

## Difference in transient ischemia-induced neuronal damage and glucose transporter-1 immunoreactivity in the hippocampus between adult and young gerbils

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### ABSTRACT

**Objective(s):** The alteration of glucose transporters is closely related with the pathogenesis of brain edema. We compared neuronal damage/death in the hippocampus between adult and young gerbils following transient cerebral ischemia/reperfusion and changes of glucose transporter-1 (GLUT-1)-immunoreactive microvessels in their ischemic hippocampal CA1 region.

**Materials and Methods:** Transient cerebral ischemia was developed by 5-min occlusion of both common carotid arteries. Neuronal damage was examined by cresyl violet staining, NeuN immunohistochemistry and Fluoro-Jade B histofluorescence staining and changes in GLUT-1 expression was carried out by immunohistochemistry.

**Results:** About 90% of pyramidal neurons only in the adult CA1 region were damaged after ischemia/reperfusion; in the young, about 53% of pyramidal neurons were damaged from 7 days after ischemia/reperfusion. The density of GLUT-1-immunoreactive microvessels was significantly higher in the young sham-group than that in the adult sham-group. In the ischemia-operated-groups, the density of GLUT-1-immunoreactive microvessels was significantly decreased in the adult and young at 1 and 4 days post-ischemia, respectively, thereafter, the density of GLUT-1-immunoreactive microvessels was gradually increased in both groups after ischemia/reperfusion.

**Conclusion:** CA1 pyramidal neurons of the young gerbil were damaged much later than that in the adult and that GLUT-1-immunoreactive microvessels were significantly decreased later in the young. These data indicate that GLUT-1 might differently contribute to neuronal damage according to age after ischemic insults.

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### Introduction

Although ischemic stroke occurs mainly in elderly individuals aged 65 years or older, nowadays, there are growing evidences of an increasing trend of childhood ischemic stroke (1, 2). Childhood ischemic stroke is generally considered as a rare and benign occurrence, with an incidence of at least 3.3/100,000 (3). However, childhood ischemic stroke is increasingly recognized as an important cause of disability and lifelong morbidity and it is among the top 10 causes of death in children (2).

Reperfusion following ischemia causes a wide

range of pathophysiological process that leads to further damage, and the process may be defined as ischemia/reperfusion injury (4, 5). Limited oxygen availability is associated with impaired endothelial cell barrier function (6) and a concomitant increase in vascular permeability and leakage (7). In addition, ischemia/reperfusion leads to the activation of cell death programs, including apoptosis, autophagy-associated cell death and necrosis (8). Some studies using ischemic animal models have shown that young animals are resistant to ischemia/reperfusion injury (9, 10). Recently, we also compared neuronal

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damage in the ischemic CA1 region between the young and adult gerbils after 5 min of transient cerebral ischemia/reperfusion and showed that the neuronal death in the hippocampal CA1 region of the young was more delayed and less than that in the adult (11-13). However, the precise mechanisms of the more delayed neuronal death in the young remain unclear.

Glucose is a major energy substrate of energy metabolism for the central nervous system (CNS). Neurons in adult brains have a higher energy demand and a continuous supply of glucose from blood (14). Glucose transporters (GLUTs) play critical roles in regulating glucose transportation and controlling the level of glucose in the brain (15, 16). Many studies have investigated that GLUT-1 is highly enriched in the endothelial cells of the blood brain barrier (BBB) (17-19).

A disruption of glucose uptake and utilization in the brain is anticipated to cause negative effects on the function and survival of the brain cells. It has been reported that the alteration of GLUT-1 is closely related with the pathogenesis of cerebral edema (20). GLUT-1 expression increases in response to focal and global ischemia in the adult rat brain (21, 22). Therefore, regulating GLUT-1 expression may ensure more glucose supply to ischemic areas. However, few studies regarding GLUT-1 changes in ischemic brains between the young and adult have been demonstrated. Therefore, in the present study, we compared chronological change of GLUT-1 immunoreactivity in the hippocampus of the young gerbil with that in the adult following 5 min of transient cerebral ischemia reperfusion.

## Materials and Methods

### Induction of transient cerebral ischemia

We used male Mongolian gerbils (*Meriones unguiculatus*) obtained from the Experimental Animal Center, Kangwon National University, Chunchon, South Korea. Gerbils were used at 1 (BW 25-30 g) and 6 months (BW 65-75 g) of age for the young and adult group. Experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Kangwon University and adhered to guidelines that are in compliance with the current international laws and policies (Guide for the Care and Use of Laboratory Animals, The National Academies Press, 8th Ed., 2011). The animals were divided into four groups: adult sham-operated-group, young sham-operated-group, adult ischemia-operated-group, and young ischemia-operated-group ( $n = 7$  at each point in time in each group).

Transient cerebral ischemia was developed according to a previously published method by us (13). In brief, the animals were anesthetized with a mixture of 2.5% isoflurane in 33% oxygen and 67% nitrous oxide. Ischemia/reperfusion was induced by

occluding both common carotid arteries for 5 min. The body (rectal) temperature under free-regulating or normothermic ( $37 \pm 0.5$  °C) conditions was monitored with a rectal temperature probe (TR-100; Fine Science Tools, Foster City, CA) and maintained using a thermometric blanket before, during and after the surgery until the animals completely recovered from anesthesia. Sham-operated animals were subjected to the same surgical procedures except that the common carotid arteries were not occluded.

### Tissue processing for histology

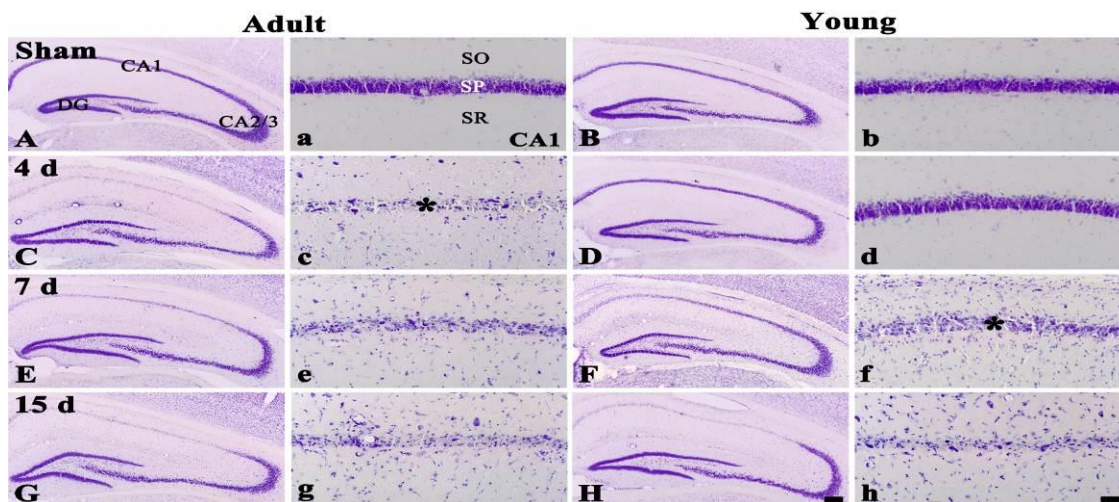
As described previously method (13), in brief, gerbils ( $n = 7$  at each point in time) in each group were sacrificed under anesthesia at designated times (1, 2, 4, 7 and 15 days after reperfusion) and perfused transcardially with 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in 0.1 M phosphate-buffer (PB, pH 7.4). The brains were serially sectioned into 30  $\mu$ m coronal sections on a cryostat (Leica, Wetzlar, Germany).

### Cresyl violet (CV) staining and Fluoro-Jade B (F-J B) histofluorescence

To investigate neuronal death in the hippocampus after ischemia-reperfusion, CV staining for normal cells and F-J B histofluorescence for dying or dead cells were performed. As described (13), in brief, the sections were stained with 1.0% (w/v) cresyl violet acetate (Sigma-Aldrich, St. Louis, MO, USA) and dehydrated by immersing in serial ethanol bath. For F-J B histofluorescence, the sections were immersed in a 0.0004% F-J B (Histochem, Jefferson, AR, USA) staining solution and examined using an epifluorescent microscope (Carl Zeiss, Göttingen, Germany) with blue (450-490 nm) excitation light and a barrier filter.

### Immunohistochemistry for neuronal nuclei (NeuN) and GLUT-1

Immunohistochemistry was carried out as described (13). In brief, the brain sections were blocked with 10% normal goat serum in 0.05 M PBS followed by staining with primary mouse anti-NeuN (a neuron-specific soluble nuclear antigen) (diluted 1:1,000, Chemicon International, Temecula, CA, USA) and rabbit anti-GLUT-1 (diluted 1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4 °C. The sections were next incubated with the secondary antibodies (Vector Laboratories Inc., Burlingame, CA, USA) and were developed using Vectastain ABC (Vector Laboratories Inc.). And they were visualized with 3,3'-diaminobenzidine in 0.1 M Tris-HCl buffer. In order to establish the specificity of the immunostaining, a negative control test was carried out with pre-immune serum instead of primary



**Figure 1.** CV staining in the hippocampus of the adult (left two columns) and young (right two columns) sham- (A, a, B and b) and ischemia-operated- (C – H and c – h) groups. In the adult ischemia-operated-groups, CV<sup>+</sup> cells are damaged in the stratum pyramidale (SP) from 4 days after ischemia/reperfusion (asterisk). However, in the young ischemia-operated-groups, CV<sup>+</sup> cells are damaged from 7 days post-ischemia (asterisk). SO, stratum oriens; SR, stratum radiatum. Scale bar = 800 (A – H) and 50 (a – h)  $\mu$ m

antibody. The negative control resulted in the absence of immunoreactivity in any structures.

#### Data analysis

As applied (13), the sections were selected according to anatomical landmarks corresponding to AP from -1.4 to -1.8 mm of gerbil brain atlas. The number of NeuN-immunoreactive and F-J B-positive cells was counted in a 250×250  $\mu$ m square, applied approximately at the center of the stratum pyramidale (SP) of the hippocampal CA1 region. Cell counts were obtained by averaging the total cell numbers from each animal per group: A ratio of the count was calibrated as %.

In order to quantitatively analyze GLUT-1 immunoreactivity, as described (23), briefly, the density of GLUT-1-immunoreactive structures was evaluated on the basis of a relative optical density (ROD), which was obtained after the transformation of the mean gray level using the formula:  $ROD = \log(256/\text{mean gray level})$ . A ratio of the ROD was calibrated as %, with the sham-group designated as 100 %.

#### Statistical analysis

Data are expressed as the mean  $\pm$  SEM. The data were evaluated by a Tukey test for *post-hoc* multiple comparisons following one-way ANOVA. Statistical significance was considered at  $P < 0.05$ .

## Results

#### CV-positive (CV<sup>+</sup>) cells

In the adult and young sham-operated-groups, CV<sup>+</sup> cells were well distributed in the hippocampus (Figures 1A, 1a, 1B and 1b). In the adult ischemia-operated-groups, a significant loss of CV<sup>+</sup> cells was observed in the stratum pyramidale (SP) of the CA1

region, not the other subregions, 4 days after ischemia/reperfusion (Figures 1C and 1c). Thereafter, the distribution pattern of CV<sup>+</sup> cells in the SP of the CA1 region was similar to that at 4 days post-ischemia (Figures 1E, 1e, 1G and 1g).

In the young ischemia-operated-groups, the distribution pattern of CV<sup>+</sup> cells in the SP of the CA1 region was not changed 4 days after ischemia/reperfusion (Figures 1D and 1d). However, at 7 days post-ischemia, the morphological damage of CV<sup>+</sup> cells was shown in the SP of the CA1 region (Figures 1F and 1f), thereafter, similar damage was found in the SP (Figures 1H and 1h).

#### NeuN+ and F-J B+ cells

In the adult sham-group, pyramidal neurons in the CA1 region were well immuno-stained with NeuN and no F-J B+ pyramidal neurons were found (Table 1, Figures 2A and 2a). In the young sham-group, the distribution pattern of NeuN+ and F-J B+ neurons in the CA1 region was similar to that in the adult sham-group (Table 1, Figures 2B and 2b).

In the adult ischemia-operated-groups, a significant loss of NeuN+ neurons (about 90% of the adult sham-group) was observed in the SP of the CA1 region at 4 days post-ischemia (Table 1, Figure 2C), and, at this point in time, many F-J B+ cells were observed in the SP of the CA1 region (Table 1, Figure 2c). Thereafter, the distribution pattern of NeuN+ and F-J B+ pyramidal neurons in the ischemic CA1 region was similar to that at 4 days post-ischemia (Table 1, Figures 2E, 2e, 2G and 2g).

In the young ischemia-operated-groups, NeuN+ pyramidal neurons in the CA1 region at 4 days post-ischemia were similar to those in the sham-group (Table 1, Figure 2D), and no F-J B+ cells were



**Table 1.** Changes in the mean average number of pyramidal neurons of the ischemic hippocampal CA1 region in the adult and young gerbils

Time after I-R	NeuN-immunoreactive neurons		F-J B-positive cells	
	Adult	Young	Adult	Young
Sham	90 ± 4.32	85 ± 3.65	0	0
4 days	9 ± 1.71*	86 ± 2.01*	50 ± 3.79*	0
7 days	10 ± 3.65*	40 ± 1.76*#	54 ± 1.24*	16 ± 1.11*#
15 days	13 ± 1.56*	42 ± 3.42*+	57 ± 3.41*	21 ± 3.03*+

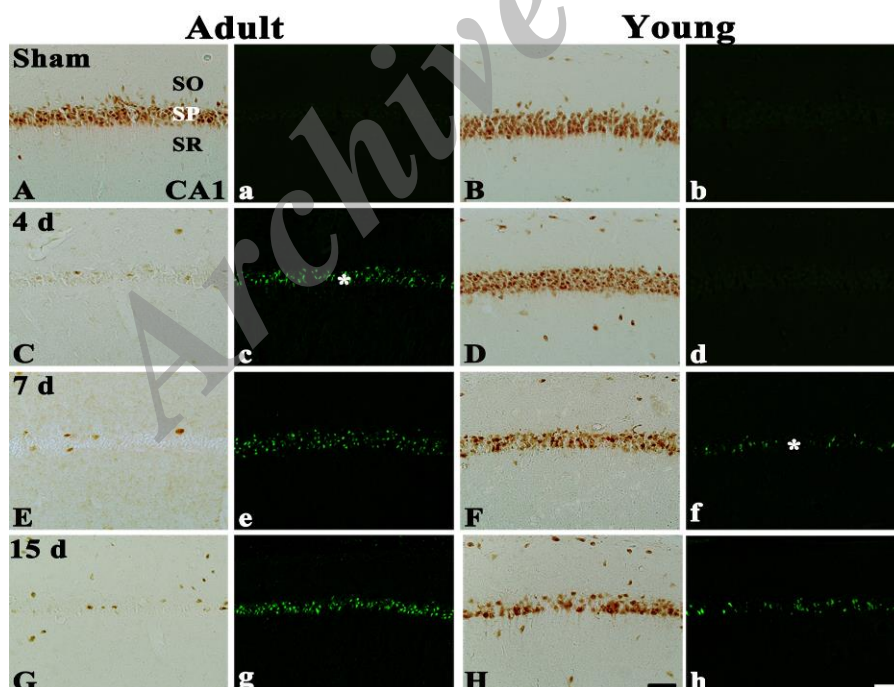
The mean number of NeuN-immunoreactive neurons and F-J B-positive cells was counted in a 250 X 250 µm square of the stratum pyramidale of the CA1 region after ischemia-reperfusion (I-R) (n=7 per group; \**P*<0.05, significantly different from the corresponding sham-group; #*P*<0.05, significantly different from the respective pre-time point group; +*P*<0.05, significantly different from the corresponding adult-group)

detected in the SP (Table 1, Fig. 2d). However, at 7 days post-ischemia, NeuN+ neurons were significantly decreased (about 52 % of the sham-group) in the SP of the CA1 region (Table 1, Figure 2F), and, at this point in time, many F-J B+ cells were found in the SP (Table 1, Figure 2f). Thereafter, distribution patterns of NeuN+ and F-J B+ pyramidal neurons were similar to those at 7 days post-ischemia (Table 1, Figures 2H and 2h).

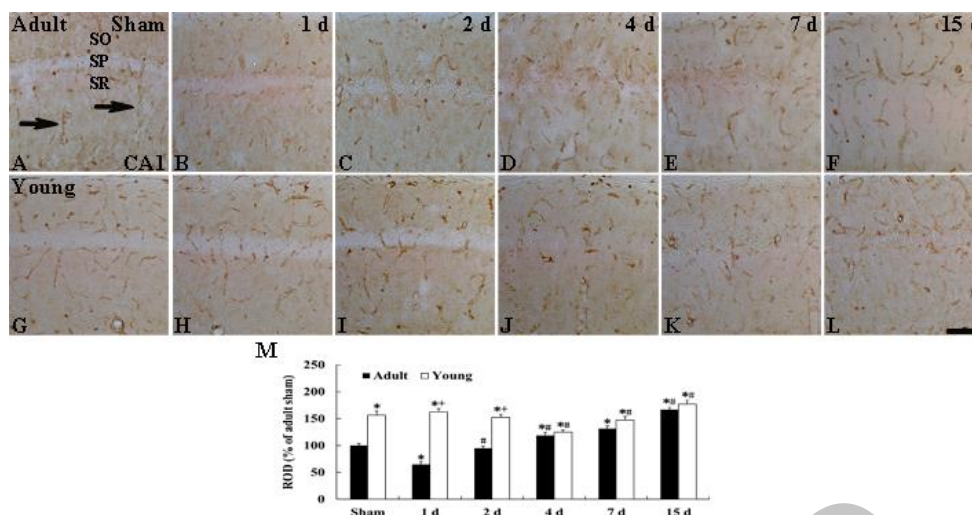
#### GLUT-1 immunoreactivity

CA1 region: GLUT-1-immunoreactive microvessels in the adult sham-group were easily observed in all layers of the CA1 region (Figure 3A).

In the young sham-group, the relative optical density (ROD) of GLUT-1-immunoreactive microvessels in the CA1 region was significantly higher (about 56% of the adult sham-group) than that in the adult sham-group (Figures 3B and 3M). In the adult ischemia-operated-groups, the ROD of GLUT-1-immunoreactive microvessels in the CA1 region was significantly decreased (about 35% of the adult sham-group) 1 day after ischemia/reperfusion compared with that in the adult sham-group (Figures 3B and 3M). At 2 days post-ischemia, the ROD of GLUT-1-immunoreactive microvessels was recovered to the ROD of the sham-group, thereafter, the ROD of GLUT-1-immunoreactive microvessels was gradually increased with time after ischemia/reperfusion (Figures 3C–3F and 3M).



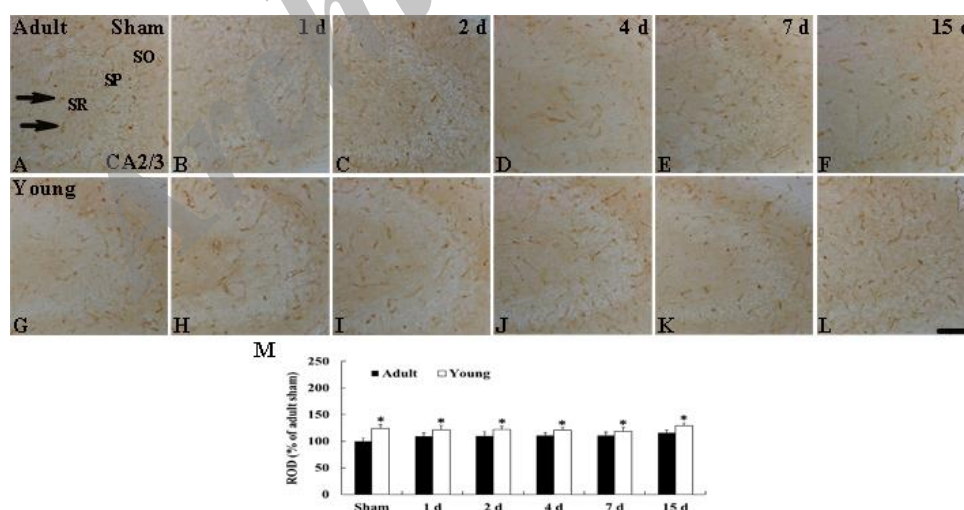
**Figure 2.** NeuN immunohistochemistry and F-J B histofluorescence staining in the CA1 region of the adult (left two columns) and young (right two columns) sham- (A, a, B, b) and ischemia-operated- (C – H and c – h) groups. In the adult ischemia-operated-groups, a few NeuN+ and many F-J B+ cells (asterisk) are shown in the stratum pyramidale (SP) from 4 days post-ischemia; however, NeuN+ cells are significantly decreased and F-J B+ cells (asterisk) are observed in the young ischemia-operated-groups from 7 days post-ischemia. SO, stratum oriens; SR, stratum radiatum. Scale bar = 50 µm



**Figure 3.** Immunohistochemistry for GLUT-1 in the CA1 region of the adult (upper column) and young (lower column) of the sham- (A and G) and ischemia-operated- (B-F, H-L) groups. GLUT-1-immunoreactive microvessels (arrows) in the young sham-group are higher in density than to those in the adult sham-group. In the ischemia-operated-groups, the density of GLUT-1-immunoreactive microvessels is distinctively decreased at 1 and 4 days post-ischemia, respectively, in the adult and young, and, thereafter, the density in both groups is gradually increased. SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum. Scale bar= 50  $\mu$ m. M: ROD as % of GLUT-1-immunoreactive structures in the adult and young groups (n= 7 per group; \* $P$ <0.05, significantly different from the young sham-group, # $P$ <0.05, significantly different from the respective pre-time point group; \* $P$ <0.05, significantly different from the corresponding adult ischemia-group). The bars indicate the means  $\pm$  SEM

In the young ischemia-operated-groups, the ROD of GLUT-1-immunoreactive microvessels in the CA1 region was not changed until 2 days post-ischemia (Figures 3H, 3I and 3M). Four days after ischemia-reperfusion, the ROD of GLUT-1-immunoreactive microvessels was decreased (about 20% of the young sham-group) (Figures 3J and 3M), thereafter, the ROD of GLUT-1-immunoreactive microvessels in the CA1 region was increased with time (Figures 3K, 3L and 3M).

**CA2/3 region:** The ROD of GLUT-1-immunoreactive microvessels in the CA2/3 region of the adult sham-group was similar to that in the CA1 region (Figure 4A). In the young sham-group, The ROD of GLUT-1-immunoreactive microvessels was also higher (about 24% of the adult sham-group) than that in the adult sham-group (Figures 4G and 4M). In the adult ischemia-groups, the ROD of GLUT-1-immunoreactive microvessels in the CA2/3 region



**Figure 4.** Immunohistochemistry for GLUT-1 in the CA2/3 region of the adult (upper column) and young (lower column) groups of the sham- (A and G) and ischemia-operated- (B-F, H-L) groups. GLUT-1-immunoreactive microvessels (arrows) in the young sham-group are higher in density than to those in the adult sham-group. In both ischemia-operated-groups, distribution patterns of GLUT-1-immunoreactive microvessels in the CA2/3 region are not significantly changed after ischemia-reperfusion. SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum. Scale bar = 50  $\mu$ m. M: ROD as % of GLUT-1-immunoreactive structures in the adult and young groups (n= 7 per group; \* $P$  < 0.05, significantly different from the young sham-group). The bars indicate the means  $\pm$  SEM

was not changed after ischemia/reperfusion (Figures 4B-4F and 4M). In addition, the pattern of GLUT-1 immunoreactivity in the young-ischemia groups was not changed after ischemia/reperfusion (Figures 4H-4L and 4M).

## Discussion

The Mongolian gerbil has been commonly used for making a good animal model to investigate mechanisms of neuronal death following transient global cerebral ischemia/reperfusion (24, 25), because about 90% of the gerbils lack the communicating arteries between the carotid and vertebral arteries. Thus, the bilateral occlusion of the common carotid arteries essentially and completely eliminates blood flow to the telencephalon while completely sparing the vegetative centers of the brain stem (26). Among the brain regions, pyramidal neurons of the hippocampal CA1 region are the most vulnerable to transient cerebral ischemic insult (27), and this neuronal death is called "delayed neuronal death" since the neuronal death occurs very slowly after ischemia/reperfusion (28-30). Therefore, we have chosen the gerbil as an animal model of transient cerebral ischemia to study this subject.

Among risk factors for ischemic stroke, age is an important in determining the outcome of cerebral ischemic injury. Until now, age-related studies have been focused on neuronal death using adult gerbils (31, 32), and some reports have demonstrated that neuronal death induced by transient cerebral ischemia occurs much later in the aged than in the adult (31-33). On the other hand, childhood ischemic stroke is increasingly recognized as an important cause of disability and lifelong morbidity, although cerebral ischemia occurs mainly in the older (2). A previous study showed a greater resistance to various periods of transient cerebral ischemia using from 2 week-old to 12 week-old gerbils (10). Some researchers have also reported that young animals are less vulnerable to brain ischemic insult (9, 34), and we recently reported that resistance to cerebral ischemic insults was different according to age; young and aged gerbils are more resistant to cerebral ischemia than the adult under the same condition (11, 32, 35). In the present study, using CV staining, NeuN immunohistochemistry and F-J B histofluorescence staining, we observed the neuronal death in the young gerbil was much more delayed and less severe than that in the adult. This result is similar to previous studies that showed that the young gerbil was resistant to ischemic damage (9, 10). In addition, we recently reported that endogenous anti-oxidants and anti-inflammatory cytokines were markedly increased and they might be related with much more delayed and lesser neuronal death in the young gerbil hippocampus

following transient cerebral ischemia/reperfusion (13, 36, 37).

It is well known that transient cerebral ischemia leads in oxygen-glucose deprivation and energy failure, which is associated with the development of neuronal cell damage/death in the hippocampal CA1 region (38). Because glucose is a major source of energy metabolism for the CNS, it is essential to keep adequate glucose supply to the brain (14). GLUT-1 plays a critical role in regulating glucose transportation and controlling the level of glucose in the brain (17, 39), and its expression is modulated in concert with metabolic demand and regional rates of cerebral glucose utilization (16). GLUT-1 is specifically localized to capillary endothelial cells of the brain (18, 19). Several studies have examined effects of global or focal ischemia on brain GLUT-1 expression in adult animal models (21, 40-42). A recent research demonstrated that the expression of GLUT-1 was increased in the hippocampus and cerebral cortex after ischemia/reperfusion injury in diabetic rats (43). On the other hand, Li *et al* (44) reported that the accumulation of GLUT-1 induced by progesterone treatment showed neuroprotective effects against cerebral ischemic insults. In the present study, we found that the density of GLUT-1-immunoreactive microvessels was significantly higher in the young than that in the adult and that, in the ischemia-operated-groups, the density of GLUT-1-immunoreactive microvessels in the young were changed later and significantly higher than that in the adult. This finding is supported by previous studies that showed that cerebral hypoxia-ischemia significantly increased the expression of GLUT-1 in the immature rat brain (45, 46). Based on these findings, high GLUT-1 expression seems to supply more glucose for ischemic brain, which may be an effective protection against cerebral ischemic insults. Although it was recently reported that hypoxia stimulated GLUT-1 expression in endothelial cells *in vitro* and *in vivo* (47, 48) and that GLUT-1 contributed to maintaining the integrity of BBB (49), it needs to study the precise mechanism of the ischemia-induced change of GLUT-1 expression in brain microvessels.

## Conclusion

Our present findings demonstrate that GLUT-1-immunoreactive microvessels in the hippocampal CA1 region of the young were more than those in the adult and that the density of the microvessels in the young following ischemic insult was significantly increased much later than that in the adult. We suggest that the ischemia-mediated increase of GLUT-1-immunoreactive microvessels in the young may contribute to less and more delayed neuronal death in the young gerbil.



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## Conflict of interest

The authors have no financial conflict of interest.

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