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Protective effects of cyclosporine and allopurinol on transient global cerebral ischemia in gerbils

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Abstract: The effects of cyclosporine and allopurinol on neuronal death following global cerebral ischemia were evaluated in Mongolian gerbils. The animals were randomly divided into four groups of 12 each: (1) sham operation as control, (2) occlusion of the bilateral common carotid arteries for 12 min and treatment with physiological saline, (3) occlusion plus treatment with 5 mg/kg of cyclosporine, and (4) occlusion plus treatment with 100 mg/kg of allopurinol 30 min before cerebral ischemia and daily thereafter for 6 days. On the 7th day after ischemia or sham operation, the gerbils' brains were removed. The number of necrotic pyramidal cells in the cortex and hippocampal CA1 was evaluated and tissue chemiluminescence (reflecting the presence of superoxide radicals) and lipid peroxides were examined. The number of necrotic pyramidal cells in each field of view (×100) of the cortex was 115 ± 79 after ischemia, which was significantly larger than 14 ± 8 in the control group, and was 45 ± 33 and 60 ± 49 after treatment with cyclosporine and allopurinol, respectively. The number of surviving pyramidal cells per mm length after ischemia in CA1 was 37 ± 14, which was significantly smaller than 174 ± 30 in the control group, but 78 ± 31 following treatment with was cyclosporine, and 108 ± 53 with allopurinol. A reduced number of necrotic pyramidal cells was associated with lower tissue chemiluminescence and lipid peroxides. The results suggest that both cyclosporine and allopurinol can inhibit neuronal death after global cerebral ischemia, and that autoimmunization and superoxide radicals are partially responsible for neuronal death.

Key words: Cerebral ischemia, Cyclosporine, Allopurinol, Superoxide radicals, Lipid peroxidation

Introduction

Recently more and more studies have implicated immunocytes such as polymorphonuclear leukocytes (PMNLs) in cerebral ischemia and reperfusion injury. PMNLs penetrate into the cerebral ischemic area as early as 24 h following ischemic insult [1, 2]. Inhibition of PMNLs with monoclonal antibody or suppressing no-reflow in animals with leukopenia [3] maintains electroencephalographic activity and preserves the cortical peak of the evoked response during and after cerebral ischemia [4].

Transplanted organs are subjected to ischemia and reperfusion injury to similar extents. It is well known that immunosuppressants such as cyclosporine and glucocorticoid play an important role in the success of organ transplantation by preventing immunocytes from being activated by tissue heterogeneity.

Activated PMNLs are also a major source of superoxide radicals. Treatment with superoxide radical scavengers such as allopurinol or superoxide dismutase were reported to improve the recovery of somatosensory evoked potentials after 4 h of reperfusion [5] and alleviate the delayed neuronal death following global cerebral ischemia [6].

Using the model of delayed neuronal death developed by Kirino [7], this study was conducted to evaluate the role of superoxide radicals generated from immunocytes in cerebral ischemic injury and to determine if cyclosporine, an immunosuppressant, and allopurinol, a superoxide radical scavenger, could protect pyramidal cells from death with respect to the histopathological changes in the cortex and hippocampal CA1, tissue superoxide radicals, and lipid peroxides.

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Materials and methods

The experimental protocol was approved by the Animal Care and Use Committee of Dokkyo University and was followed with strict adherence to the NIH guidelines for the use of experimental animals.

Preparation of agents

Allopurinol (Sigma Chemical, St. Louis, MO, USA) was dissolved in 1 N NaOH and its pH was adjusted to 10.47 by adding 1 N HCl. Cyclosporine (Sandimmun R, Sando, Tokyo, Japan) was diluted with distilled water.

Experiment protocol

Forty-eight Mongolian gerbils weighing 80–90 g which supplied from animal experimentation center of Saitama were used. Anesthesia was induced with diethyl ether and maintained with 1%–2% halothane during surgery. The animals were randomly assigned to one of four groups: the control and those treated with intraperitoneal injection of physiological saline, 5 mg/kg of cyclosporine, or 100 mg/kg of allopurinol 30 min before global cerebral ischemia and daily thereafter for 6 days.

A radiation lamp was used to keep the gerbils' body temperature at 37°C during the surgical operation. A 1-cm incision was made at the middle line of the anterior neck. Bilateral common carotid arteries were carefully freed from the surrounding tissues by microsurgical techniques and were clamped, except for the sham-operated gerbils, for 12 min using two Heifetz clips simultaneously. The clips were then released and the spontaneous circulatory reperfusion was identified by gross observation. On the 7th day following ischemia or sham operation, gerbils were decapitated under ether anesthesia and their forebrains were removed within 2 min. Each forebrain was cut roughly into three portions in the coronal direction: the front portion was used for measuring superoxide radicals in situ by chemiluminescence, the middle portion for examining neuronal death in the cortex and CA1, and the back portion for determining lipid peroxides in cerebral tissue.

Measurement of superoxide radicals

The removed middle portion of forebrain was immediately immersed in liquid nitrogen for 5 min, and then four tissue sections 18 μm thick were removed at −20°C using a cryostat.

Superoxide radicals were determined indirectly according to the method of Hayashi et al. [8]. This determination is based on the principle that chemiluminescence may be emitted when 2-methyl-6-phenyl-3,7-dihydroimidazo[1,2a]-prazin-3-one solution (CLA-phenyl) reacts with superoxide radicals. The specificity of this method has been confirmed by Nakano et al. [9]. The detailed procedure is as follows: The bottom of a small transparent plastic box, Tissue-Tek (Miles IN, USA) was covered with a piece of millipore filter (CA 250/0, Schuell Dassel, Germany) wetted with 0.1 ml of saturated CLA-phenyl (A5307, Kasei Kogyo Tokyo, Japan). Each frozen cerebral tissue slice was directly placed on the millipore filter paper, thawing and becoming trapped in the millipores at room temperature. An ultrasensitive Polaroid film was placed under the Tissue-Tek and exposed to chemiluminescence of 380 nm given off from CLA-phenyl in the presence of superoxide radicals for 10 min in an entirely dark room. The light intensity and the exposed area not the films, which should correlate positively with the amount of superoxide radicals, were quantified by a camera–microcomputer picture analysis system equipped with TIAS software (ACI, Chiba, Japan) and were expressed as integrated light absorption (ILA, the product of the exposed area and light intensity). The ILA obtained from the four sections of sliced tissue were summed, representing the overall ILA of the removed forebrain.

Determination of lipid peroxides

The lipid peroxides in cerebral tissue was determined according to the method of Ohkawa et al. [10] and its level was expressed as nanomoles of malondialdehyde per gram of wet tissue.

Examination of histopathological changes

The middle portion of removed forebrain was fixed by 10% formaldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 week and then embedded in paraffin, sliced into 5-μm sections in the coronal direction, and stained with hematoxylin and eosin. Histopathological changes of the CA1 subfield in the hippocampus were examined by light microscopy (Olympus BH-2, Olympus, Tokyo, Japan) in a blind fashion and quantified as the number of surviving pyramidal neurons per mm length in CA1. Necrotic pyramidal neurons in the six layers of cortex were counted in three random fields (×100) and averaged as the number of necrotic neurons in each field.

Statistical analysis

The experimental data (mean ± SD) were analyzed by the Kruskal-Wallis test. If the value of H exceeded the level of significance ($P < 0.05$), the Wilcoxon multiple comparison method was used to evaluate the differences among the four groups. Linear regression was used to determine the correlation between the tissue

chemiluminescence and lipid peroxides. The level of significance was set at $P < 0.05$.

Results

The number of necrotic pyramidal cells in each cortex field (×100) was 14 ± 8 for the sham operation group, but was 115 ± 79 for the ischemia group treated with physiological saline. There was a significant difference between the two numbers. Compared with the ischemia group treated with physiological saline, significantly fewer necrotic pyramidal cells were found in the treatment with either cyclosporine or allopurinol: 45 ± 33 for cyclosporine and 60 ± 49 for allopurinol. The findings in the CA1 were similar to those in the cortex: the number of surviving pyramidal cells per mm of length was 174 ± 30 in the sham operation group, but was 37 ± 14 for the ischemia group treated with physiological saline ($P < 0.05$). Significantly more surviving pyramidal cells were found in the groups treated with cyclosporine or allopurinol (78 ± 31 and 108 ± 53, respectively, Table 1).

Although 7 days had elapsed since cerebral ischemia, a relatively strong chemiluminescence could still be detected in the ischemic animals treated with physiological saline. Such chemiluminescence was suppressed following the administration of cyclosporine, especially allopurinol (Table 1). The lipid peroxide level was also significantly lower than that in the sham operation group (Table 1).

A significantly positive correlation was found between the measurements of chemiluminescence and lipid peroxides in all groups except the sham operation group (Fig. 1). This verified the reliability of chemiluminescence measurement as a method for determining superoxide radicals.

Typical histopathological changes in CA1 of the four experimental groups are shown in Fig. 2. The CA1 subfield in the sham operation group was clear and intact, but in the group treated with physiological saline it was completely destroyed by the ischemia. In the groups

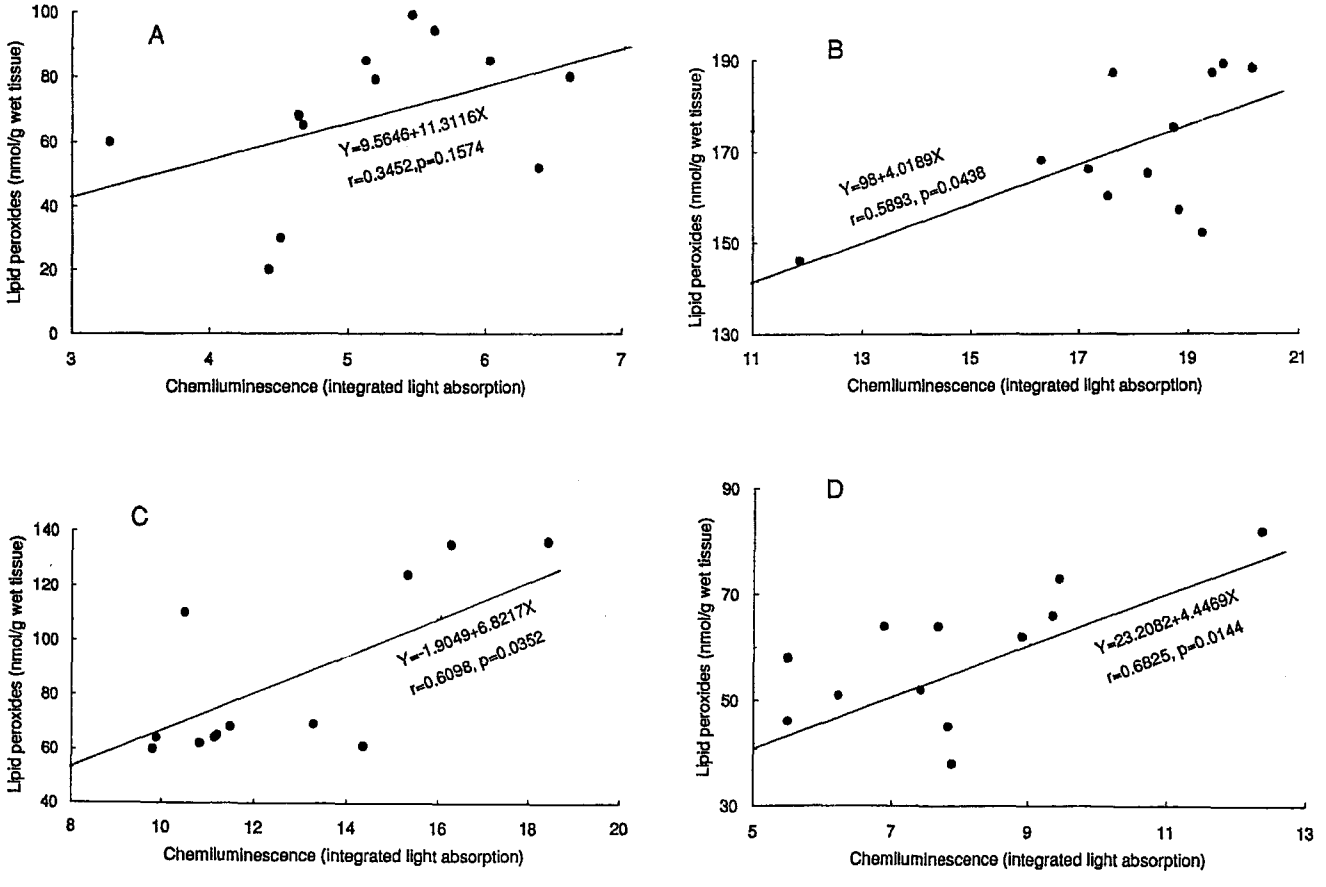

Fig. 1A–D The correlation between chemiluminescence and lipid peroxides in the four experimental groups. **A** The group treated with sham operation, **B** the ischemia group treated with physiological saline, **C** the ischemia group treated with cyclosporine, and **D** the ischemia group treated with allopurinol

Table 1. The effects of cyclosporine and allopurinol on neuronal death in the cortex and CA1, intensity of chemiluminescence, and lipid peroxides from cerebral tissue in gerbils subjected to 12 min of global ischemia and 7-day spontaneous reperfusion

	Cerebral ischemia group			
	Control	Saline	Cyclosporine	Allopurinol
Number of neuronal deaths in cortex (per ×100 field)	14 ± 8*	115 ± 79@	45 ± 33	60 ± 49
Number of neuronal cells surviving in CA1 (per mm length)	174 ± 30*	37 ± 14@	78 ± 31#	108 ± 53
Intensity of chemiluminescence from cerebral tissue	5.18 ± 0.27*	17.91 ± 0.64@	12.72 ± 0.81#	7.92 ± 0.56
Lipid peroxides in cerebral tissue (malondialdehyde nmol/g wet tissue)	68 ± 7†	170 ± 4@	85 ± 9#	56 ± 4

* $P < 0.05$ vs other three treatments in the same variable, @$P < 0.05$ vs cyclosporine and allopurinol treatments in the same variable, #$P < 0.05$ vs allopurinol treatment in the same variable, †$P < 0.01$ vs physiological saline treatment in the same variable.

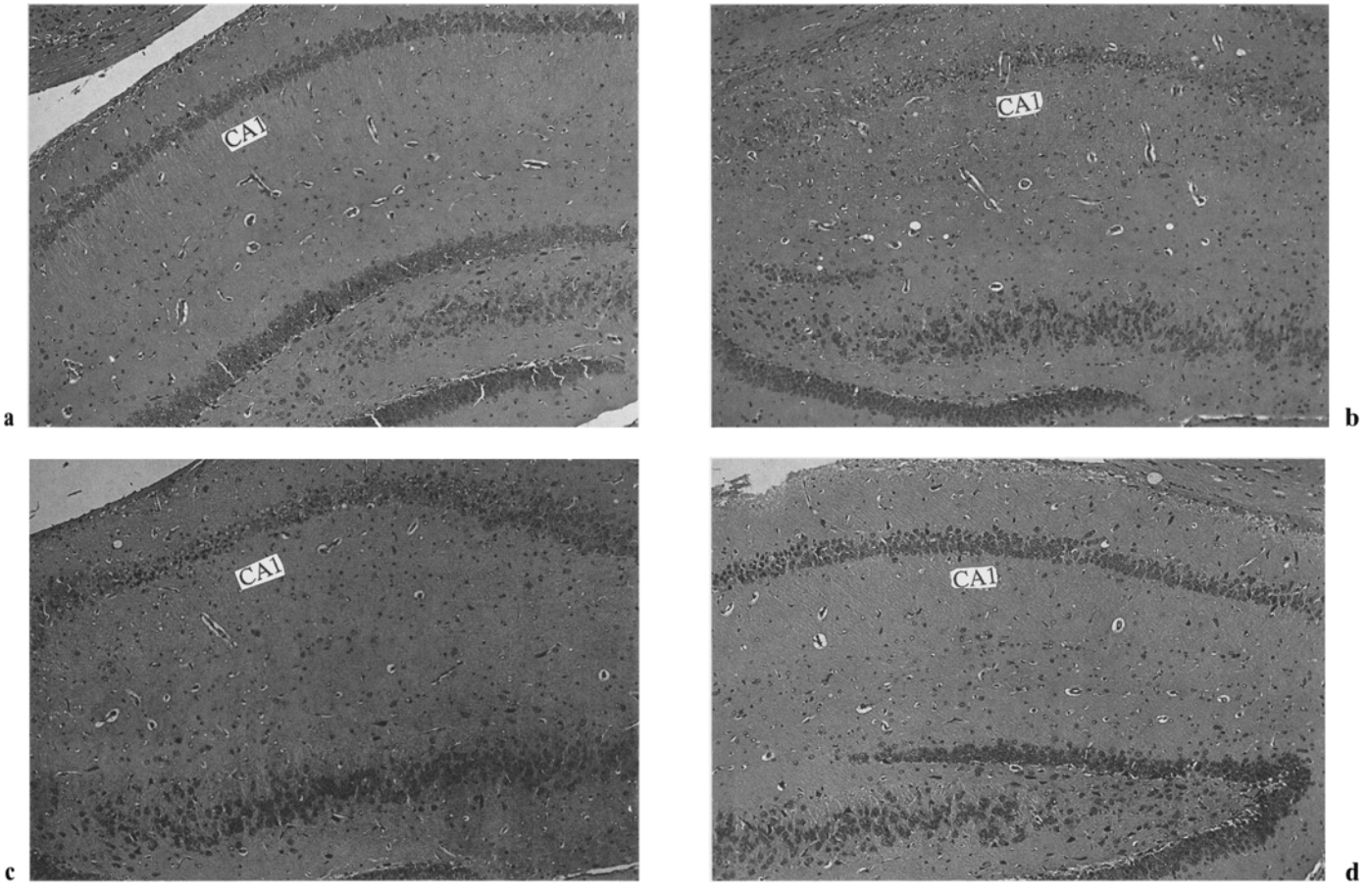

Fig. 2. **a** The hippocampus of a gerbil in the sham operation group. The pyramidal cells look normal and the subfield of CA1 is intact. **b** In the hippocampus of a gerbil subjected to 12-min global ischemia and 7-day spontaneous reperfusion and treated with physiological saline, almost all of the pyramidal cells in CA1 had died. **c** In the hippocampus of a gerbil subjected to ischemia and treated with cyclosporine, some pyramidal cells in CA1 died but some appear normal. **d** In the hippocampus of a gerbil subjected to same ischemia but treated ip with 100 mg/kg allopurinol daily, the pyramidal cells in CA1 appear normal. (H & E, ×100)

treated with cyclosporine or allopurinol, however, neuronal death was alleviated and CA1 was generally kept intact.

Discussion

In this study, the generation of superoxide radicals and lipid peroxidation were observed on the 7th day after global cerebral ischemia. Their changes following treatment with cyclosporine or allopurinol were associated with the suppression of neuronal death in both the cortex and CA1, suggesting that superoxide radicals play an important role in the pathogenesis of neuronal death.

Resuscitation of the brain after a period of global ischemia is determined by the degree of normalization of hemodynamic and metabolic disturbances. The former includes the no-reflow phenomenon [11] and postischemic hypoperfusion syndrome [12]. The no-reflow phenomenon is complicated and is associated with many factors such as increased blood viscosity [13], microcirculatory compression by swollen perivascular glial cells [14, 15], and disseminated intravascular coagulopathy [16]. Although the extent and severity of no-reflow depends on the type and duration of ischemia, it is a constant finding when ischemia is 10 min or longer [17]. The postischemic hypoperfusion syndrome always develops following the phase of reactive hyperemia after initiating cerebral reperfusion. It is also one of the causes of the non-flow phenomenon [18].

Although the improvement of hemodynamic disturbances may lead to the recovery of energy metabolism, evoked potential, spontaneous electrocortical activity, and even integrated neuronal function [19–21] from global ischemia as long as 1 h, "delayed neuronal death" in pathological terms is still seen from the 4th day after 5 min of global ischemia [7]. Until now the mechanism of delayed neuronal death has not been understood, but many studies have provided evidence that immunocytes if the particular type of immunocytes discussed here are believed to produce cytotoxins, may be involved in the pathogenesis of damage to the central nervous system during ischemia [22, 23]. These immunocytes, including neutrophils, mononuclear phagocytes, and microglia, infiltrated the ischemic area early after initiating ischemia [24–26]. In addition, administration of inhibitors to these cells has been shown to be effective in reducing ischemic injuries [25, 27]. The mechanism by which immunocytes add to ischemic injury is much more likely to be associated with their release of cytotoxins including superoxide radicals, leukotrienes, and prostaglandins [28, 29].

Cyclosporine is a potent immunosuppressant which inhibits T-lymphocyte activation [30], however it can also prevent neurological deterioration of patients with subarachnoid hemorrhage caused by the rupture of an aneurysm [31] because it relaxes cerebral vasospasm [32]. Ogawa et al. also reported that it could prevent the late onset reduction of muscarinic acetylcholine receptors which occurred in the hippocampus 7 days after 5 min of cerebral ischemia. This suggested that the immunological mechanism might be involved in progressive brain damage [33], however he found no attenuation of delayed neuron death after administering cyclosporine. The cause of the discrepancy between our study and his is not clear, although it may be attributed to the different dose of cyclosporine or the method of determination of pathological changes in pyramidal cells.

Allopurinol is a xanthine oxidase inhibitor with oxygen radical scavenging activity [34]. Administration of allopurinol before cerebral ischemia improved the recovery of somatosensory evoked potentials [5], brain edema, and infarct volume [35]. However, whether its mechanism is associated with scavenging oxygen free radicals is controversial [36, 37]. As the neuronal sensitivity to ischemia varies at different sites in the brain, the necrotic mechanism for the pyramidal cells in CA1 and in the cortex may also be different. The findings in this study showed that the alleviation of neuronal death in both the cortex and CA1 is commonly associated with the reduction of superoxide radicals and the associated lipid peroxidation. This not only suggests that superoxide radicals play an important role in neuronal death in both the cortex and CA1 but may also support the assumption that allopurinol alleviates ischemic injury by scavenging superoxide radicals.

Although cyclosporine is an immunosupressor, it is almost as effective as allopurinol, a superoxide radical scavenger, in reducing the superoxide radicals, the associated tissue peroxidation, and ischemic injuries to pyramidal cells. This implies that the action mechanism of cyclosporine may also relate to superoxide radicals and that auto-immunization may be involved in the pathogenesis of delayed neuronal death. Ischemic insult may alter the membrane antigenicity of the neurons that are susceptible to ischemia. Inflammatory cells such as lymphocytes and leukocytes may be activated and many cyctotoxic factors including superoxide radicals may be released and thus cause damage to the ischemic cells.

The improvement of neuronal necrosis by cyclosporine or allopurinol was still far from complete, underlining the complexity of the mechanism of neuronal death. We therefore conclude that superoxide radicals are partially responsible for neuronal death following ischemia.

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