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Protective effect of adenosine against neuronal injury induced by middle cerebral artery occlusion in rats as evidenced by diffusion-weighted imaging

Y.K. Gupta[a,*], Kusum Sinha[a], Geeta Chaudhary[a], N.R. Jagannathan[b]

[a]*Department of Pharmacology, All India Institute of Medical Sciences, New Delhi 110029, India*
[b]*Department of NMR, All India Institute of Medical Sciences, New Delhi 110029, India*

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Abstract

In the present study, adenosine, an inhibitory neuromodulator, was studied in male Wistar rats subjected to 2 h of transient middle cerebral artery (MCA) occlusion. Adenosine (500 mg/kg ip) was administered twice — once at the time of MCA occlusion and again at the time of reperfusion—and evaluated for its protective effect by using diffusion-weighted imaging (DWI) (30 min after reperfusion). After the DWI experiments, one group of animals was euthanized 2 h after reperfusion for the estimation of oxidative stress markers, while in another group, neurological deficit was assessed 24 h after MCA occlusion. In the adenosine-treated group, percent hemispheric lesion area (%HLA) in DWI was significantly attenuated (11.7 ± 5.2) as compared to vehicle-treated group (21.4 ± 4.7). The level of malondialdehyde (MDA) (301.8 ± 22 nmol/g wet tissue) in the adenosine-treated group was significantly decreased as compared to that in the vehicle-treated MCA-occluded rats (420 ± 20 nmol/g wet tissue). An insignificant change was observed in the levels of glutathione in both the vehicle-treated MCA-occluded and the adenosine-treated groups. The neurological deficit was significantly improved in the adenosine-treated group (1.8 ± 0.06) as compared to the vehicle-treated (2.9 ± 0.38) group. This is the first study to demonstrate the effectiveness of adenosine using DWI in the MCA-occluded rats.

Keywords: Stroke; Adenosine; Middle cerebral artery occlusion; Neuroprotection

1. Introduction

Ischemia causes irreversible and fatal damage to the affected neurons, and reperfusion of oxygen that follows may further be detrimental. The mechanisms that give rise to ischemic brain damage have not yet been definitely determined, but considerable evidence say that both the release of excitatory amino acids (EAA), as well as the massive generation of free radicals during reperfusion, are important in the ischemic brain (Chan, 1996; Mason et al., 2000). Consistent with this, agents that inhibit the release of EAA and/or having free radical scavenging activity have been investigated in experimental models of cerebral ischemia (Kawamoto et al., 1997; Sinha et al., in press; Schmid-Elsaesser et al., 1999).

Adenosine is an endogenous inhibitory neuromodulator in central nervous system. During ischemia–hypoxia, metabolic stress is induced and the extracellular level of adenosine is increased as a result of intense degradation of ATP, and has been suggested to cause the neuroprotective effect (Karl et al., 1992). The mechanism underlying this neuroprotective activity is not clear, however, studies have shown that adenosine inhibits the ischemia-evoked release of EAA (Masino et al., 1999). Yavuz et al. (1997) reported that exogenously administered adenosine protects the neurons against oxidative stress in global ischemia–reperfusion injury in gerbils. Therefore, adenosine appears to exert a dual action, i.e., inhibiting the release of EAA and scavenging the free radicals. Thus, it has been suggested that the dual approach could be more beneficial than an EAA receptor antagonist or free radical scavenger used alone.

Middle cerebral artery (MCA) occlusion followed by reperfusion is a model of focal ischemia in rats, which resembles to that of stroke in humans and is being increas-

* Corresponding author. Tel.: +91-11-6593684; fax: +91-11-6864789.
E-mail address: ykg@hotmail.com (Y.K. Gupta).

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ingly used for evaluation of therapeutic intervention (Koizumi et al., 1986). In the present study, the effect of adenosine treatment was evaluated in MCA occlusion–reperfusion model of acute ischemic stroke in rats.

We used diffusion-weighted imaging (DWI), a novel imaging technique based on the principle of magnetic resonance to study the effect of adenosine. As, the ultimate goal of experimental research is to improve the functional outcome of human recovery from stroke, the neurological evaluation was also done. The levels of markers of oxidative stress were also estimated in the adenosine-treated rats.

2. Material and methods

2.1. Animals

Albino male Wistar rats procured from the central animal facility at All India Institute of Medical Sciences, New Delhi, were group housed in polypropylene cages ($38 \times 23 \times 10$ cm) with not more than five animals per cage. They were maintained under standard laboratory conditions with natural dark–light cycle (14 ± 1 h light; 10 ± 1 h dark) and allowed free access to standard dry rat diet (Golden Feeds, India) and tap water ad libitum. All experimental procedures in rats described were reviewed and approved by the Institutional Animal Ethics Committee.

2.2. Drugs and experimental protocol

Adenosine (Sigma, St. Louis, USA) was dissolved in 8% Tween 20. It was administered at a dose of 500 mg/kg ip. The dose was chosen on the basis of our preliminary studies (data not shown). The dose was administered two times, once immediately after MCA occlusion and again immediately after reperfusion.

Rats were divided into two groups consisting of 10 rats each. In one group, DWI was performed 30 min after reperfusion and then the rats were sacrificed 2 h after reperfusion for estimation of oxidative stress markers, i.e., malondialdehyde (MDA) and glutathione. In the other group, neurological evaluation was performed 24 h after MCA occlusion. Vehicle-treated MCA-occluded rats and sham-operated rats were run parallel using the same experimental protocol.

2.3. MCA occlusion to induce focal cerebral ischemia

Rats were anesthetized with chloral hydrate 400 mg/kg ip (dissolved in distilled water). Core temperature (rectal) was maintained around 37 ± 1 °C throughout the surgical procedure using heating lamp and the thermocontrolled base of the operating table. A midline incision was made and the right common carotid artery, external carotid artery and internal carotid artery were exposed. A 4.0 monofilament nylon thread (Ethicon, Johnson & Johnson) with its tip rounded by heating quickly by bringing it near a flame was used to occlude the MCA. The filament was advanced from the external carotid artery into the lumen of the internal carotid artery until a resistance was felt which ensured the occlusion of the origin of MCA. The nylon filament was allowed to remain in the place for 2 h after which it was gently retracted so as to allow the reperfusion of the ischemic region (Koizumi et al., 1986).

In the sham-operated rats the external artery was exposed and there after rats were sutured without touching the internal carotid artery.

2.4. Assessment of cerebral infarction

2.4.1. Magnetic resonance imaging (MRI)

Magnetic resonance studies were carried out using an animal MRI/ MRI scanner (Bruker, BIOSPEC). Experiments were carried out at 4.7 T using a 69-mm circularly polarized birdcage volume resonator. Identification of the ischemic region made through multislice T_2-weighted pilot images using rapid acquisition with rapid enhancement sequence (TR = 2000 ms, TE = 25 ms, slice thickness = 2 mm, number of slices = 7). After identification of the site of the ischemia i.e., the region of interest, diffusion-weighted images were acquired using stimulated echo diffusion-weighted pulse sequence. Diffusion-weighted images were acquired at three different b values with following acquisition parameters: TR = 2000 ms, TE = 40 ms, TM = 30 ms, gradient duration = 10 ms, b = 25, 50, 75 mT/m. The area of ischemic tissue damage was calculated from the diffusion-weighted images as the number of pixels with hyperintensity of 15% relative to the corresponding anatomic structures in the contralateral hemisphere and expressed as percent hemisphere lesion area. All seven imaged slices were summed to provide the total volume of ischemic tissue injury.

The threshold value of 15% was found to be the lowest cutoff value that did not include pixels in the contralateral nonischemic hemisphere.

The percent (%) volume of ischemic region was calculated as the volume of hyperintense region divided by the volume of ipsilateral hemisphere

2.5. Neurological evaluation

Twenty-four hours after MCA occlusion, the animals were subjected to neurological evaluation using a six-point scale (Tatlisumak et al., 1998). Briefly, the scoring was as follows: 0 = no deficits, 1 = failure to extend left forepaw fully, 2 = circling to the left, 3 = paresis to the left, 4 = no spontaneous walking, 5 = death.

2.6. Estimation of markers of oxidative stress

Malondialdehyde (MDA) and reduced glutathione (GSH) were estimated 2 h after reperfusion. Simultaneous control experiments were also run. The rats were decapitated under

ether anesthesia and the brains quickly removed, cleaned by rinsing with chilled saline and stored at −70 °C. The biochemical analysis was performed within 48 h.

2.7. Measurement of lipid peroxidation

MDA (indicator of lipid peroxidation) was estimated as described by Okhawa et al. (1979). Briefly, brain tissues were homogenized with 10 times (w/v) 0.1 sodium phosphate buffer (pH 7.4). The reagents, acetic acid 1.5 ml (20%) pH 3.5, 1.5 ml thiobarbituric acid (0.8%) and 0.2 ml sodium dodecyl sulfate (8.1%), were added to 0.1 ml of processed tissue sample. The mixture was then heated at 100 °C for 60 min. The mixture was cooled with tap water and 5 ml of *n*-butanol: pyridine (15:1% v/v) and 1 ml of distilled water was added .The mixture was shaken vigorously. After centrifugation at 4000 rpm for 10 min, the organic layer was withdrawn and absorbance was measured at 532 nm using a spectrophotometer.

2.8. Measurement of glutathione

Glutathione was measured according to the method of Ellman (1959) with minor modification. Briefly, brain tissues were homogenized with 10 times (w/v) 0.1 sodium phosphate buffer (pH 7.4). This homogenate was then centrifuged with 5% trichloroacetic acid to centrifuge out the proteins. To 0.1 ml of this homogenate, 2 ml of phosphate buffer (pH 8.4), 0.5 ml of 5′5 dithiobis (2-nitrobenzoic acid) (DTNB) and 0.4 ml of double distilled water were added. The mixture was vortexed and the absorbance read at 412 nm within 15 min.

2.9. Statistical analysis

The data has been represented as mean ± S.E.M. Two-way analysis of variance (ANOVA) with post hoc comparison (Tukey) was used for comparing the signal intensities in DWI, whereas one way (ANOVA) with post hoc comparison was used for statistical analysis of the other parameters.

3. Results

3.1. Effect of adenosine treatment on DWI

Focal ischemia was evident in all MCA-occluded rats. Ischemic regions were manifested as increased signal intensity on DWI scans with a high *b* value.

The MR signal intensity of the ischemic region (right hemisphere) was compared with the corresponding identical region of the brain in the contralateral hemisphere. In the

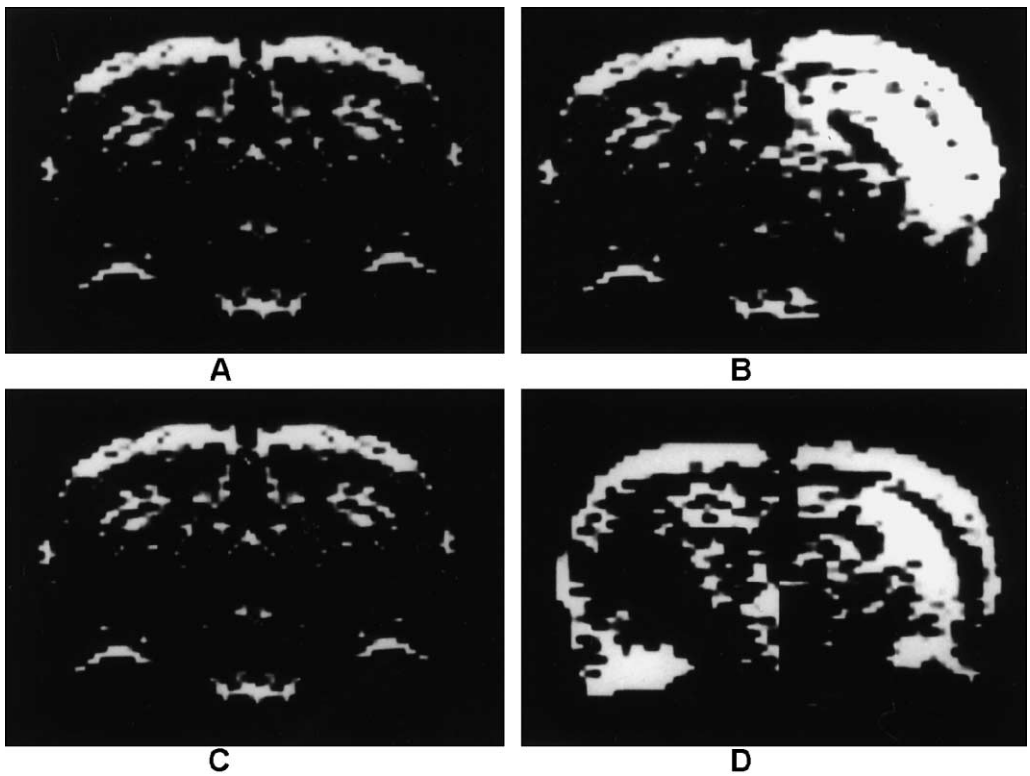

Fig. 1. DWI scans demonstrating (A) normal and (B) vehicle-treated middle cerebral artery-occluded rats (30 min after reperfusion), and (C) normal and (D) adenosine-treated middle cerebral artery-occluded rats (30 min after reperfusion). Focal ischemia was evident as regions of increased signal intensity in the right cerebral cortex. Adenosine treatment appeared to ameliorate lesions.

ischemic region, in the peripheral parietal cortex, as well as in the caudate putamen, there was significantly high intensity (228.7 ± 13.2, 227.5 ± 22.8, arb.) as compared to the contralateral hemisphere (186.2 ± 41.2, 182.6 ± 27.4, arb.) for the same slice of brain (Fig. 1B). In the adenosine-treated group, the signal intensity was not significantly different as compared to contralateral side. The mean values of signal intensity in the cortex, as well as caudate putamen in the right cerebral hemisphere, were 188.4 ± 35.2 and 182.1 ± 24.6, respectively, as compared to 180.7 ± 15.5 and 179.8 ± 22 for the contralateral side (Fig. 1D).

There was significant difference in the signal intensity of the caudate putamen and cortex of the contralateral and ipsilateral hemisphere of the vehicle-treated group and between the ipsilateral hemispheres of the vehicle and the adenosine-treated group. The value was significant between the contralateral and ipsilateral hemisphere of the adenosine-treated group and the contralateral hemispheres of the vehicle and the adenosine-treated group.

The percent hemispheric lesion area (%HLA) in vehicle-treated MCA-occluded rats after 30 min of reperfusion was 21.4 ± 4.7%. In adenosine-treated rats, the %HLA was (11.7 ± 5.2%) and was significantly less as compared to the vehicle-treated MCA-occluded rats ($P < .05$).

3.2. Neurological evaluation

Twenty-four after middle cerebral artery occlusion animals were scored according to a 6-point neurological scale. In the normal rats the mean neurological score was 0. In the vehicle-treated middle cerebral artery-occluded rats the mean neurological score increased to 2.9 ± 0.38 indicating neurological deficit. The rats in this group failed to extend their left paw fully, circled to the left and hind limb paresis was also seen. The neurological score in adenosine treated group improved significantly ($P < 0.05$) as compared to vehicle-treated middle cerebral artery-occluded rats. The value being (1.92 ± 0.4). None of the rats in the drug treated group showed hind limb paresis.

3.3. Effect of adenosine on brain MDA levels after 2 h of reperfusion

In vehicle-treated rats, 2 h after reperfusion, the levels of brain MDA were found to be significantly raised (420 ± 20 nmol/g wet tissue) as compared to the sham-operated rats (195 ± 26 nmol/g wet tissue) ($P < .05$). In the adenosine-treated group, the levels of MDA were significantly attenuated (301.8 ± 22 nmol/g wet tissue) ($P < .05$) as compared to the vehicle-treated MCA-occluded rats (Fig. 2).

3.4. Effect of adenosine on brain glutathione levels after 2 h of reperfusion

Similar to the MDA protocol, the brain glutathione levels were estimated 2 h after reperfusion in vehicle and adenosine-treated rats. Levels of glutathione in vehicle-treated MCA-occluded rats were not significantly changed as compared to the sham-operated rats. The values being 94.2 ± 14.9 and 88.7 ± 9.2 μg/g wet tissue, respectively. The levels of

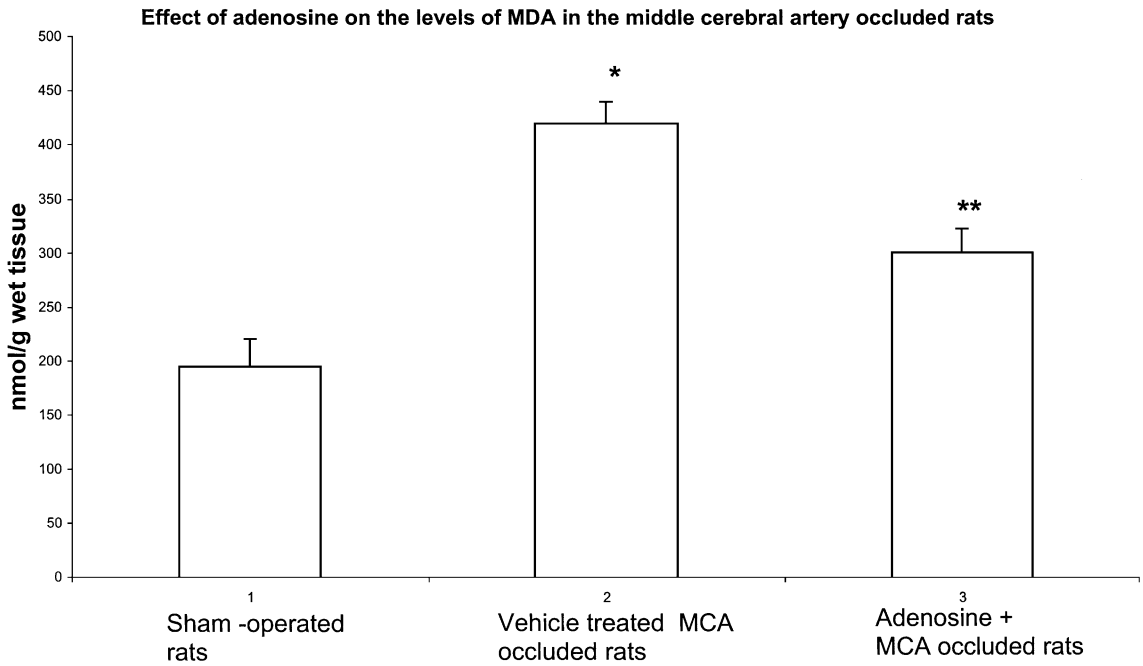

Fig. 2. Effect of adenosine (500 mg/kg ip) on the level of MDA in middle cerebral artery-occluded rats. The values are expressed as nanomole per gram of wet tissue. * $P < .05$ vs. sham-operated rats. ** $P < .05$ vs. vehicle-treated middle cerebral artery-occluded rats.

glutathione in the adenosine-treated group (103.2 ± 6.5 μg/g wet tissue) were also not changed significantly as compared to sham and also vehicle-treated MCA-occluded group.

4. Discussion

Brain injury resulting from stroke is a major problem, however the treatment for limiting the neuronal injury has proven to be elusive, primarily because the pathophysiology involved therein is not yet well understood (Read et al., 1999). With the upsurge in the information, that EAA and free radicals play a major role in stroke, new vistas has been opened for the potential use of EAA and antioxidant in stroke therapy. Reports have suggested that free radicals themselves cause EAA release, which in turn further release, the free radicals (Scultz et al., 1995). Therefore, agents which block the EAA mediated action, as well as those having free radical scavenging, could be a better approach as compared to the individual approach alone (Schmid-Elsaesser et al., 1999).

Adenosine, a neuromodulator has been reported to possess dual action, in that it inhibits the release of EAA, as well as scavenges the free radicals (Masino et al., 1999; Yavuz et al., 1997). Thus, it was considered worthwhile to investigate the effect of adenosine against acute ischemic stroke induced by MCA occlusion in rats.

In humans ischemic stroke, recirculation occurs frequently after cerebral ischemia. Resembling to this clinical setting, ischemia–reperfusion is induced in rats by mechanical clipping of MCA or photothrombotic occlusion of the vessels. However, the limitation of these models is that these involved craniotomy. MCA occlusion by intraluminal thread, which is relatively noninvasive method of inducing cerebral ischemia (Koizumi et al., 1986) was, therefore, used in the present study and the effect of adenosine was evaluated by DWI, neurological deficits and by estimation of oxidative stress markers.

DWI is sensitive to early changes due to ischemia and is superior to routine T_1 and T_2 MR-weighted imaging which is not best suited to detect infarcts during the initial hours after the onset of stroke. DWI reveals early ischemic lesions as regions of increased signal intensity, i.e., decreased water diffusitivity (Lo et al., 1997). Several studies have reported a similar significant correlation between 2, 3, 5 triphenyltetrazolium hydrochloride (TTC) derived postmortem infarct volume and the DWI derived ischemic lesion after reperfusion and suggested that early in vivo estimation by DWI could be used for evaluation of therapeutic efficacy of neuroprotective agents (Tatlisumak et al., 1998; Minematsu et al., 1992a,b).

In the present study, DWI exhibits hyperintensity in the lateral caudate putamen, and parts of the lower frontoparietal cortex after MCA occlusion followed by reperfusion. The signal intensity in right cerebral hemisphere was significantly enhanced which reflects the ischemic changes after occlusion of right MCA. This may be attributed to the failure of energy metabolism which leads to an influx of Na^+ and osmotically driven water into cells. The shift of water into cell results in restricted diffusion of water protons leading to hyperintensity in DWI which is confined to the region of MCA (Lo et al., 1997). In adenosine-treated rats, the signal intensity of the right cerebral hemisphere was not as significantly high in comparison to contralateral hemisphere. The infarct volume was also significantly decreased as compared to the vehicle-treated MCA-occluded rats. These results might be due to the beneficial effect of adenosine in acute phase of ischemia. The findings are in accordance with the observation of Muller et al. (1996) who reported that U743899, a free radical scavenger attenuated the enhanced signal intensity and decreased volume of infarct, which occurred due to MCA occlusion. Various other studies have also demonstrated the protective effect of drugs using diffusion-weighted imaging (Ebisu et al., 2001; Shi et al., 2001).

MCA occlusion significantly increased the level of MDA at 2 h after reperfusion as compared to that in the sham-operated rats. Increased level of MDA in the postischemic period suggests the increased load of free radicals during reperfusion, which could be contributing, if not solely responsible to neuronal injury. Earlier studies have also reported the increased level of MDA after reperfusion in model of bilateral carotid occlusion in gerbil (Yavuz et al., 1997). In rats, which were treated with adenosine, there was a significant decrease in the levels of MDA suggesting that adenosine would have scavenged the free radicals and thereby decreasing the oxidative stress. There was insignificant change in the level of glutathione at 2 h after reperfusion as compared to that in the sham-operated rats. Glutathione is an endogenous antioxidant found in all animal cells and reacts with the free radicals and can protect from singlet oxygen, hydroxide and superoxide radical damage. The insignificant change in the glutathione could be because there must be adequate cytosolic glutathione stores available in the brain to scavenge free radicals, so as to get depleted at least during the acute phase of oxidative stress.

MCA occlusion caused significant impairment in motor performance, which was evident from the increased neurological score. This corroborates clinically the neuronal damage in the territory of MCA occlusion, i.e., caudate putamen and cortex that control the motor function. Adenosine-treated MCA-occluded rats showed improvement in neurological function as compared to the vehicle-treated MCA-occluded rats. The finding suggests that the improvement in neurological function could be due to decreased size of infarction in territory of MCA as is also seen in the DWI images in the present study.

This is the first study to demonstrate the protective effect of adenosine against ischemia reperfusion injury using DWI. The decrease in ischemic volume demonstrated in DWI was further substantiated by the neurological evaluation and the decrease in the markers of oxidative stress and

that the antioxidant property of adenosine can be one of the mechanism involved in the neuronal protection.

In recent clinical trials with thrombolytic therapy, e.g., recombinant tissue plasminogen activator, though reperfusion could be established, a significant pan necrosis occurred due to sudden oxidative stress. The present study is of clinical significance since adenosine has been shown to have neuroprotective effect in reperfusion injury. Thus, the protection of neurons shown by adenosine after reperfusion indicates the beneficial effect of pharmacological intervention with adenosine. Therefore, neuroprotective treatment with adenosine in combination with thrombolytic may prove more beneficial in the treatment of acute ischemic stroke.

References

Chan PH. Role of oxidants in ischemic brain damage. Stroke 1996; 27:1124–9.

Ebisu T, Katsuta K, Fujikawa A, Aoki I, Umeda M, Naruse S, Tanaka C. Early and delayed neuroprotective effects of FK506 on experimental focal ischemia quantitatively assessed by diffusion-weighted MRI. Magn Reson Imaging 2001;19:153–60.

Ellman GL. Tissue sulphyldryl groups. Arch Biochem Biophys 1959; 82:70–3.

Karl A, Rudolphi PS, Fiona E, Bertil B. Neuroprotective role of adenosine in cerebral ischemia. TIPS 1992;13:439–45.

Kawamoto T, Ikeda Y, Teramoto A. Protective effect of L-histidine (singlet oxygen scavenger) on transient forebrain ischemia in rats. No-To-Shinkei 1997;49:612–8.

Koizumi J, Yoshida Y, Nazakawa T, Ooneda G. Experimental studies of ischemic bran edema: a new experimental model of cerebral embolism in rats in which recirculation can be introduced in the ischemic area. Jpn J Stroke 1986;8:1–8.

Lo EH, Pierce AR, Mandeville JB, Rosen BR. Neuroprotection with NBQX in rat's focal cerebral ischemia: effects on ADC probability distribution functions and diffusion perfusion relationship. Stroke 1997;28:439–47.

Masino SA, Mesches MH, Bickford PC, Dunwiddie T. Acute peroxide treatment of rat hippocampal slices induces adenosine mediated inhibition of excitatory transmission in area CA1. Neurosci Lett 1999;274: 91–7.

Mason RB, Pluta RM, Walbridge S, Wink DA, Oldfield EH, Boock RJ. Production of reactive oxygen species after reperfusion in vitro and in vivo. J Neurosurg 2000;93:99–107.

Minematsu K, Li L, Fisher M, Sotak CH, Davis MA, Fiandaca MS. Diffusion weighted magnetic resonance imaging: rapid and quantitative detection of focal brain ischemia. Neurology 1992a;42:235–40.

Minematsu K, Li L, Sotak CH, Davis MA, Fisher M. Reversible focal ischemic injury demonstrated by diffusion weighted magnetic resonance imaging in rats. Stroke 1992b;23:1304–11.

Muller TB, Haraldseth O, Jones RA, Sebastiani G, Lindhoe CF, Unsgard G, Oksendal AN. Perfusion and diffusion weighted MR imaging for in vivo evaluation of treatment with U74389G in a rat stroke model. Stroke 1996;26:1453–8.

Okhawa H, Ohishi N, Yagi K. Assay of lipid peroxides in animals tissue by thiobarbituraic acid reaction. Anal Biochem 1979;95:351–8.

Read JS, Hirano T, Davis SM, Donnan GA. Limiting neurological damage after stroke. Drugs Aging 1999;14:11–39.

Schmid-Elsaesser R, Zausinger S, Hungerhuber E. Neuroprotective effects of combination therapy with trilazad and magnesium in rats subjected to reversible focal cerebral ischemia. Neurosurgery 1999;44:163–71.

Scultz JB, Henshaw DR, Siwek D, Jenkins BG, Ferrante RJ, Cipolloni PB, Kowall NW, Rosen BR, Beal MF. Involvement of free radicals in excitotoxicity in vivo. J Neurochem 1995;64:2239–47.

Shi J, Bui JD, Yang SH, He Z, Lucas TH, Buckley DL, Blackband SJ, King MA, Day AL, Simpkins JW. Estrogens decrease reperfusion-associated cortical ischemic damage: an MRI analysis in a transient focal ischemia model. Stroke 2001;32:987–92.

Sinha K, Deogonakar M, Jagannathan NR, Gupta YK. Effect of melatonin on ischemia reperfusion injury induced by middle cerebral artery occlusion in rats. Eur J Pharmacol. (in press).

Tatlisumak T, Carano RAD, Takano K, Sotak CH, Fisher H. A novel endothelium antagonist, A-127722, attenuates ischemic lesion size in rats with temporary middle cerebral artery occlusion a diffusion and perfusion MRI study. Stroke 1998;29:850–7.

Yavuz O, Turkozkan N, Bilgihan A, Dogulu F, Aykol S. The effect of 2-chloro adenosine on lipid peroxide level during experimental cerebral ischemia–reperfusion in gerbils. Free Radical Biol Med 1997;22: 337–41.