Neuroprotective Effect of Cilostazol against Focal Cerebral Ischemia via Antiapoptotic Action in Rats

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ABSTRACT

This study examined the protective effects of cilostazol on cerebral infarcts produced by subjecting rats to 2-h occlusion of the left middle cerebral artery followed by 24-h reperfusion. The ischemic cerebral infarct consistently involved the cortex and striatum. The infarct size was significantly reduced, when rats received 10 mg/kg cilostazol intravenously 5 min or 1 h after the completion of 2-h ischemia. Cyclic AMP level was significantly elevated in the cortex of 4- and 12-h reperfusion (P < 0.01) following treatment with cilostazol (10 mg/kg, 5 min after 2-h ischemia) accompanied by decreased tumor necrosis factor-α level. Samples from the regions corresponding to the penumbra showed markedly reduced Bcl-2 protein level and, in contrast, high levels of Bax protein and cytochrome c release. Cilostazol decreased Bax protein and cytochrome c release and increased the levels of Bcl-2 protein. Cilostazol (10^-7–10^-5 M) potently and concentration dependently scavenged hydroxyl and peroxyl radicals. In conclusion, cilostazol treatment decreases ischemic brain infarction in association with inhibition of apoptotic and oxidative cell death.

Ischemic neuronal death including development of an infarct has been recently ascribed in part to the programmed cell death (Linnik et al., 1995; Chopp and Li, 1996). Transient focal ischemia initiates a cascade of detrimental events including accumulation of intracellular calcium, formation of free radicals and cytokines (tumor necrosis factor-α and interleukin-1β), which lead to disruption of cellular homeostasis and structural damage of ischemic brain tissue (Kochanek and Hallenbeck, 1992; Feuerstein et al., 1994). Ischemia results in the activation of cytosine proteases of the caspase family, alterations in plasma membrane phospholipids, and nuclear DNA condensation and fragmentation (Bredesen, 1995). During apoptosis, free radicals are known to induce lipid peroxidation, DNA damage (Dirnagl et al., 1999), and open the mitochondrial membrane permeability transition pore opens, mediating release of cytochrome c from mitochondria that activates caspasases, finally producing apoptosis (Chen et al., 1997; Kluck et al., 1997). Therefore, treatment with antioxidants is effectively useful in suppressing neuronal damage (Huh et al., 2000).

During apoptosis, Bcl-2 allows cells to adapt to an increased state of oxidative stress by suppressing the programmed cell death, either by counteracting the radical overproduction imposed by cell death stimuli or by fortifying the cellular antioxidant defenses (Hockenbery, 1995; Chen et al., 1997). On the other hand, Bax is involved in the programmed cell death (Oliva et al., 1993; Hockenbery, 1995).

Cilostazol was introduced to increase the intracellular level of cyclic AMP by blocking its hydrolysis by type III phosphodiesterase (Kimura et al., 1985) and is approved for use for treating intermittent claudication by the Food and Drug Administration (Dawson et al., 1998). Its principal actions include inhibition of platelet aggregation (Kimura et al., 1985; Kohda et al., 1999), antithrombosis in feline cerebral ischemia, and vasodilation via mediation of increased cyclic AMP level (Tanaka et al., 1989).

In the current study, we sought to examine the potential neuroprotective effects of cilostazol on the cerebral infarct size and on the DNA fragmentation after subjecting the rats to 2-h occlusion of MCA and 24-h reperfusion. We performed DNA fragment assay to identify the apoptotic cell damage, and analyzed the changes in Bcl-2, as well as Bax level and cytochrome c release from mitochondria after treatment with cilostazol.

Materials and Methods

Preparation of Animals. All animal studies conformed to the guidelines outlined in the Guide for Animal Experiments edited by...

ABBREVIATIONS: MCA, middle cerebral artery; TNF, tumor necrosis factor; EPR, electron paramagnetic resonance; PRAC, peroxyl radical absorbing capacity; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; ROS, reactive oxygen species; Ro-201724, 4-[3-butoxy-4-methoxy-benzyl]-2-imidazolidinone.
Focal cerebral ischemia was induced by occlusion of the left MCA as described elsewhere. Surgical nylon suture thread (3–0 in size) was inserted into the lumen of the internal carotid artery to block the flow of the middle cerebral artery. Two hours after middle cerebral artery occlusion, reperfusion was allowed by withdrawal of the suture thread until the tip cleared the internal carotid artery. Mean arterial blood pressure and blood gas and pH were not significantly different from those in control.

The cilostazol was dissolved in dimethylsulfoxide as a 30 mg/ml stock solution and diluted to 10 mg/ml with phosphate-buffered saline.

**Analysis of Cerebral Infarct.** At 24 h of reperfusion after 2-h MCA occlusion, rats were given an overdose of thiopental sodium and decapitated, and then the brain was quickly removed and frozen in liquid nitrogen. The brain was cut in a 2-mm thick coronal block. The brain slices were immersed in 2% solution of 2,3,5-triphenyltetrazolium chloride in normal saline at 37°C for 30 min and then fixed in 10% phosphate-buffered formalin at 4°C. The 2,3,5-triphenyltetrazolium chloride-stained brain slices were photographed using a charge-coupled device video camera and the size of an infarct was calculated with the image analysis system (Image-Pro Plus; Media Cybernetics LP, Silver Spring, MD) and expressed as the percentage of ipsilateral hemisphere. Infarct volume (in cubic millimeters) was determined by measuring the appropriate area by the interval thickness of each hemisphere. Infarct volume (in cubic millimeters) was determined by calculating the area under curve of fluorescence just prior to addition of the 2,2'-azobis(2-amidinopropane) hydrochloride (2.2′-azobis(2-amidinopropane) hydrochloride was estimated as the 100% value for that sample. The PRAC values were calculated as follows: PRAC = [area of compound – area of blank]/area of 1 μM trolox – area of blank, where 1 PRAC unit is equivalent to the value of 1 μM trolox.

**DNA Fragmentation Assay.** After 2-h MCA occlusion/24-h reperfusion, samples were dissected from the region corresponding to the penumbra zone and contralateral control areas. The brain was cut in a cryostat to produce a standard coronal block. For oligonucleosomal fragmentation of genomic DNA, cells were lysed in 1 ml of lysis buffer (10 mM Tris-Cl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1% sodium dodecyl sulfate, and 0.5 mg/ml proteinase K). Digestion was continued for 1 to 3 h at 55°C, followed by addition of ribonuclease A to 0.1 mg/ml and running dye (10 mM EDTA, 0.25% bromophenol blue, 50% glycerol). Equivalent amounts of DNA (15–20 μg) were loaded into wells of a 1.6% agarose gel and electrophoresed in 0.5 × Tris-acetate EDTA buffer (40 mM Tris-acetate, 1 mM EDTA) for 2 h at 6 V/cm. DNA was visualized by ethidium bromide staining. Gel pictures were taken by the UV transillumination with a Polaroid camera.

**Western Blot Analyses.** After 2-h MCA occlusion/24-h reperfusion, the samples corresponding to the penumbra zone were homogenized, and cells were lysed in lysis buffer containing 50 mM Tris-Cl (pH 8.0); 150 mM NaCl; 0.02% sodium azide; 100 μg/ml phenylmethylsulfonyl fluoride; 1 μg/ml aprotenin, and 1% Triton X-100. Following centrifugation at 12,000 rpm, 50 μg of protein of each sample was loaded into 12% SDS-polyacrylamide electrophoresis gel and transferred to nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ). The blocked membranes were then incubated with the antibody to Bcl-2 and Bax (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Mitochondrial cytochrome c was prepared following procedures. After MCA occlusion-reperfusion, the samples corresponding to the penumbra zone were washed in ice-cold phosphate-buffered saline and homogenized in buffer A (20 mM Hepes-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl2, 1 mM Na-EDTA, 1 mM Na-EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride) containing 250 mM sucrose and then centrifuged twice at 750g for 10 min at 4°C. The harvested supernatants were again centrifuged at 10,000g for 10 min at 4°C, and the resulting mitochondrial pellets were dissolved in the 1× SDS sample buffer. Western blots were preformed as described above with the antibody to cytochrome c (Santa Cruz Biotechnology, Inc.). The immunoreactive bands were visualized using chemiluminescent reagent of the SuperSignal West Dura Extended Duration Substrate kit (Pierce Chemical, Rockford, IL).

**Drugs.** Cilostazol (6-[4-(1-cyclohexyl-1H-tetrazol-5-yl)butoxy]-3,4-dihydroxy-2-[1H]-quinolinone) was generously donated from Otsuka Pharmaceutical Co. Ltd (Tokushima, Japan). Trolox (6-hydroxy-2,5,7,8-tetramethoxychroman-2-carboxylic acid; Alexis Biochemicals, San Diego, CA) was dissolved in dimethylsulfoxide as a stock of 10 mM. DMPO (Sigma-Aldrich, Seoul, Korea) was purified by double distillation and stored at −70°C before use. β-Phycoerythrin (Sigma-


Statistical Analysis. Differences between data of infracted area and volume in each section between groups were evaluated by performing the Wilcoxon test. Two-way repeated measures analysis of variance were used for comparison of the results of PRAC assay. Other data were analyzed with Student’s t test for comparison of two means. Results are expressed as means ± S.E.M. Differences were considered to be significant when P < 0.05.

Results

Effect of Cilostazol on Infarct Size and Volume. The ischemic zone was consistently identified in the cortex and striatum of the left cerebral hemisphere as a distinct pale-stained area in the rats subjected to 2-h ischemia/24-h reperfusion. The infarct area was significantly reduced when the animals received cilostazol (10 mg/kg) 5 min or 1 h after the completion of 2-h ischemia, respectively. It was, however, not the same case when rats received the drug 3 h after 2-h ischemia (Fig. 1). Accordingly, the infarct volume (vehicle, 162.1 ± 31.1 mm³) was significantly diminished to 67.2 ± 28.2 mm³ and 70.5 ± 17.6 mm³ in the cilostazol-treated group when administered at 5 min or 1 h after the completion of 2-h ischemia, respectively (Fig. 1, Inset).

Figure 2 shows the comparison of 3 mg/kg with 10 mg/kg cilostazol on the hemispheric infarct area. Cilostazol, 10 mg/kg, but not 3 mg/kg, showed a significant suppression of hemispheric infarct size, when cilostazol was administered at 5 min after 2-h ischemia.

Antiapoptotic Effect. At 24-h of reperfusion after MCA occlusion, the samples corresponding to the penumbra zone were obtained. A strong staining for terminal deoxynucleotidyl transferase-mediated dUTP-biotin in situ nick-end labeling was present in a moderate to large number of cells in the vehicle-treated ischemic brain, which became more conspicuous at 48 h after reperfusion. The number of terminal deoxynucleotidyl transferase-mediated dUTP-biotin in situ nick-end labeling-positive stained cells was significantly reduced in the cilostazol-treated ischemic brains (data not shown).

The DNA was segmented at 180 to 200 base-pair intervals reflecting the activity of endonuclease cleavage of DNA at internucleosomal sites. DNA fragmentation was slowly increased when the time of cilostazol administration was increased from 5 min to 1 h or 3 h after the completion of 2-h ischemia. Reduction in DNA fragmentation was more prominent when cilostazol was administered at 5 min or 1 h rather than 3 h after the completion of 2-h ischemia (Fig. 3A). Treatment with 10 mg/kg cilostazol strongly suppressed the oligonucleosomal DNA laddering in contrast to the effect of 3 mg/kg cilostazol (Fig. 3B).

Fig. 2. Dose-dependent effect of cilostazol on the infarct area of each coronal section between vehicle- and cilostazol-treated rats subjected to 2-h ischemia/24-h reperfusion. Cilostazol (3 or 10 mg/kg, i.v.) was injected at 5 min after the completion of 2-h ischemia. Infarct area significantly decreased in the 2nd, 3rd, and 4th coronal sections by treatment with 10 mg/kg cilostazol. Results are expressed as means ± S.E.M. of six to seven experiments. +, P < 0.05; **, P < 0.01 versus vehicle.

Western Blot Analyses. Figure 4 shows Western blot results for Bcl-2 and Bax protein and release of cytochrome c following treatment with cilostazol. Samples from normal rats showed a considerable amount of Bcl-2 protein but trace level of Bax protein and cytochrome c release. When samples were obtained from rats subjected to 2-h MCA occlusion and 24-h reperfusion, Bcl-2 protein showed a markedly reduced level, whereas Bax protein and cytochrome c release greatly increased. Following treatment with cilostazol, both Bax protein and cytochrome c release were significantly reduced with increasing doses of cilostazol. In contrast, Bcl-2 level dose dependently increased.

Figure 5 illustrates the densitometric analyses of Western blot biochemical results. After MCA occlusion/reperfusion, Bcl-2 level was lowered to 13.6 ± 0.5% of normal control level, which was prominently recovered by treatment with 3 and 10 mg/kg cilostazol to 145.5 ± 14.1% (P < 0.001) and 250.5 ± 15.7% (P < 0.001), respectively, indicative of a full reverse of Bcl-2 protein by cilostazol (Fig. 5A). In contrast, Bax protein was markedly increased by focal ischemia reperfusion to 650.6 ± 33.8% of control, and this level was strongly suppressed by 10 mg/kg cilostazol to 206.1 ± 19.8% relative density (Fig. 5B). Cytochrome c release from mitochondria was also significantly increased by focal ischemia to 1187.8 ±
83.5% of control, which was dose dependently suppressed by 3 and 10 mg/kg cilostazol as shown in Fig. 5C.

Effect of Cilostazol on Cyclic AMP Level. The cyclic AMP levels in the hemispheres of untreated, nonischemic control rats were 3.1 \pm 0.5 \text{ pmol/mg of protein}, which was significantly elevated to 7.2 \pm 0.2 \text{ pmol/mg of protein} (P < 0.01) following treatment with cilostazol (10 mg/kg, i.v.). Following 2-h occlusion, rats were decapitated at 1, 4, 12, and 24 h, respectively. The cyclic AMP levels from brains subjected to 2-h ischemia followed by 4-h (1.3 \pm 0.3 \text{ pmol/mg of protein}) and 12-h reperfusion (1.3 \pm 0.4 \text{ pmol/mg of protein}) were significantly elevated to 4.9 \pm 0.4 and 3.8 \pm 1.0 \text{ pmol/mg of protein} (P < 0.01) by pretreatment with cilostazol, respectively (Fig. 6). However, the cyclic AMP levels after cilostazol treatment were little different between ipsilateral and contralateral side of brains subjected to MCA occlusion (data not shown).

Effect of Cilostazol on TNF-\(\alpha\) Levels. The level of TNF-\(\alpha\) in the untreated, nonischemic control hemispheres was 4.7 \pm 8.7 \text{ pg/mg of protein}. In the ischemic hemispheres, the level of TNF-\(\alpha\) was highly elevated to 48.7 \pm 5.3, 56.9 \pm 7.1, and 105.8 \pm 4.9 \text{ pg/mg of protein} at 1-, 4-, 12-h reperfusion after 2-h occlusion, respectively, and then returned to the basal level at 24-h reperfusion. Those increases were significantly suppressed by treatment with cilostazol (10 mg/kg, i.v., 5 min after the completion of 2-h ischemia) as shown in Fig. 7.

Hydroxyl Radical Scavenging Effect of Cilostazol. Figure 8 shows the EPR spectra of the spin adduct of DMPO

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**Fig. 3.** Representative findings of agarose gel electrophoresis showing DNA laddering. At 24-h of reperfusion after 2-h MCA occlusion, the samples corresponding to the penumbra zone were obtained. DNA from penumbra lesion showed signs of internucleosomal fragmentations evidenced by a ladder pattern. A, rats received cilostazol (10 mg/kg) at 5 min, 1 h, or 3 h after the completion of 2-h ischemia. Reduction in DNA fragmentation was more prominent in the samples obtained from rats that received cilostazol at 5 min rather than 1 h or 3 h after ischemia. B, cilostazol (3 and 10 mg/kg; CSZ-3 and CSZ-10) was administered at 5 min after 2-h MCA occlusion. The experiment was done in duplicate with different samples from different rats. M, Lambda DNA/HindIII marker; CSZ, cilostazol.

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**Fig. 4.** Representative findings of Western blot for Bcl-2, Bax proteins, and cytochrome c release from mitochondria in the penumbra regions isolated from normal control brains and cilostazol (3 and 10 mg/kg; CSZ-3 and CSZ-10)-treated brains. Cilostazol was administered at 5 min after the completion of 2-h occlusion of MCA. Both Bax protein and cytochrome c immunoreactivities were markedly increased, whereas Bcl-2 protein was decreased in the vehicle-treated ischemic brains. These variables were efficiently reversed by cilostazol treatment dose dependently. The experiment was conducted in duplicate with different samples from different rats. CSZ, cilostazol.

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**Fig. 5.** Densitometric analyses of Bcl-2, Bax proteins, and cytochrome c release following treatment with cilostazol (3 and 10 mg/kg; CSZ-3 and CSZ-10) in comparison with vehicle-treated group. Cilostazol was administered at 5 min after the completion of 2-h occlusion of MCA. Increased Bax protein and cytochrome c and decreased Bcl-2 protein shown in the vehicle group were substantially reversed by cilostazol treatment. Results are expressed as means ± S.E.M. of four experiments. CSZ, cilostazol. ###, P < 0.001 versus control; ***, P < 0.01 versus vehicle.
with the hydroxyl radical, DMPO/•OH, which was observed when DMPO reacted with hydroxyl radical generated by the Fenton system. Scavenging of the hydroxyl radicals was confirmed by using catalase (0.5–10 U/ml). Cilostazol potently inhibited the DMPO/•OH adduct formation in a concentration-dependent manner. The signals were almost completely suppressed by 10^{-5} M cilostazol. The concentration required for inhibiting the hydroxyl radical formation by 50% (IC_{50}) was 2.58 ± 0.07 μM. However, cilostazol did not inhibit the formation of DMPO/OOH (data not shown).

**Peroxyl Radical Absorbing Capacity (PRAC).** Peroxyl radical absorbing ability of cilostazol was examined using β-phycocerythrin. 2,2’-Azobis(2-amidinopropane) hydrochloride was used as a source of peroxyl radicals. Figure 9 shows the time-dependent decrease of β-phycocerythrin (β-PE) fluorescence in the absence (blank) and presence of different concentrations of cilostazol and trolox. When 2,2’-azobis(2-amidinopropane) hydrochloride was used as a source of peroxyl radicals, peroxyl radical absorbing ability of cilostazol was determined by employing β-PE. Inset, PRAC values were calculated. Each point represents means ± S.E.M. of four experiments. **, P < 0.01 versus control.
Discussion

The current study shows that cilostazol decrease of infarct size was associated with decreased oligonucleosomal DNA fragmentation, increased Bcl-2, decreased Bax protein, and reduced cytochrome c release from mitochondria. Furthermore, cilostazol increased cyclic AMP production and suppressed TNF-α in the ischemic cortex. This compound additionally showed a potent ability to scavenge hydroxyl and peroxyl radicals in in vitro experiments.

The present results showed that cerebral infarct volume was significantly reduced when the animals received cilostazol at 5 min and 1 h, but not 3 h, after the 2-h ischemia. Cilostazol is known to inhibit platelet aggregation and produce vasorelaxation via activation of cyclic AMP as a type III phosphodiesterase inhibitor (Tanaka et al., 1989). Cilostazol was recently approved by the Food and Drug Administration for treatment of intermittent claudication (Dawson et al., 1998). Gotoh et al. (2000) reported that cilostazol treatment achieves a considerable risk reduction (about 41.7%) in patients with recurrent cerebral infarction. An elevation of cyclic AMP was demonstrated to suppress the generation of superoxide anion and hydrogen peroxide in alveolar macrophages (Takei et al., 1998). The present results show that cilostazol effectively scavenged hydroxyl and peroxyl radicals. Previous work (Kim et al., 2002) showed that cilostazol reduces intracellular hydrogen peroxide, highlighting the ability of cilostazol to react with a wide spectrum of radical molecules.

Yang et al. (1994) showed that increased infarct size observed at 24 h after MCA occlusion was significantly decreased in transgenic mice overexpressing human copper-zinc superoxide dismutase, suggestive of the importance of the oxygen free radicals in the ischemic brain injury. Cilostazol-induced reduction of cerebral infarct size may correlate with both ROS and increasing cyclic AMP. It is likely that the unique pharmacological profile of cilostazol, both increased cyclic AMP and scavenging effect of oxygen radicals, contributes to the current findings of inhibition of apoptosis and decreased cerebral infarct. The fact that increased cyclic AMP suppressed the generation of superoxide and hydrogen peroxide (Takei et al., 1998) might suggest the synergistic effect of cyclic AMP and oxygen radical scavengers. Whether cyclic AMP is mechanistically involved is uncertain because the rat cerebral cortex has low levels of phosphodiesterase III (Challiss and Nicholson, 1990).

TNF-α, a deleterious cytokine in stroke, was demonstrated to mediate inflammatory, thrombogenic, and vascular changes in association with brain injury (Kochanek and Hallenbeck, 1992; Feuerstein et al., 1994). Increased level of TNF-α in brain tissue has been found in cerebral ischemia (Lavine et al., 1998), causing neuronal cell death via induction of free radicals in glial cells (Hu et al., 1997) and apoptosis (Bühler et al., 2000). Recently, cyclic AMP elevating agents such as Ro-201724, amrinone, milrinone, and pentoxiphylline inhibited TNF-α production in rat hearts and glial cells (Yoshikawa et al., 1999). The ROS including H₂O₂ and its derived form, hydroxyl radical (Li et al., 1997), are implicated in the signaling pathways initiated by TNF-α, which is in turn involved in apoptosis (Kroemer et al., 1995; Bühler et al., 2000). In concert with these reports, it is suggested that decreased TNF-α level was closely related with increased cyclic AMP levels, and the free radical-scavenging action of cilostazol further ameliorates the consequences observed after cerebral ischemia by reducing TNF-α levels.

Recently, accumulating evidence points to a significant role for Bcl-2 and related proteins in promoting cell survival and cell death (Bredesen, 1995). Martinou et al. (1994) showed that overexpression of Bcl-2 in transgenic mice protects neurons from ischemia-induced cell death. ROS, including H₂O₂ and hydroxyl radical, and lipid hydroperoxides are all implicated in the processes of apoptosis (Kroemer et al., 1995; Li et al., 1997), and they mediate cytokine (i.e., TNF-α and IL-1α)-induced apoptosis in rat mesangial cells (Bühler et al., 2000). Bcl-2 protects the integrity of mitochondrial oxidative phosphorylation and thus limits mitochondrial dysfunction induced by several apoptosis stimuli (Kluck et al., 1997). In our results, low levels of the Bcl-2 and high levels of Bax were found in the penumbra regions of ischemic brains in association with increased cytochrome c release. Interestingly, even postischemic administration of cilostazol could reverse these increased levels of Bax and cytochrome c as well as the decreased Bcl-2 levels induced by MCA occlusion.

Bax is believed to be a cell-death effector, the activity of which is neutralized by binding of Bcl-2 (Sato et al., 1994). Our results that cilostazol strongly suppressed MCA occlusion-induced up-regulation of Bax protein provide support for the postulate that cilostazol can protect against anti- ischemia-induced apoptosis. Mitochondria is an important regulatory site of apoptosis (Kroemer, 1998), especially in relation to the rise of cytochrome c release from mitochondria to cytosol, thereby governing apoptosis (Zhang et al., 2000). Most recently, it was suggested that Bcl-2 prevents the loss of the mitochondrial membrane potential and the release of cytochrome c to cytosol (Gross et al., 1999), whereas Bax protein promotes apoptosis by triggering the release of cytochrome c from mitochondria and activation of caspase cascade (Jürgensmeier et al., 1998). ROS produced endogenously are known to enhance the permeability of the mitochondrial membrane and the release of cytochrome c to the cytosol (Shimizu et al., 1999). Consistent with other reports, our results show up-regulation of Bcl-2 and down-regulation of Bax protein and cytochrome c release that co-occur with an impressive neuroprotective effect of cilostazol to suppress the DNA fragmentation and brain infarct due to ischemic injury after MCA occlusion.

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References


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