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The Effects of Peripheral Leukocytes on the Hippocampal Neuronal Changes in Transient Global Ischemia and Unilateral Cerebral Hemispheric Infarction

The participation of activated leukocytes and subsequent production of chemical mediators has been well accepted in the pathophysiology of hypoxic-ischemic injury. This study was performed to see the effects of leukocytes on hippocampal neuronal damage in transient global ischemia induced by 10-min occlusion of bilateral common carotid arteries (CCAs) with reperfusion for various times, and in complete unilateral ischemia induced by 24-hr ligation of left CCA. Leukopenia was induced by intraperitoneal injection of cyclophosphamide for 4 days. The results showed that hippocampal neuronal damages were worse at 6-hr reperfusion in leukopenic experimental group than in the control group. In comparison, 24-hr and 3-day reperfusion leukopenic groups showed less numbers of damaged neurons and milder changes. The 5-day reperfusion group showed inconsistent changes. Unilateral CCA occlusion showed extensive infarction in 83.3% of gerbils in the control group, compared to 25% of gerbils in the experimental group ($p<0.05$). These results strongly suggest that the number of peripheral leukocytes were closely related to the development of delayed neuronal damage of hippocampus in transient global ischemia and the incidence of infarction induced by 24-hr unilateral CCA ligation.

Key Words: *Leukocytes, Delayed neuronal damage, Hippocampus, Ischemia, Infarction*

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INTRODUCTION

In the past decades, there has been tremendous expansion in our understanding of cellular and biochemical events accompanied by ischemic brain injury. Contemporary data suggest that leukocytes are implicated in the pathogenesis of cerebral ischemia and stroke. Many studies demonstrated the delayed accumulation of leukocytes 24 hr after the onset of stroke symptoms in clinical settings (1) as well as early leukocyte accumulation during the first four hr of reperfusion in experimental studies (2). Although their actual role in these processes remains undefined, the postulated effects include limitation of cerebral blood flow (CBF) by mechanical vessel plugging, exacerbation of breakdown of blood-brain barrier or parenchymal injury via release of hydrolytic enzyme, production of lipid mediator or oxygen radical, and initiation of thrombosis (3). Recently, breakthroughs in understanding the mechanisms of leukocyte adhesion and migration to the ischemic area have bugun to be made. Most notably, leukocyte adhesion has been shown to occur via highly specific receptor-ligand interactions with the endothelium and extravascular matrix, mediated by adhesion molecules (4-6). In many studies, anti-leukocyte intervention produced beneficial effects on postischemic injury, such as attenuation of postischemic hypoperfusion (7), cerebral blood flow improvement with decrease of infarct size (8), reduction of cerebral edema (9), and blunting increased intracranial pressure (10). In contrast, other studies failed to show any beneficial effects of anti-leukocyte intervention (11-13). Several studies showed even the beneficial effects of activated leukocytes in ischemic brain injury via local elaboration of interleukin-1 or nerve growth factor for homeostasis and regeneration (14-15).

Hippocampus, a well-known target for hypoxic and ischemic damage, shows characteristic delayed neuronal damage (DND) by 10-min occlusion of common carotid arteries (CCAs) with reperfusion. Excitatory amino acids (EAAs) and their receptors have been mainly used to explain the pathophysiologic mechanism of DND (16). Since the hippocampus has small numbers of blood vessels

and leukocytes are rarely noted in DND, the role of leukocytes and blood vessels in hypoxic encephalopathy have attracted little attention in the hippocampus. As previously described, infiltration of the leukocytes in that area is thought to be through the specific receptor-ligand interaction mediated by adhesion molecules, special attention should be refocused.

So, this study was performed to see the effects of activated leukocytes on hippocampal neuronal damages in transient global ischemia induced by 10-min occlusion of bilateral CCAs with reperfusion for various times, 6 and 24 hr, 3, 5 and 7 days, respectively. In addition, the effects of leukocytes on unilateral cerebral hemispheric infarction induced by 24 hr of ligation of left CCA were also observed.

MATERIALS AND METHODS

Classification of experimental animals and production of leukopenia

Mongolian gerbils (*Meriones unguiculatus*) of both sexes weighing 50-70 g were used for the study. Each gerbil was allowed free access to food and water before and after surgery. The experimental animals were divided into two groups. The first group received four consecutive daily intraperitoneal injections of cyclophosphamide 50 mg/kg in order to reduce their peripheral blood leukocyte count, and the control group received the same number of injections of normal saline. At the time of injection, an additional intraperitoneal injection of Mesna (sodium 2 mercaptoethanesulphonate) 50 mg/kg was given to limit the nephrotoxic effects of cyclophosphamide. The control and leukopenic groups were further divided into bilateral transient global ischemia with reperfusion group and complete unilateral carotid ligation group.

Peripheral blood sampling and leukocyte count

Peripheral blood sampling was done from the caudal artery just before the carotid ligation, and by cardiac puncture before transcardiac perfusion fixation. The peripheral leukocytes were counted manually. The blood was diluted 10 times with 0.5% acetic acid solution. The leukocytes were counted in four 1 mm^2 squares at four corners of counting chamber, and the numbers were multiplied by 10.

Induction of transient global ischemia and unilateral cerebral infarction

Bilateral transient global ischemia with reperfusion was induced as follows. Under ketamine (10 mg/mL) anesthesia (intraperitoneal injection, 0.09 mL/kg), both CCAs of each gerbil were exposed through a midline incision in the neck and were occluded for 10 min using miniature Mayfield aneurysm clips. Restoration of carotid blood flow was verified under surgical microscopy after their removal. In the preliminary study with bilateral carotid ligation for five min and reperfusion, only few hippocampal neurons showed ischemic changes. To evaluate various postischemic periods, the gerbils were sacrificed after six and 24 hr, three, five, and seven days of reperfusion, respectively. The rectal temperature of the gerbils was maintained at 36 to 37℃ by a heating pad and heating lamp during the procedure.

Unilateral cerebral infarction was induced by permanent ligation of left CCA with silk and the brains were removed after 24 hr.

Tissue preparation and immunohistochemical method

At the scheduled time, each gerbil was anesthesized and the brain was fixed with a transcardiac infusion of 4% paraformaldehyde following perfusion with isotonic saline to remove blood from the cerebral vasculatures. The brains were removed and fixed in the same solution for a further 24 hr. Coronal sections of the supratentorial portion of each brain were taken and embedded in paraffin.

All brains were stained with hematoxylin-eosin (H-E) and cresyl-violet (C-V) as well as immunohistochemical study with anti-tubulin antibody. The immunohistochemical reaction was accomplished using the peroxidase-antiperoxidase method described with some modifications (17). Briefly, each deparaffinized five μm coronal section was reacted with a primary antiserum for 60 min before reaction with the peroxidase-antiperoxidase complex by LSAB kit from DAKO (Santa Babara, CA, U.S.A.). The peroxidase reaction was carried by incubation with link antibody and streptavidin for 20 min, respectively, and subsequently with 3-aminoethyl 9-carbasol (AEC). The antiserum for tubulin from rat brain, raised in a mouse, was purchased from Zymed (San Francisco, CA, U.S.A.) and diluted to 1:50.

Evaluation of neuronal damage

In bilateral transient ischemia with reperfusion group, the neuronal damages were evaluated at each region of the hippocampus, which was divided into subiculum-CA1, lateral CA1, and CA2-3. The severity of neuronal changes was graded according to the percentage of damaged neurons as follows: none=0; less than 25% of neurons involved=1+; between 25% and 75% of neurons

Table 1. Criteria for the identification of viable neuronal cells

1. Sharply delineated nucleus with ellipsoid or round shape
2. Clearly distinguishable nucleolus located centrally within the nucleus
3. Nucleus slightly darker than surrounding neuropil
4. Neuronal cytoplasm clearly demonstrated from surrounding neuropil
5. Less than one third of the neuron surrounded by confluent vacuolization

involved=2+; greater than 75% of neurons involved=3+. Only cells with a complete presentation of all listed criteria by Stummer et al. (18) were classified as viable neuronal cells (Table 1). In addition, the extent of the damage process in neurons was also observed.

In the unilateral cerebral infarction group, the incidence rate of infarction was compared between the control and leukopenic groups, since the morphological change and extent of necrosis were quite similar.

RESULTS

Peripheral leukocytes counts

Peripheral leukocyte counts were quite varied, ranging from 5,100-17,800/μL (mean 7,400$\pm$3,340/μL) in the control group, compared to 400-2,200/μL (mean 1,020 $\pm$552/μL) in the leukopenic group. The difference between the control and experimental groups was statistically significant ($p<0.001$).

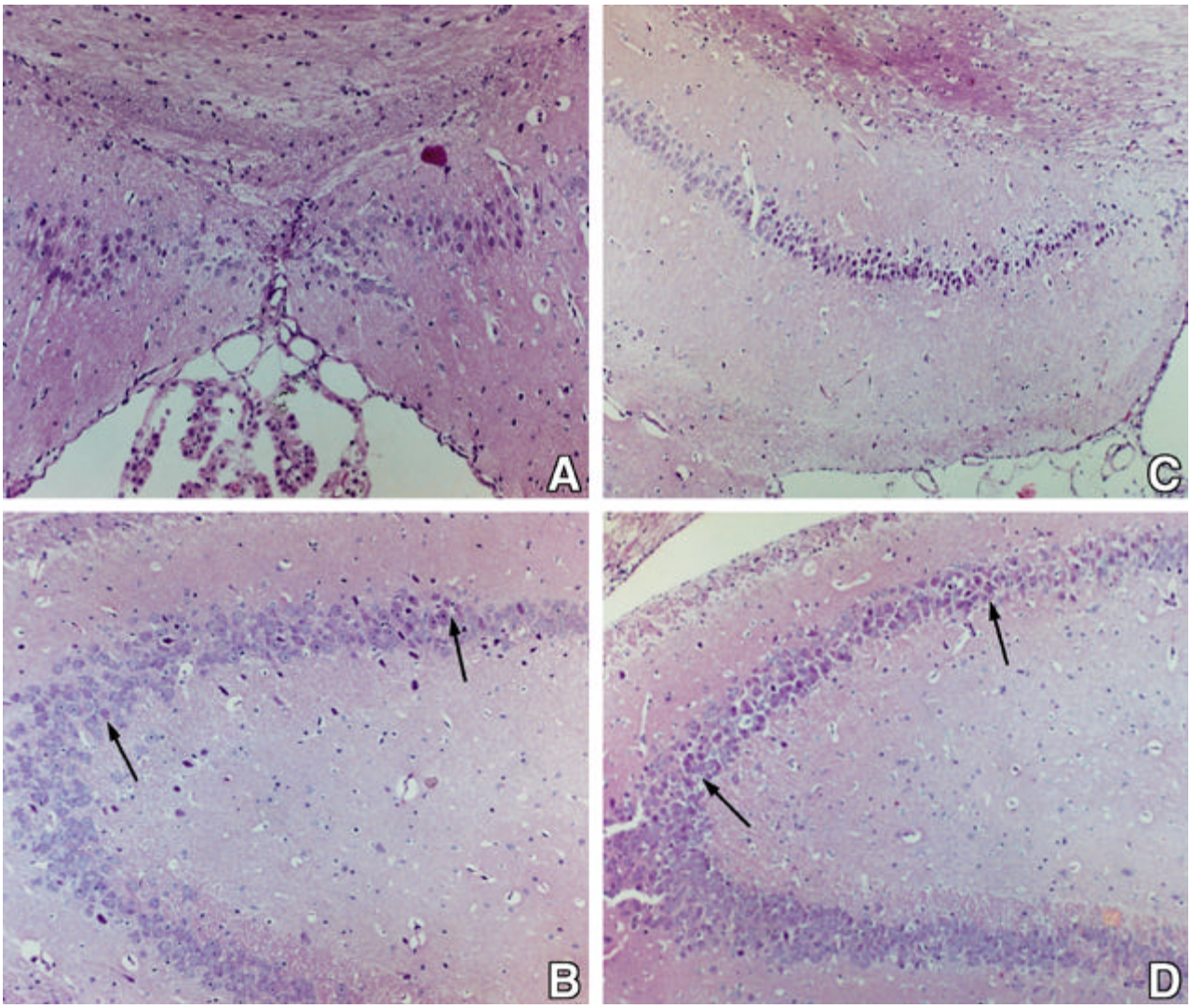

Fig. 1. Neuronal changes seen in 10-min bilateral global ischemia with reperfusion for six hr in control (A, B) and experimental (leukopenia) group (C, D). A, C: Neurons in subiculum-CA1 show red neurons with dark eosinophilic cytoplasm (×40, ×25, H-E). B, D: Neurons in CA2-3 show eosinophilic inclusion (EI) bodies (arrows) (×33, ×25, H-E).

Morphological changes

Bilateral transient global ischemia with reperfusion

Ischemic damages of hippocampal neurons in the control group were quite similar to previously reported findings (17). The earliest changes seen in this study was eosinophilic neurons in subiculum-CA1 and eosinophilic inclusion (EI) in CA2-3 at six hr of reperfusion (Fig. 1A, B). At 24 hr of reperfusion, eosinophilic neurons in CA1 showed fragmentation, clumping, and pyknosis of nucleus, which proceeded to chromatolysis and loss of neurons with ghost cell formation at three days of reperfusion (Fig. 3A, B). Degenerated neurons were frequently noted at lateral CA1 at three days and all the neurons in CA1 were necrotic and disappeared after five days (Fig. 5). The CA3 area showed largely well preserved neurons.

Eosinophilic neuron and intracytoplasmic EI bodies showed faint blue cytoplasm with loss of Nissl substance by C-V stain (Fig. 2B, C). Pyknotic neurons showed bluish homogenous cytoplasm with dot-like nucleus by C-V stain. Loss of reaction for tubulin in nerve cell bodies and/or dendrites and background stain in neuropil were used as criteria for degenerating neurons (Fig. 2D). EI bodies also showed loss of reaction to anti-tubulin antibody, but showed relatively preserved cell processes compared to eosinophilic neurons. EI bodies seemed somewhat reversible, because they were partly reduced in number during the course of time.

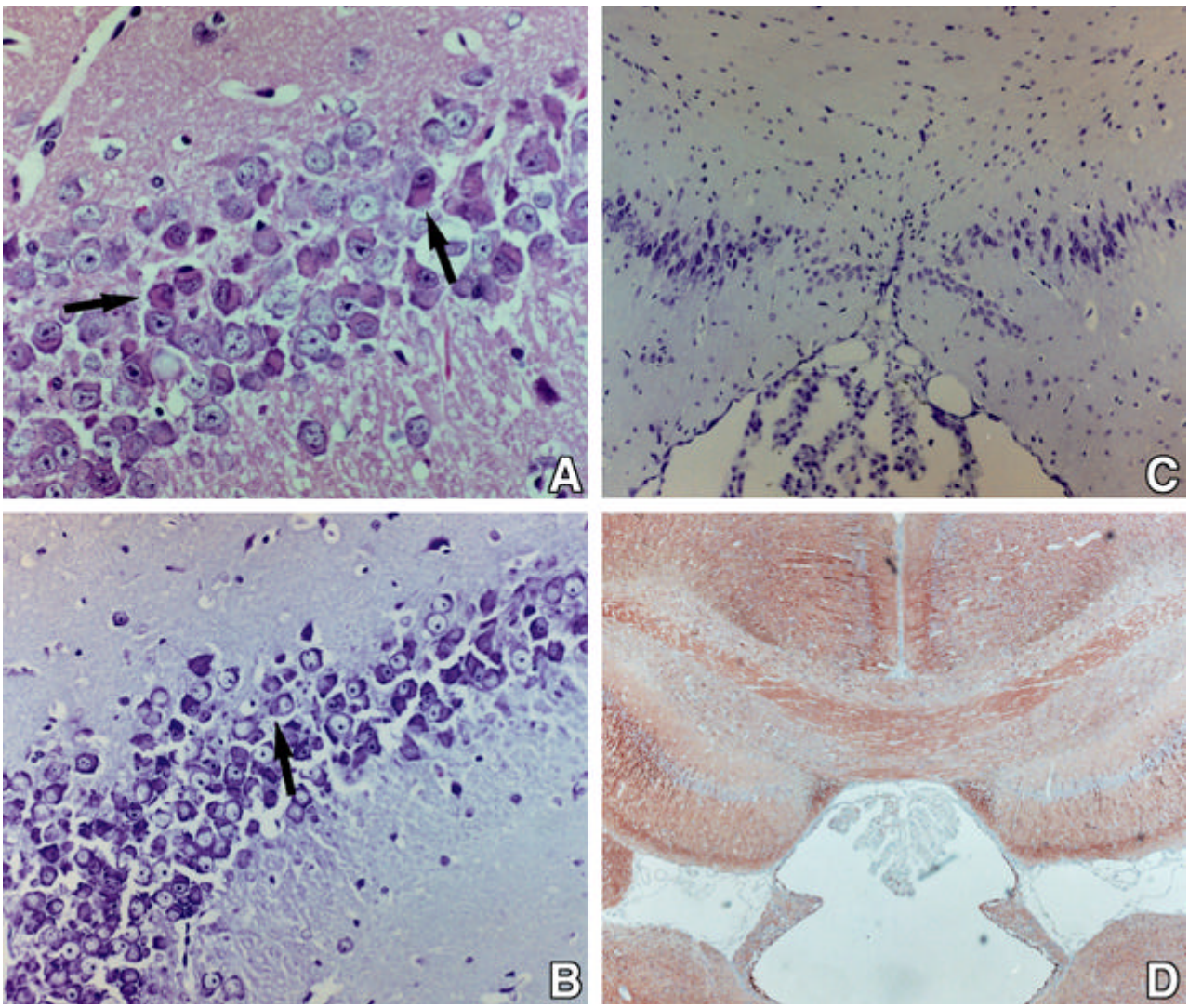

Fig. 2. Characteristic neuronal changes of hippocampal neurons in 10-min bilateral global ischemia with reperfusion for six hr in control (C) and experimental (leukopenia) group (A, B, D). A: Neurons in CA3 show EI bodies (arrows) (×100, H-E). B: Pale blue homogenous stain of EI bodies in CA2-3 with cresyl violet stain (arrow) (×66, C-V). C: Dark blue homogenous stain of eosinophilic neurons in CA1 by cresyl violet stain (×40, C-V). D: Loss of tubulin in cell bodies and dendrites in subiculum-CA1 (×10, ABC).

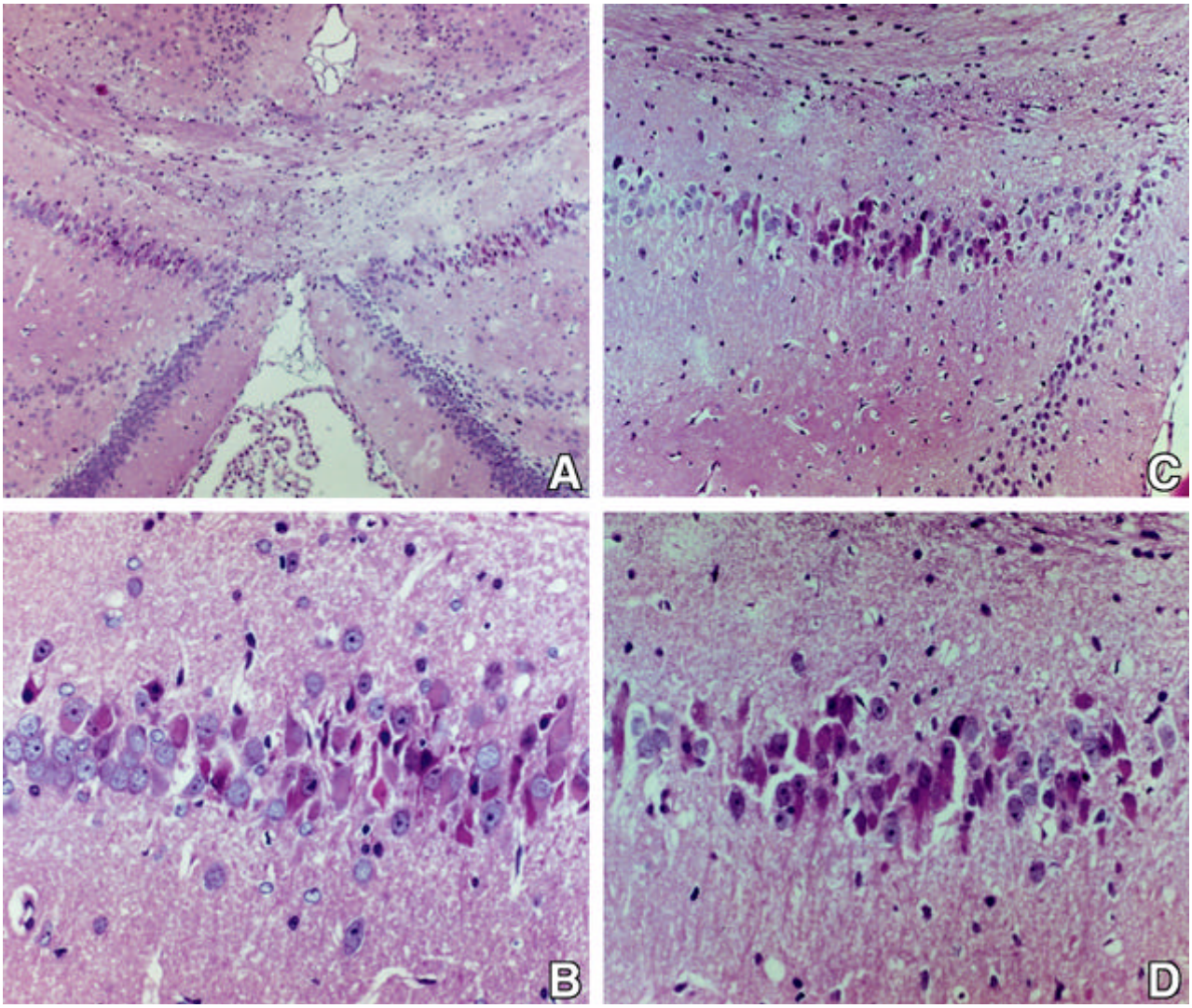

Fig. 3. Neuronal changes seen in 10-min bilateral global ischemia with reperfusion for 24 hr in control (A, B) and experimental (leukopenia) group (C, D). A, B: Neurons in subiculum-CA1 show fragmentation, clumping, and pyknosis of nucleus (×25, ×100, H-E). C, D: Neurons in subiculum-CA1 show dark eosinophilic condensed cytoplasm with occasional pyknotic nuclei (×40, ×80, H-E).

Table 2. Neuronal changes in hippocampus with bilateral CCA ligation with 6-hr reperfusion

Group	WBC (/μL)	Sub-CA1	Lateral CA1	CA2-3
Control	6,500	1+	0	1+
	5,900	1+	0	1+
	7,400	1+	0	2+
Experimental	500	3+	0	3+
	900	2+	0	3+
	500	2+	0	3+
	400	3+	0	2+

Sub, subiculum; CA, cornus ammonis

The comparison of neuronal changes between the control and leukopenic groups was as follows. At six hr of reperfusion (Table 2), the control group showed 1+ of eosinophilic neurons and EI bodies in subiculum-CA1 and in CA2-3, respectively (Fig. 1A, B). The leukopenic group showed more numbers of eosinophilic neurons and EI bodies (2-3+), compared to the control group (Fig. 1C, D). After 24 hr of reperfusion, the degree of neu-

Table 3. Neuronal changes in hippocampus with bilateral CCA ligation with 24-hr reperfusion

Group	WBC (/μL)	Sub-CA1	Lateral CA1	CA2-3
Control	6,100	2+	0	2+
	7,600	3+	0	2+
	5,700	3+	0	3+
Experimental	900	3+	0	3+
	700	3+	0	3+
	600	2+	0	2+
	1,200	3+	0	3+

Sub, subiculum; CA, cornus ammonis

ronal damage severity (2-3+) in the leukopenic group was similar to the control group (Table 3). But, the extent of the degenerating process in neurons in the leukopenic group, showing relatively preserved dark eosinophilic condensed cytoplasm and pyknotic nuclei, was milder compared to the control group, showing marked fragmentation, clumping, and pyknosis of nucleus (Fig. 3). At three days of reperfusion, the control group showed many (3+) markedly necrotic neurons in subiculum-CA1 and scattered necrotic neurons (1+) in lateral CA1 as well as many EI bodies (2-3+) in CA2-3 (Table 4, Fig. 4). The leukopenic group showed less numbers

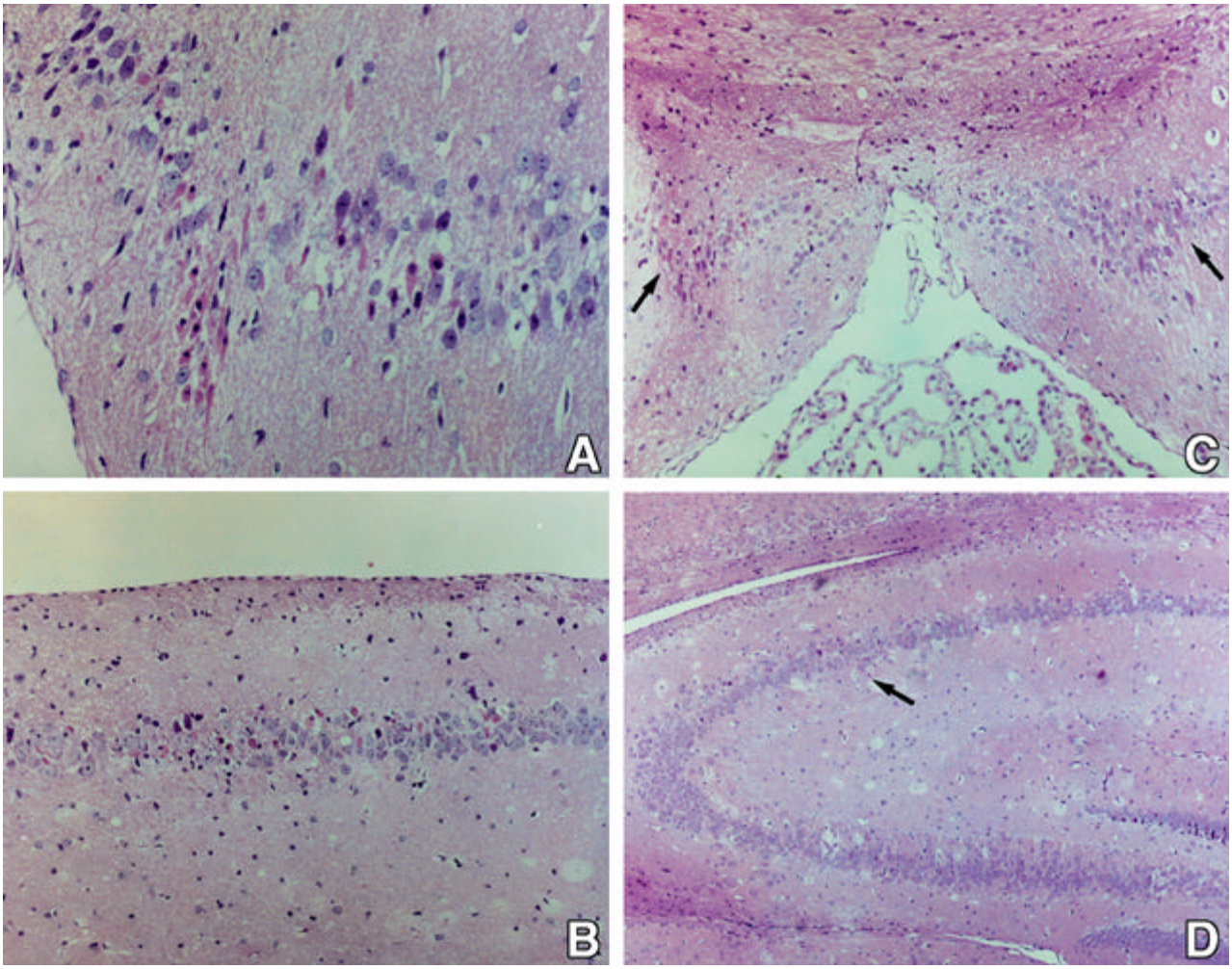

Fig. 4. Neuronal changes seen in 10-min bilateral global ischemia with reperfusion for three days in control (A, B) and experimental (leukopenia) group (C, D). A: Neurons in subiculum-CA1 show pyknosis and karyorrhexis of nuclei (×80, H-E). B: Neurons in lateral CA1 show similar degeneration (×40, H-E). C, D: Neurons in subiculum-CA1 (C) and CA2-3 (D) show degeneration with pyknotic nuclei and eosinophilic cytoplasm (arrows) (×40, ×20, H-E).

Table 4. Neuronal changes in hippocampus with bilateral CCA ligation with three days reperfusion

Group	WBC (/μL)	Sub-CA1	Lateral CA1	CA2-3
Control	10,800	3+	1+	2+
	5,100	3+	1+	2+
	5,600	3+	1+	3+
Experimental	1,900	2+	1+	1+
	1,200	2+	1+	1+
	800	3+	1+	2+
	1,500	2+	1+	1+

Sub, subiculum; CA, cornus ammonis

Table 5. Neuronal changes in hippocampus with bilateral CCA ligation with five days reperfusion

Group	WBC (/μL)	Sub-CA1	Lateral CA1	CA2-3
Control	5,700	3+	3+	1+
	7,200	2+	2+	3+
	5,200	3+	3+	3+
	8,200	3+	3+	3+
Experimental	2,200	2+	1+	1+
	500	2+	1+	1+
	950	3+	3+	1+
	1,100	3+	3+	1+
	1,600	2+	2+	1+

Sub, subiculum; CA, cornus ammonis

of necrotic neurons (2-3+) and EI bodies (1-2+) in sub-CA1 and CA2-3, respectively. The extent of the degenerating process was also much less in the leukopenic group compared to the control group. At five days of reperfusion (Table 5), the control group showed diffuse degeneration of neurons with numerous ghost cells in subiculum-CA1 and lateral CA1, which was seen as a diffuse loss of tubulin in immunohistochemical study (Fig. 5). In the leukopenic group, the results were inconsistent. Two out of five gerbils showed fewer damaged

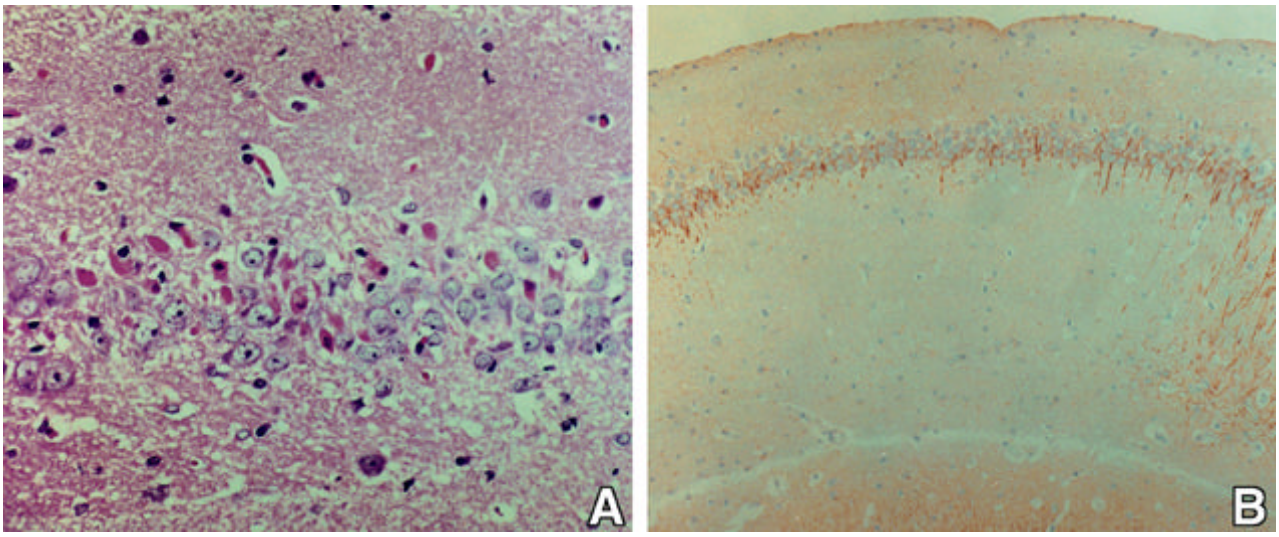

Fig. 5. Neuronal changes seen in 10-min bilateral global ischemia with reperfusion for five days in control group. A: Neurons in lateral CA1 show pyknosis and karyorrhexis of nuclei and fragmentation of cytoplasm (×80, H-E). B: Neurons in lateral CA1 show diffuse loss of tubulin (×33, ABC). In experimental (leukopenia) group, the results were inconsistent. Two out of five gerbils showed fewer damaged neurons and one gerbil showed similar numbers of degenerating neurons. The other two animals showed diffuse neuronal degeneration similar to controls.

neurons (2+ in sub-CA1 and 1+ in lateral CA1) compared with the control group and one gerbil showed similar numbers of degenerating neurons (2+) but a lesser extent of neuronal damage. The other two animals showed diffuse neuronal degeneration similar to controls. At seven days of reperfusion, the control group showed diffuse loss of neurons with numerous ghost cells in CA1 (Fig. 6). The leukopenic group at seven days of reperfusion was not available, since all the gerbils were dead by that time.

Unilateral cerebral hemispheric infarction

After complete left carotid artery occlusion for 24 hr without reperfusion, infarction in the left cerebral hemisphere occurred in five out of six gerbils (83.3%) in the control group, compared to only one out of four gerbils (25%) in the leukopenic group. The difference was statistically significant ($p<0.05$). The infarction involved the left cerebral cortex, hippocampus, basal ganglia, and thalamus (Fig. 7).

DISCUSSION

This study was performed to compare the pathologic findings in the leukopenic and control groups and to see

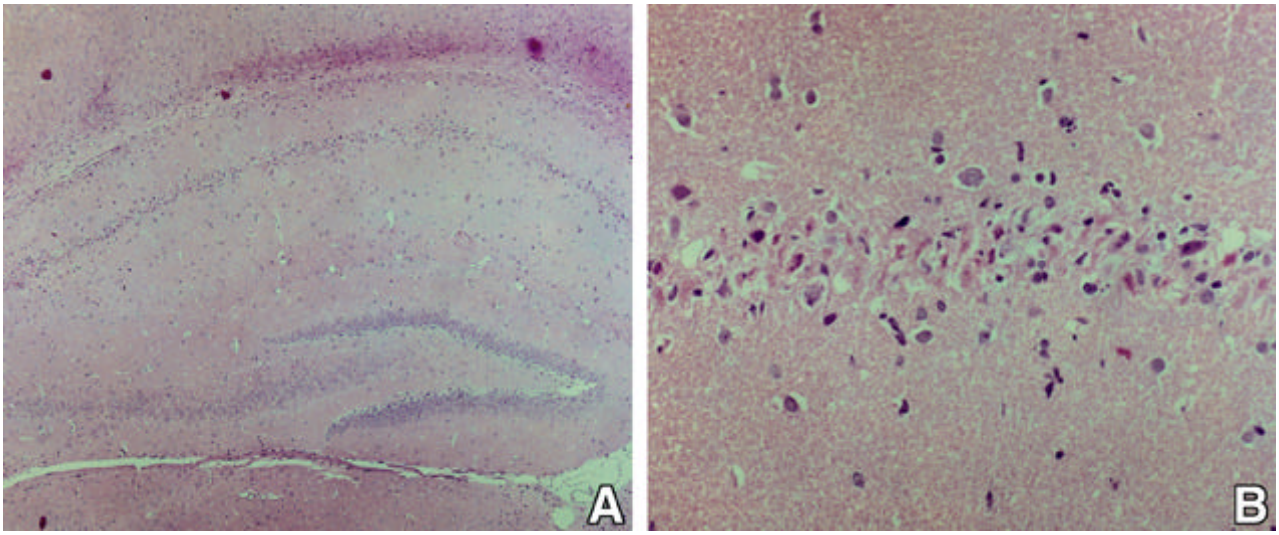

Fig. 6. Neuronal changes seen in 10-min bilateral global ischemia with reperfusion for seven days in control group. A: Diffuse degeneration of neurons in CA1 (×13, H-E). B: Necrotic neurons with fragmented cytoplasm (×80, H-E).

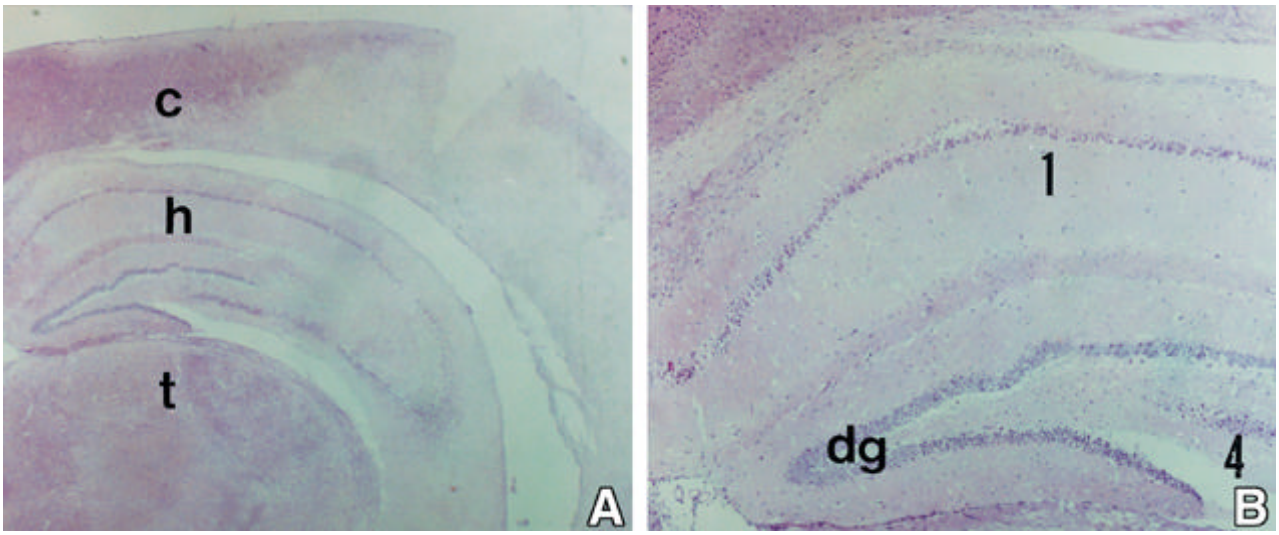

Fig. 7. Changes of left cerebral hemisphere in unilateral progressive ischemia for one day. A: Extensive necrosis of neuropil with edema involving cerebral cortex (marked as "c"), hippocampus (h), and thalamus (t) (×5, H-E). B: Hippocampus with numerous pyknotic neurons in CA1 (1), CA4 (4), and dentate gyrus (dg) (×13, H-E).

the effects of leukocytes on neuronal damages after ischemic brain injury. The study demonstrated that activated leukocytes were closely related to the development of delayed neuronal death of hippocampus after transient global ischemia as well as neuronal loss following cerebral infarction, but not to early ischemic insults.

Transient global ischemia in gerbils showed typical sequential damage in hippocampal neurons depending on the duration of ischemia, presence or absence of reperfusion, and anatomical locations (16, 19). Selective vulnerability of neurons in different areas of the central nervous system has been an object of great interest and experimental studies. Neurons in the hippocampus show a different pattern of reaction or degeneration according to the location as well as the type of injury. The neurons of the cornus ammonis (CA) 1 subfield are especially well known to be selectively vulnerable to ischemic insult and slowly progressing neuronal death after transient global ischemia, that is "delayed neuronal death" (19). Within the CA1 region, subiculum CA1 and medial CA1 showed different immunohistochemical reactions, suggesting differences in cellular vulnerability between these subfields (20). The neurons in CA3 subfield are well known to show intraneuronal EI bodies during reperfusion after transient ischemia (19). In previous studies, the authors showed that immunohistochemical investigation for tubulin and creatine kinase (CK) BB-isoenzyme was the best method for detecting neuronal changes in the early ischemic stage. And, subiculum-CA1 region just medial to CA1 region was the most vulnerable subregion of the hippocampus and showed positive reaction in 100% by anti-tubulin and anti-CK antibodies at 0 min reperfusion after five min ischemia (21, 22). In the other study with the same conditions, the earliest change was scattered HC cells at five min after bilateral CCA ligation. They showed a loss of tubulin reaction in cell bodies and dendrites. They were found in haphazard pattern in the early stage of ischemia (up to one day), which suggests the nonspecific or reversible nature of the changes. With H-E stain, intraneuronal eosinophilic inclusion bodies in the CA3 region were first detected at three hr after reperfusion. Neurons with eosinophilic cytoplasm (eosinophilic neurons) were first noted in subiculum-CA1 at six hr by H-E stain, increased in numbers at 48 hr and full blown at one wk (16, 19). All specimens of gerbil brains in the present study were stained with H-E, C-V, and immunohistochemical study with anti-tubulin antibody, to evaluate neuronal changes in detail. As previously mentioned, the very early stage of ischemic neuronal damage when even nearly no change is found with H-E or C-V stain, can be recognized with anti-tubulin antibody due to the loss of tubulin reaction in cell bodies or dendrites. In this study, the degree of injury in each damaged neuron is as important as or even more important than the percentage of changed neurons. At six hr of reperfusion, an early ischemic period, the eosinophilic neurons seen in hippocampal CA1, underwent further degeneration showing fragmentation, clumping, pyknotic nuclei and chromatolysis, karyorrhexis of nuclei with fragmentation of cytoplasm, finally changing into ghost neurons, a typical "delayed neuronal death". In contrast, some of the EI bodies, the early changes in CA3, showed somewhat reversible changes. So, more EI bodies, that is a higher grade of neuronal change, do not always mean more severe damage, and the speed of degeneration progressed into or how many neurons changed into pyknotic or ghost neurons are more important indicators.

Neuronal injury after cerebral ischemia is a complex subject, which includes redundant mechanisms such as excitatory amino acids (23), intracellular calcium accumulation (24), leukocyte infiltration (25), derangement of genomic expression (26), toxic zinc influx (27) and so on. Each of the mechanisms, not a part of them, should be blocked for therapeutic strategy. Of these, activated leukocytes has recently been refocused due to our understanding of the mechanisms of leukocyte adhesion and migration, and thus raising the possibility of clinical usefulness of antileukocyte therapy. Activated polymorphonuclear leukocytes appear to play a crucial role in neuronal injury after ischemia and reperfusion, probably through release of proteolytic enzymes such as NP4 and NGAL, release of oxygen free radicals, mechanical obstruction of capillaries and collateral microvasculature, impairment of microcirculatory blood flow (28), and inducing vasoconstriction through activation of thrombin or complement system (29), supported by increased plasma levels of some cytokines (30). Activated mononuclear cells, through the secretion of cytokines such as TNF with subsequent activation of receptor proteins, may exert procoagulant/prothrombotic effects by impairing the normal nonthrombogenicity of the endothelial wall (31), causing activation of other leukocytes (32), inducing activation and aggregation of platelets, and causing activation of certain coagulation factors (33). This may cause further injury to the atherosclerotic endothelium and may play a crucial role in the initiation, formation and progression of arterial thrombosis. Current research also suggests that underlying stroke risk factors such as atherosclerosis, hypertension, diabetes, advancing age, may be critical determinants of the contribution of leukocytes to stroke (34, 35). As a consequence of breakthroughs in leukocyte biology, selective and relatively nontoxic antibodies inhibiting leukocyte adhesion, anti-intercellular adhesion molecule (anti-ICAM) or neutrophil inhibitory factor, for example, have begun to be applied to experimental stroke (36).

As in many studies previously described, we can see that activated leukocytes are involved in delayed neuronal death after global ischemia and established cerebral infarction. The leukopenic group 24 hr after reperfusion showed slower and milder damage, and the three-day reperfusion leukopenic group revealed fewer damaged neurons. However, it was quite interesting that, at six hr after reperfusion, hippocampal neuronal changes in the leukopenic group were faster and more extensive than in the controls. The phenomenon cannot be easily understood, but may suggest the different effects of leukocytes during the early ischemic stage, and even the possibility of some beneficial effects of leukocytes during the early period. Several investigators have also reported the beneficial effects of leukocytes in stroke. Cell-mediated immunity is depressed during the first week after stroke. Reductions in both lymphocyte number and function are observed (37). In addition, inhibition of postischemic leukocytes accumulation and their function might create disturbances in complex interactions between immune response and regenerative response in the CNS. Recent work suggests that IL-1 (a product of monocyte/macrophage) is an important inflammatory mediator produced after traumatic and ischemic brain injury (38). Although detrimental effects on permeability of blood-brain barrier may result from its production (39), IL-1 is also a signal for the local elaboration of the homeostatic and regenerative molecule nerve growth factor (14, 15).

However, several limitations of this study can be pointed out. The selective inhibition of leukocyte adhesion was not performed. Leukocyte depletion by cyclophosphamide showed high mortality rate in leukopenic group leading to incomplete observation of the longer postischemic period and one could not completely rule out the possibility of any unknown action of the agent itself. And selecting subpopulations of leukocytes was not done. Continuing studies in this area should be done to exactly clarify the role of leukocytes in cerebral ischemia and stroke as well as to gauge the prophylactic and therapeutic potential of antileukocyte agents.

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