic (arrows) expressions. Typical cytoplasmic stainings are shown in magnified **insets**. Scale bars = 20 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

ratios of cytoplasmic positive pyramidal neurons were 14.0% ± 7.4%, 23.0% ± 9.1%, 36.1% ± 13.3%, 26.8% ± 12.1%, 25.8% ± 9.3%, and 21.1% ± 8.8% at 1, 3, and 7 days ($§§P < 0.01$ vs. 1 day) and 1 ($§P < 0.05$ vs. 1 day), 3, and 6 months, respectively.

HSP70 and Aβ Immunohistochemistries

HSP70 and Aβ immunohistochemical results are shown in Figure 6. The expression of HSP70 was not detected in the CA1 pyramidal neurons of SC brains (Fig. 6A, top) or after 1 × 2-min tCCAO (Fig. 6A, left, B, left). Even after 3 × 2-min tCCAO, the HSP70 immunoreactivity was induced only at 7 days (50.0 ± 7.8 cells/mm^2, $**P < 0.01$ vs. SC), which quickly disappeared from 1 to 6 months (Fig. 6A, right, B, right). In contrast, gerbils of the SC group showed no expression of Aβ, nor did both groups of 1 × and 2 × 2-min tCCAO (Fig. 6C,D).

DISCUSSION

The present study shows that CA1 pyramidal neurons in the gerbils were extensively lost at 7 days, which then recovered from 1 to 6 months in the group subjected to 3 × 2-min tCCAO (Figs. 1, 2). Time-dependent changes in the TDP43- and FUS/TLS-positive cells were observed after 1 × 2-min tCCAO, and the peak time of TDP43 and FUS/TLS cell number and cytoplasmic redistribution accelerated to an earlier period of reperfusion after 3 × 2-min tCCAO (Figs. 3–5). In contrast, HSP70 was induced only at 7 days after 3 × 2-min tCCAO, without any such induction in Aβ (Fig. 6).

Ischemic tolerance was observed originally in the gerbil model of transient global brain ischemia (Kitagawa et al., 1990; Kato et al., 1991; Kirino et al., 1991; Lehotský et al., 2009). In these gerbils, ischemic preconditioning of 2 min ischemia was extremely effective in protecting against ischemic cell death. A single 2-min ischemia depletes high-energy phosphate compounds and perturbs the protein synthesis but never causes neuronal death and therefore was chosen as the mild ischemic treatment for subsequent neuroprotection (Kitagawa et al., 1990; Maysami et al., 2008). In our study, although a single 2-min ischemia induced no neuronal damage (Fig. 2, left), three 2-min ischemic insults caused neuronal damage in the selectively vulnerable region CA1 in hippocampus (Fig. 2, right), as in a previous study (Kato et al., 1989). However, the three times mild ischemia group exhibited a significant recovery of the CA1 pyramidal neurons after 7 days to 6 months (Fig. 2B, right).

In the present study, significant changes in TDP43 and FUS/TLS were shown in the ischemic brains (Figs. 3–5). Upregulation of TDP43 expression with prominent cytosolic localization was found in motor neurons of ALS, which may represent an appropriate response to neuronal injury (Moisse et al., 2009). Cytoplasmic redistribution and altered nuclear distribution of TDP43 were also observed in ischemic rat neurons (Kanazawa et al., 2011) after 90 min of middle cerebral artery occlusion (MCAO). Taken together with the present study (Fig. 3, 5), both degenerative and ischemic neuronal injuries have common characteristics with respect to altered subcellular localization of TDP43. The cytoplasmic redistribution of TDP43 may be caused by perturbation of its nuclear import of the shuttling between the nucleus and the cytosol (Strong et al., 2007; Winton et al., 2008; Kanazawa et al., 2011). The colocalization of TDP43 with markers for RNA transport and