The effect of chronic cerebral hypoperfusion on middle cerebral artery occlusion-induced cellular damage in spontaneous hypertensive rats

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This certifies that the Doctoral Dissertation of
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I dedicate the dissertation to my loving family.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Abstract

The effect of chronic cerebral hypoperfusion on middle cerebral artery occlusion-induced cellular damage in spontaneous hypertensive rats

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An ischemic stroke can result from various mechanisms such as athero-thrombosis, caridoembolism or hemodynamic compromise. It has been reported that patients with atherothrombotic stroke have less severe neurological deficits and smaller cerebral infarction than those with cardioembolic stroke. When exposed to a sufficient but sublethal alteration of their environment, most living organisms acquire transient tolerance to subsequent and otherwise lethal environmental changes. To test the hypothesis that chronic cerebral hypoperfusion induces tolerance to the subsequent severe ischemia, we examined the effect of chronic cerebral hypoperfusion on brains subjected to acute focal ischemia by means of two well- known animal models, namely middle cerebral artery occlusion/reperfusion (MCAO/R) and bilateral common carotid arteries ligation (BCAL). Chronic cerebral hypoperfusion was successfully induced in the male spontaneous hypertensive rat (SHR) by BCAL. Rats that were bred for 4 weeks after BCAL or sham operation were subjected to MCAO/R using a nylon suture model. The animals' brains were then prepared for paraffin blocks or frozen blocks. Subsequently an *in situ* nick translation study, immunohistochemical staining for apurinic/apyrimidinic endonuclease/ redox factor-1 (APE/Ref-1) and matrix metalloproteinase (MMP) -9 and zymography were performed.

Thirteen of the 45 rats that underwent BCAL died while all 27 rats that underwent the sham operation survived. The number of positive cells in the *in situ* nick translation study, which was taken as an indication of cellular injury, was significantly reduced in those rats that were subjected to chronic cerebral hypoperfusion. Immunoreactivity for APE/Ref-1, which plays a role in DNA repair, was markedly increased in the brain tissues of those rats subjected to chronic cerebral hypoperfusion. Indirect evidence of extracellular matrix remodeling, which might be associated with adaptive arteriogenesis or angiogenesis, was obtained in the form of increased MMP-2 activity in the hypoperfused brain. The findings of this study provide experimental evidence for the hypothesis that chronic sublethal cerebral hypoperfusion is protective for subsequent severe ischemic insults, which was in part supported by increased DNA repair activity and evidence of extracellular matrix remodeling in tissue with chronic cerebral hypoperfusion.

Key Words: Chronic cerebral hypoperfusion, Middle cerebral artery occlusion, Ischemic tolerance

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I. INTRODUCTION

Ischemic stroke can result from various mechanisms, such as atheroth rombosis, caridoembolism or hemodynamic compromise. Many stroke victims demonstrate atherosclerosis of the extracranial and/or intracranial arteries on angiographic studies. Atherosclerosis may result in a predisposition to stroke by *in situ* thrombotic occlusion, distal thrombotic embolism or hemodynamic compromise. It has been reported that patients with atherothrombotic stroke have less severe neurological deficits and smaller cerebral infarction than those with cardioembolic stroke.[1,2,3] Although the growth of collateral channels due to the chronic cerebral hypoperfusion caused by atherosclerotic narrowing of the intracranial and/or extracranial arteries has been suggested to be one of the possible causes, the exact mechanism remains unknown.

When exposed to a sufficient but sublethal alteration of their environment, most living organisms acquire transient tolerance to subsequent and otherwise lethal environmental changes.[4] In cerebral ischemia, the concept of ischemic tolerance, in which a short ischemic event can result in subsequent resistance to severe ischemic

tissue injury, has been suggested.[4] Under experimental conditions, not only mild global but also brief and repetitive focal ischemic insults significantly decreased the infarction size after permanent or prolonged middle cerebral artery occlusion.[5,6,7,8,9,10] In human studies, patients who had experienced a number of transient ischemic attacks (TIAs) showed better recovery than those who suffered a single TIA, which suggests the presence of ischemic tolerance in clinical settings.[4,8,9]

Patients with atherothrombotic stroke are more likely to have significant stenosis of the artery proximal to an occlusion, which might have caused prior chronic cerebral hypoperfusion, previously minor or clinically insignificant infarctions, and/or a history of transient ischemic attacks, when compared with those with cardioembolic stroke. Thus, brain tissues in the vicinity of significant atherosclerotic narrowing or an occlusion may be preconditioned by minor or sublethal ischemic insults. While many strokes occur in brain tissues which have been in a chronically hypoperfused state or have been mildly injured by previous TIAs or minor ischemic strokes, the fate of brain tissues and cellular responses upon being subjected to an abrupt arterial occlusion, which may be different from the occlusions which were occurring in the previously normal tissues, is unknown. Previously, the animal models for ischemic stroke were developed by occluding an artery either mechanically or by injecting *ex-vivo* thrombi into an artery.[3,5,10] These animal models mimic human cardioembolic infarction which is characterized by an abrupt occlusion of a previously normal artery.

Chronic cerebral hypoperfusion has been successfully induced in the rat by bilateral common carotid arteries ligation (BCAL). This model is known to exhibit long-standing cerebral hypoperfusion and white matter changes with behavioral disturbances, without causing significant ischemic damage. Hence, this model has served as an experimental model for studying vascular dementia.[11,12,13,14]

From clinical observations in atherothrombotic strokes and based on the theoretical background of ischemic tolerance, we hypothesized that chronic cerebral

hypoperfusion would induce tolerance to subsequent severe ischemia. To test this hypothesis, two well-known animal models, namely MCAO/R using a nylon suture and BCAL, were used. By sequential application of these two models, the effect of chronic cerebral hypoperfusion on brains suffering from acute focal ischemia was investigated.

II. MATERIALS AND METHODS

1. Experimental animals and preparation

Male spontaneous hypertensive rats (SHRs) weighting 250 to 340 g were used as the subjects in this experiment. The care and use of laboratory animals in this experiment were based on the Guidelines and Regulations for Use and Care of Animals in Yonsei University. All animals were allowed free access to food and water. The environmental temperature was maintained at 22.0±2.0℃, the humidity at 50±10%, the noise level below 40-50 phons, and the light cycle at 12 hours on/12 hours off. A barrier system that had regular pad change (twice a week), and monitoring of microorganism for specific pathogen free animal were used for the operative procedure. For the operative procedures, the animals were anesthetized with 5% isoflurane delivered in oxygen at 0.5 L/min with air for 5 minutes. Anesthesia was maintained with 2% isoflurane. During the operative procedures, body temperature was monitored continuously with a rectal probe and was maintained at 37.0±0.5℃ by means of a heating pad (Harvard Apparatus, Holliston, MA, USA).

2. Induction of chronic cerebral hypoperfusion

Chronic cerebral hypoperfusion was induced by means of BCAL as previously described.[12,15,16] Briefly, after making a midline cervical incision, the

common carotid arteries were exposed bilaterally and were double-ligated with 5-0 black silk sutures. The sham SHRs underwent the same operation, except that BCAL was not performed. Both groups of animals, those that underwent BCAL and those that underwent the sham operation, were then bred for 4 weeks, before being subjected to acute focal cerebral ischemia.

3. Induction of acute focal cerebral ischemia and reperfusion

Both the animals that underwent BCAL and those that underwent the sham operation were subjected to MCAO/R using a nylon suture model.[10,17] Briefly, under general anesthesia with isoflurane, the left common carotid artery (CCA) was exposed through a midline neck incision and was carefully isolated. After dissection, ligation, and coagulation of the external carotid artery (ECA) and arteries from the ECA, the internal carotid artery (ICA) was isolated. The ICA was carefully separated from the adjacent vagus nerve, and the pterygopalatine artery was ligated. The CCA was double-ligated with 5-0 black silk in the previously sham operated animals. Next, in both groups of animals, 5-0 black silk was tied loosely around the ICA, and a microvascular clip was placed across the ICA. A 4-0 nylon monofilament with its tip rounded near a flame and coated with 0.1% poly-L-lysine (Sigma, St. Louis, MO, USA), was introduced through a cut into the ICA. After tightening the silk, which was loosely tied around the ICA, and displacing the clip, the monofilament was advanced into the ICA approximately 23 mm distal to the carotid bifurcation. The incision was closed leaving 1 cm of the nylon suture protruding. Reperfusion was achieved by pulling back the suture until resistance was felt. The animals were subjected to MCAO/R for two hours and reperfusion for various durations (2, 3, 5, 10, and 18 hours).

4. Motor disability test

The animals were accorded a score of 1 point for flexion of the forelimb contralateral to the stroke when they were hung by the tail. Two points were scored for adduction of the shoulder contralateral to the stroke. An additional 3 points were scored for a circling motion toward the paretic side when attempting to walk. The animals scored 4 points when they fell toward the lateral side when pushed gently. Thus, an animal with maximal deficits scored 10 points, while an animal with no deficits scored 0 points.

5. Brain tissue preparation

After MCAO and subsequent reperfusion for various durations, the animals were sacrificed by transcardiac perfusion using a peristaltic pump under deep anesthesia with intraperitoneal urethane injection. The brains, which were fixed by transcardiac perfusion with 4% paraformaldehyde (PFA) solution, were cut coronally into 2 mm-thick blocks using a rat brain matrix and harvested for the purpose of preparing paraffin blocks. In other experiments, the brains were immediately removed after transcardiac perfusion with cold heparinized normal saline, and sectioned into 2 mm-thick coronal slices using a rat brain matrix. The 1^{st}, 3^{rd}, 5^{th}, and 7^{th} blocks were fixed with 4% paraformaldehyde. The 2^{nd}, 4^{th}, and 6^{th} blocks were embedded in Tissue-Tek OCT compound (Miles Inc., Elkhart, IN, USA), frozen in 2-methylbutane/dry ice, and stored at 80℃.

6. *In situ* nick translation (ISNT) study

Evidence of nuclear DNA scission was taken as an indication of cellular

injury, as previously reported.[18] The incorporation of digoxigenin (DIG) dUTP on the 3 μm paraffin sections was detected using DNA polymerase I. The 4th paraffin block was used for this experiment, because the MCAO employed in this model had previously been found to produce the most consistent and largest infarction in the 4th block. After deparaffinization and rehydration using graded ethanol, sections were treated with proteinase-K for 5 minutes, 2% H_2O_2, and incubated with 0.1 units/L of DNA polymerase I (Promega, Madison, WI, USA) and DIG DNA labeling mixture (Boeringer Mannheim Corp., Indianapolis, IN, USA) in translation buffer [50 mmol/L Tris-HCl] ph 7.5, 10 mmol/L $MgSO_4$, and 50 g/ml BSA) at 37℃ for 60 minutes. After washing with phosphate buffered saline (PBS), the sections were incubated with horseradish peroxidase-conjugated anti-DIG antibody (Boeringer Mannheim Corp., Indianapolis, IN, USA) for 30 minutes. Color was developed using diaminobenzidine (DAB) (Sigma, St. Louis, MO, USA) solution.

7. Immunohistochemistry

For the immunohistochemical studies, a rabbit anti-apurinic/apyrimidinic endonuclease/redox factor-1 (APE/Ref-1) polyclonal antibody (1:200, Novus, Littleton, CO, USA) in 3 μm paraffin sections and a mouse anti-MMP-9 monoclonal antibody (1:200, Oncogene, Darmstadt, Germany) in 10 μm frozen sections were used.

The sections used were from the 4th block. The frozen sections were fixed with acetone for 10 minutes and then immersed in 100 mmol/L glycine in PBS (100 mmol/L Na_2HPO_4 and 140 mmol/L NaCl, pH 7.4) for 10 minutes. This was followed by a rinsing them in PBS solution and incubation with Blotto (5% hydrated nonfat dry milk with 1% BSA) for 30 minutes to reduce nonspecific binding. Under humidified conditions, primary antibody was applied to each section followed by a 2 hours incubation period at 37℃. After washing with PBS, the biotinylated secondary

antibody was incubated for 30 minutes at 37℃ (Vector Laboratories, Burlingame, CA, USA). The peroxidase signal was developed with the use of 0.01% DAB in 0.05 mol/L Tris buffer (pH 7.6). To clarify the spatial relationship between APE/Ref-1 expression and DNA damage, we performed double staining of anti APE/ Ref-1 antibody and an ISNT study, as previously described.[7] Briefly, fixed sections with 5% PFA solutions were immunostained with anti-APE/Ref-1 antibody as described above, the sections were passed through ethanol (70%, 95%, 100%), and then immersed in chloroform for 5 minutes. The sections were rehydrated by passage through a decreasing ethanol series and rinsed in PBS. After eliminating peroxidase activity with 3% H_2O_2 in PBS, an ISNT study was performed as described above. The slides were rinsed with water, stained with methyl green, dehydrated, and mounted.

8. Extraction of protein and purification of matrix metalloproteinases (MMPs)

The 4th frozen blocks were divided into the ischemic and nonischemic hemispheres. Cryostat 10 μm-thick 45-50 frozen sections were homogenized with 400 μl working buffer (50 mM Tris HCl [pH7.5], 150 ml NaCl, 5 mM $CaCl_2$, 0.05% BRIJ-35, 0.02% NaN3, 1% Triton X-100) containing 1 mM phenylmethyl sulfonyl fluoride and centrifuged at 4℃, 9000 rpm for 20 minutes. The supernatant was stored at -80℃. The protein concentration was measured based on the Bradford method using bovine gamma globulin (Bio-Rad Laboratories, Herculus, CA, USA). MMP was purified based on a method using gelatin-sepharose 4B, as previously reported.[19] After rinsing 50 μl of gelatin-sepharose 4B (Pharmacia Biotech, Uppsala, Sweden) with 200 μl of working buffer 3 times, the protein extracts were mixed and incubated on the rocking plate for 30 minutes as 4℃. Then, the samples were centrifuged at 7000 rpm for 5 minutes, rinsed with 200 μl of working buffer, and

were centrifuged again. The precipitants were added along with 70 μl of elusion buffer (working buffer to which 10% DMSO was added) and incubated for 30 minutes with rocking at 4℃. After centrifugation, the supernatant was taken and stored at -80℃ until needed for gelatin zymography.

9. Gelatin Zymography

The purified protein extracts were mixed with an equal volume of sample buffer (80 mM Tris-HCL [pH6.8], 4% sodium dodecyl sulfate [SDS], 10% glycerol, 0.01% bromophenol blue) and were subjected to electrophoresis. The gelatinolytic activity of these samples was detected by 8% SDS-polyacrylamide gel containing 1% gelatin. Sample gels were rinsed in 150 ml of 2.5% Triton X-100 (15 minutes×3) and incubated with 250 ml of 50 mmol/L Tris-HCl buffer (pH 7.5, 10 mM $CaCl_2$, 0.02% NaN_3) for 42 hours at 37℃. After incubation, the gels were stained with 0.1% amino black containing acetic acid, methanol, distilled water (1:3:6) for 1 hour and then destained by four changes with the same solution without amino black for 130 minutes. These gels were scanned using a flatbed scanner (Scanmaker 9600 XL, Mic- rotek, Taiwan). The gelatinolytic bands were analyzed and quantified by means of a gel plotting macro using the Scion Image program.

10. Statistical analysis

The differences between the two groups were determined by means of the Wilcoxon rank sums test. The significance of temporal changes in the ISNT positive cells was analyzed by linear regression. The values were presented as mean±standard deviation. $p<0.05$ was considered as being significant.

III. RESULTS

1. Mortality and neurological outcome

Thirteen of the 45 rats that underwent BCAL died while all 27 rats that underwent the sham operation survived. Three of the 45 rats that underwent BCAL were used for evaluation of cerebral infarction by 2, 3, 5 triphenyltetrazolium chloride (TTC). The other rats were used for the ISNT study, immunohistochemical staining and zymography (Table 1). The scores of the motor disability scale were higher in the MCAO subjects that underwent the sham operation (5.07±1.08) than those that were subjected to chronic cerebral hypoperfusion (4.15±0.31) ($p=0.0038$).

Table 1. Experimental groups and tissues used in the present experiment

Acute focal ischemia		Chronic cerebral hypoperfusion				
MCAO[1]	Reperfusion	Bilateral ligation of CCA[2]			Sham operation	
		Perfusion fixation[3]	TTC[4] staining	Fresh frozen[5]	Perfusion fixation	Fresh frozen
0 hr	0 hr	3	3	3	3	3
2 hr	2 hr	3		0	3	0
2 hr	3 hr	3		3	3	3
2 hr	5 hr	3		0	3	0
2 hr	10 hr	3		0	3	0
2 hr	18 hr	3		5	3	3
Subtotal		18	3	11	18	9
Total		32			27	

[1]middle cerebral artery occlusion
[2]common carotid artery
[3]by transcardiac perfusion with 4% paraformaldehyde solution
[4]2, 3, 5-triphenyltetrazolium chloride
[5]frozen in 2-methylbutane/dry ice, and stored at 80℃

2. DNA scission

Very few ISNT positive cells could be found in the brains of the control animals that were subjected either to BCAL or to the sham operation. On the other hand, many ISNT positive cells were observed in the brains of the sham operated subjects in whom focal ischemia was induced by MCAO/R (Fig. 1), and the number of positive cells increased as the reperfusion time increased (Fig. 2). The number of positive cells was markedly less in the MCAO/R groups that were subjected to chronic cerebral hypoperfusion when compared with those that did not undergo chronic cerebral hypoperfusion (Fig. 2) ($p=0.0457$).

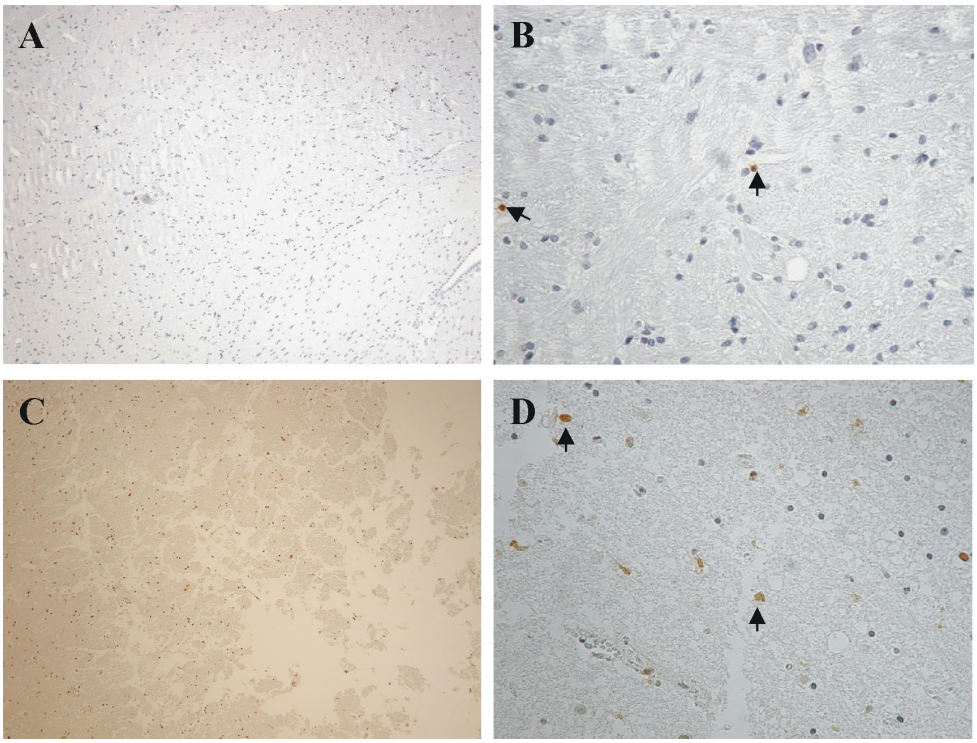

Figure 1. *In situ* nick translation (ISNT) study in the subjects that underwent middle cerebral artery occlusion for 2 hours/reperfusion for 18 hours (MCAO/R). **A**, **B** were pretreated by bilateral common carotid arteries ligation, and **C**, **D** were sham-operated. Many ISNT positive cells were observed in the brains of the sham operated subjects in which focal ischemia was induced by MCAO/R (**A**, **C** ×100; **B**, **D** ×400).

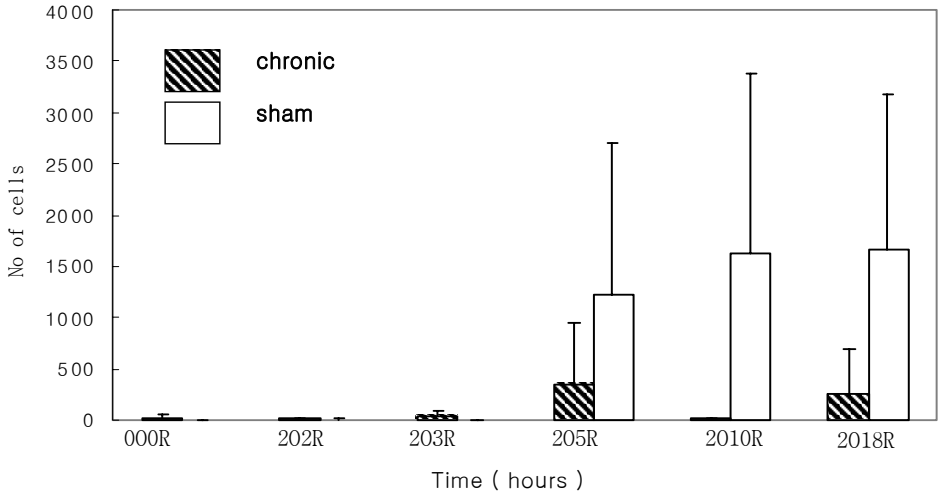

Figure 2. The number of *in situ* nick translation(ISNT) positive cells at the different time points of reperfusion following middle cerebral artery occlusion for 2 hours. A few ISNT positive cells were observed during the early time point of reperfusion in MCAO subjects that were subjected to bilateral common carotid arteries ligation (BCAL). As reperfusion time increased, the number of the positive cells in MCAO subjects that underwent sham operation were markedly increased when compared with those that underwent BCAL (O; occlusion, R; reperfusion).

3. DNA repair activity

APE/Ref-1 immunoreactivity, which was taken as evidence of DNA repair activity and ischemic resistance, differed between the groups. APE/Ref-1 positive cells were found in the control subjects that underwent chronic cerebral hypoperfusion, as well as in those that underwent sham operation. The intensity of APE/Ref-1 immunoreactivity increased markedly in the control subjects that underwent chronic cerebral hypoperfusion (Fig. 3). Fewer APE/ Ref-1 positive cells were found in the

ischemic core of the sham operated animals in which MCAO/R was induced, when compared with those that were subjected to chronic cerebral hypoperfusion (Fig. 4).

In the double staining of ISNT study and APE/Ref-1, those cells which exhibited immunoreactivity to APE/Ref-1 were not stained by the ISNT study (Fig. 5).

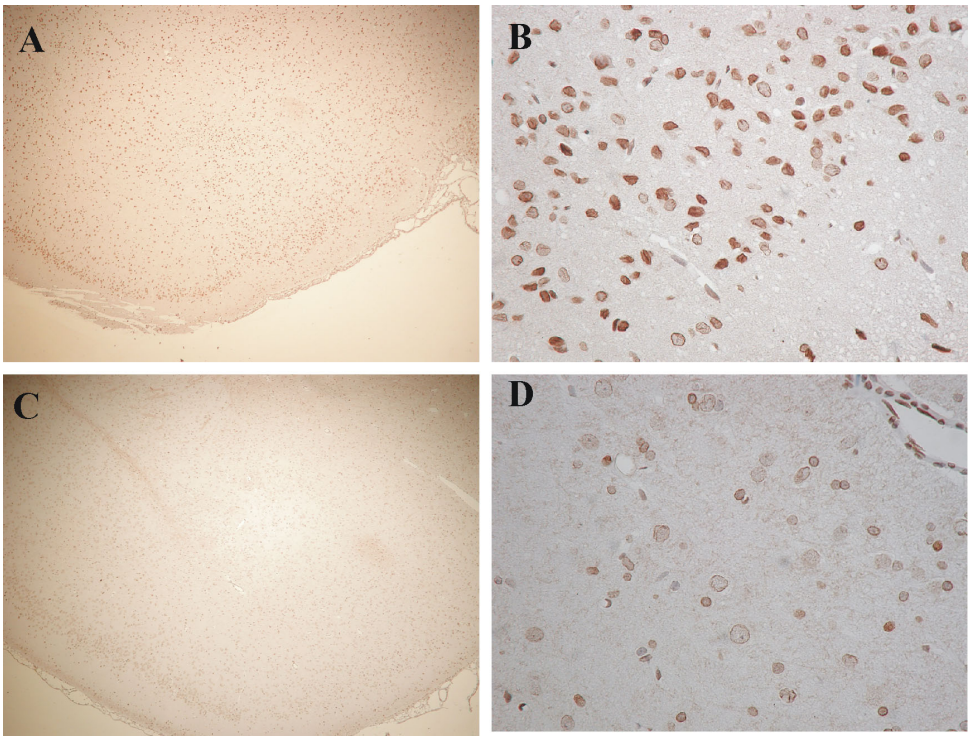

Figure 3. Immunohistochemistry for apurinic/apyrimidinic endonuclease/redox factor-1 in the control animals which did not undergo middle cerebral artery occlusion either after bilateral common carotid arteries ligation (BCAL) (**A, B**) or sham operation (**C, D**). APE/Ref-1 immunoreactivity was markedly increased in the control subjects that underwent chronic cerebral hypoperfusion (**A, C** ×40; **B, D** ×400).

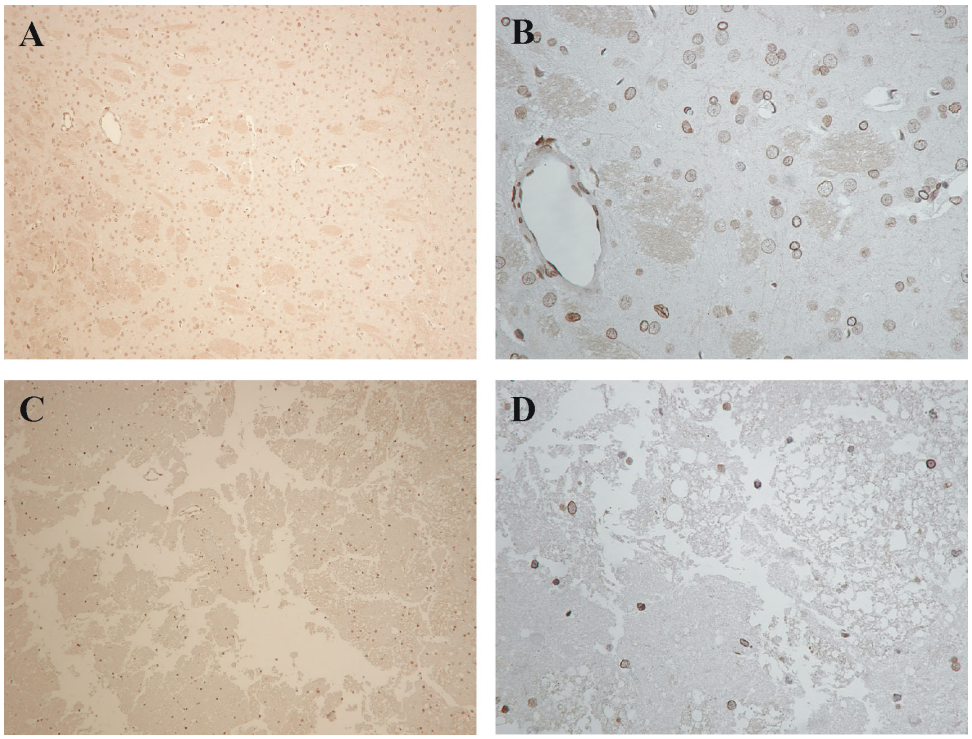

Figure 4. Immunohistochemistry for apurinic/apyrimidinic endonuclease/redox factor-1 (APE/Ref-1) in the subjects that underwent middle cerebral artery occlusion for 2 hours/reperfusion for 18 hours (MCAO/R) either after bilateral common carotid arteries ligation (BCAL) (**A, B**) or the sham operation (**C, D**). APE/Ref-1 immunoreactivity was markedly decreased in the subjects that underwent sham operation in which MCAO/R was induced, when compared with those that underwent BCAL in which MCAO/R was induced (**A, C** ×100; **B, D** ×400).

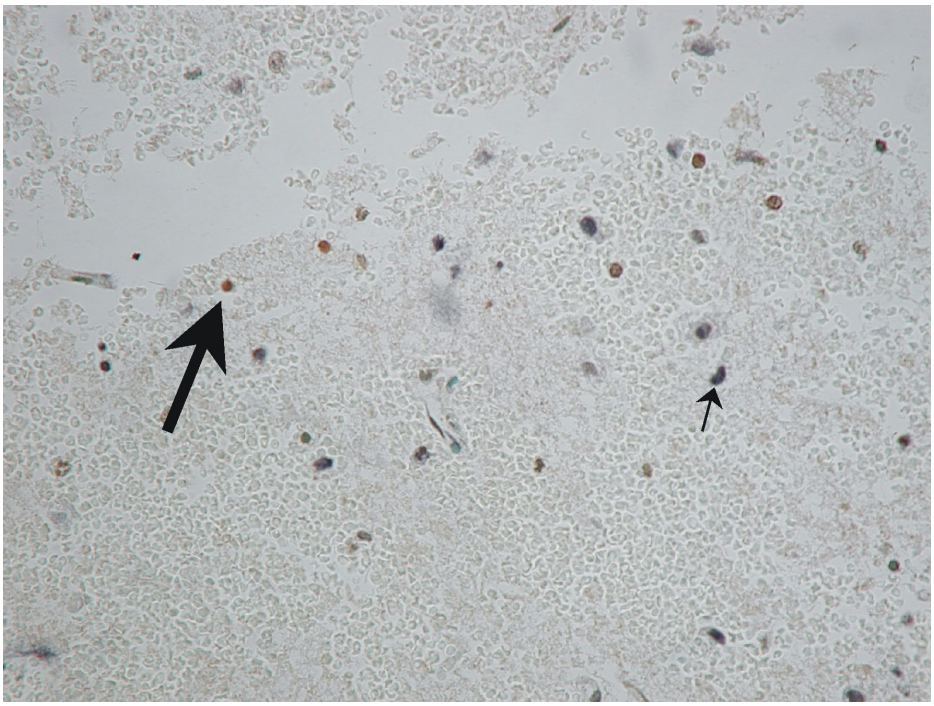

Figure 5. Double immunohistochemical staining for apurinic/apyrimidinic endonuclease/ redox factor-1 (APE/Ref-1) and dUTP incorporation by *in situ* nick translation study in the subjects in which middle cerebral artery occlusion/reperfusion was induced. APE/Ref-1 positive cells (small arrow) were not stained by ISNT study (large arrow) (×400).

4. MMP activity

In the gelatin zymograms, MMP-2 activity was observed in all tested samples, while MMP-9 activity was only observed in those subjects that underwent MCAO/R (Fig. 6).

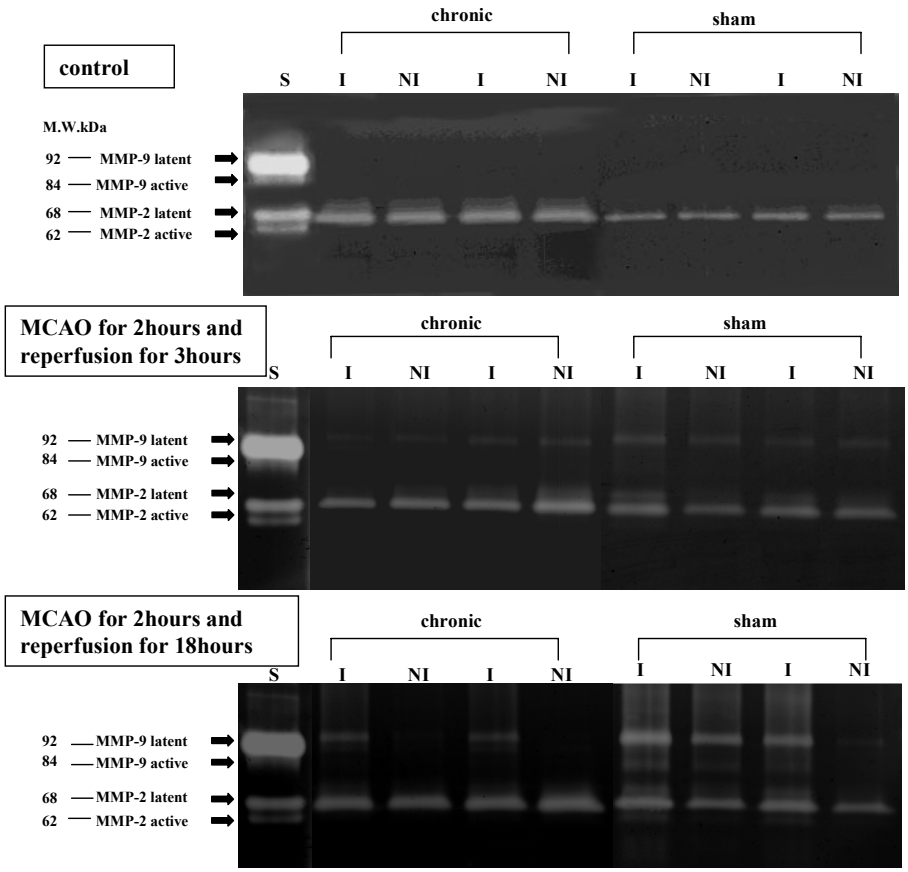

Figure 6. Representitive zymograms. Matrix metalloproteinase (MMP)-2 bands were consistently observed in the non ischemic (NI) and ischemic (I) hemispheres of all subjects while those of MMP-9 were in subjects undergoing middle cerebral artery occlusion/reperfusion. The MMP-2 activity increased in the control subjects that were subjected to chronic cerebral hypoperfusion.

4 weeks after BCAL, the MMP-2 activity was found to be increased when compared with the sham operated-animals, but did not reach statistical significance (BCAL; 7.5±6.24 vs. sham operation; 5.5±6.24, p=0.3785). In those animals in which MCAO/R was induced, the MMP-2 activity increased as the reperfusion time increased in the sham operated subjects (p=0.0001, β=97.99), but this was not the case in those animals that underwent BCAL (p=0.8467) (Fig. 7).

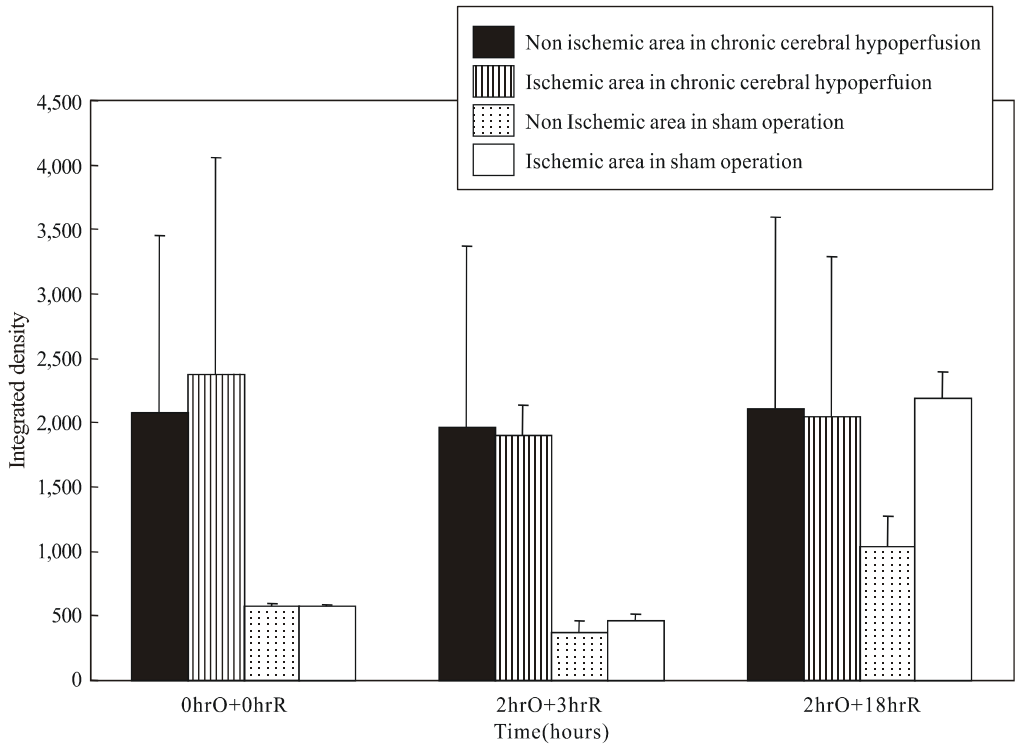

Figure 7. Matrix metalloproteinase (MMP) -2 activity in the subjects undergoing middle cerebral artery occlusion/reperfusion and the control subjects. The MMP-2 activity increased in the subjects that were subjected to chronic cerebral hypoperfusion (O; middle cerebral artery occlusion, R; reperfusion).

In those subjects that underwent MCAO/R, the MMP-9 activities increased as the reperfusion time increased (BCAL, $p=0.0022$, $\beta=19.82$; sham operation, $p=0.0006$, $\beta=59.89$) (Fig. 8).

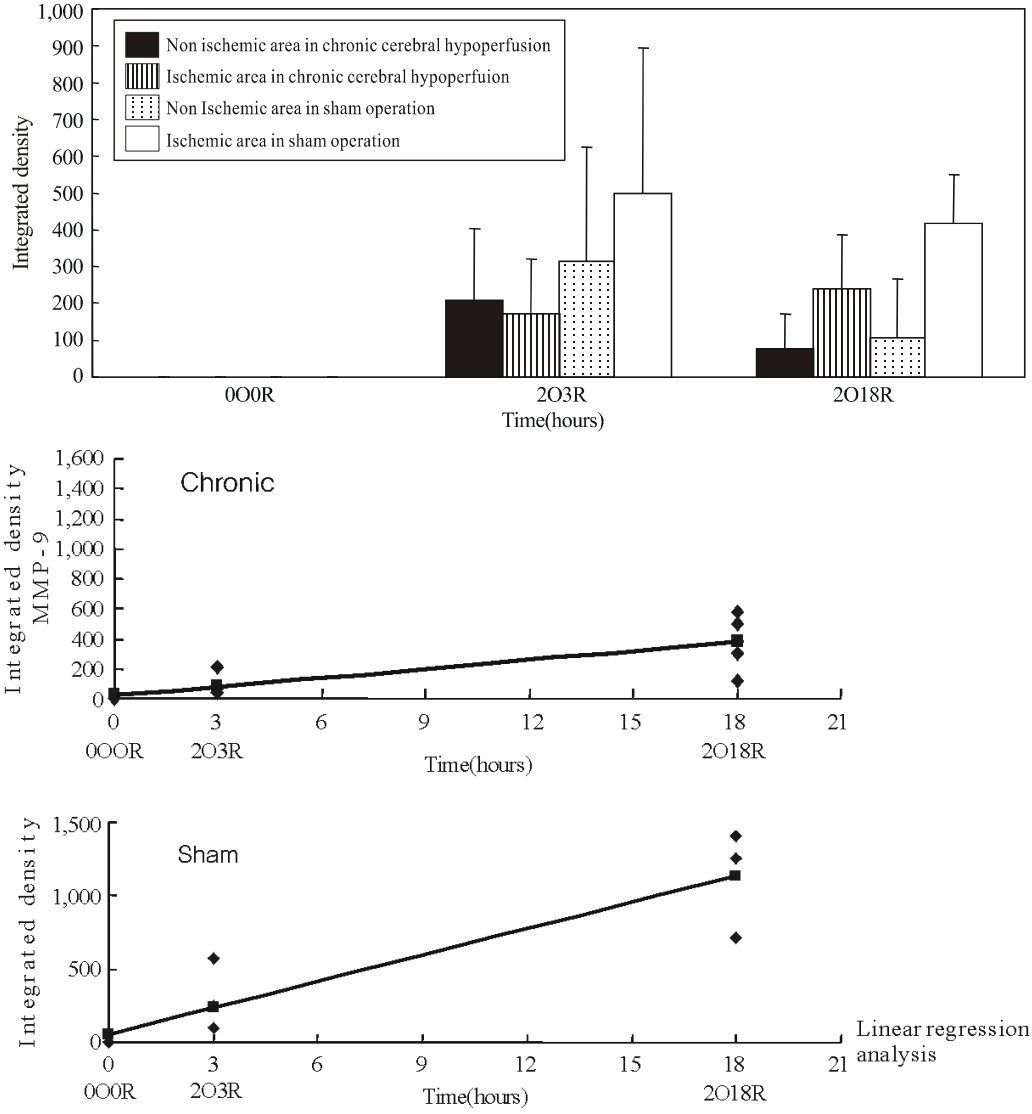

Figure 8. Matrix metalloproteinases (MMP) -9 activity in the subjects that underwent middle cerebral artery occlusion/reperfusion (MCAO/R) and the control subjects. The MMP-9 activity increased after MCAO/R. The increase was more evident in the subjects that underwent sham operation than those that underwent chronic cerebral hypoperfusion (O; middle cerebral artery occlusion, R; reperfusion).

In the histochemical studies, MMP-9 immunoreactivity was confirmed in the microvessels of the ischemic brain tissues (Fig. 9).

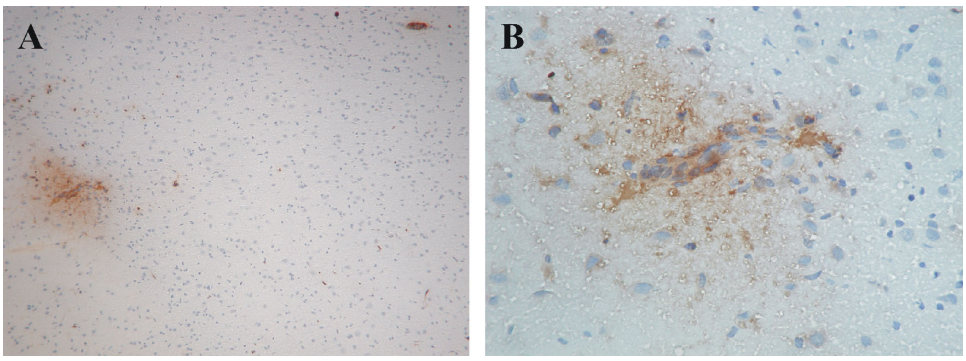

Figure 9. Immunohistochemistry for matrix metalloproteinase (MMP) -9 in a subject that underwent middle cerebral artery occlusion for 2 hours/reperfusion for 18 hours. MMP-9 immunoreactivity was found in the microvesseles of the ischemic area (A × 100, B ×400).

IV. DISCUSSION

In the present study, we investigated the effect of the chronic cerebral hypoperfusion induced by BCAL on acute severe ischemic injury. Ischemic injury, which was examined by detecting DNA scission, was markedly reduced by pretreatment with BCAL. The findings in the present study demonstrated for the first time that minor ischemic injury or chronic sublethal hypoperfusion of the brain may provide protection against subsequent severe ischemic insults in experimental conditions. The BCAL model, which was used to induce cerebral hypoperfusion in the present study, has been extensively in previous studies.[11,13,14,15,16] Although the cerebral blood flow (CBF) was not measured in this study, the CBF in this model is known to be decreased to 30-50% of the baseline after 2 days, and to remain significantly reduced in the occipital cortex, white matter, globus pallidus and subtantia nigra after 4 weeks.[21,22] Twenty-nine percent of the rats that underwent BCAL died in this study, while the mortality rate caused by BCAL in rats was 10 to 20% in previous studies.[22,23,24] The mortality rate may vary between different strains of rats. The reduction of local CBF in the cortex and thalamus after BCAL was greater in the SHRs than in the normotensive rats,[25,26,27] which may partly account for the higher mortality observed in this experiment. Although BCAL caused death in a portion of the rats, no significant focal ischemic tissue damage, except for white matter changes or glial reactions in specific areas such as the optic tract, corpus callosum, and internal capsules, were found in the brains of survivors in previous reports.[11,13,15,17] Therefore, this model has been used for the study of vascular dementia. In our preliminary experiments, no definite area of infarctions was

observed in the TTC staining results obtained from the 3 surviving rats, as would have been expected based on the previous report.[11,13,15,17] Ischemic preconditioning or ischemic tolerance, which has been termed as any stimulus capable of causing injury, can, when applied close to (but below) the threshold of damage, protect the brain against subsequent ischemia.[4,28,29,30] Thus, in the present study, it is conceivable that the brain tissues were preconditioned by chronic cerebral hypoperfusion and acquired tolerance to subsequent ischemia.

Chronic cerebral hypoperfusion may predispose the affected vicinity to certain cellular responses and lead to the redistribution of blood flow and vascular remodeling to adapt to the altered environment. In this study, APE/ Ref-1 immunoreactivity was examined to show that the evidence of reduction in DNA damage in hypoperfused tissues was coupled with a known mechanism of the cellular defense. The DNA repair enzyme, APE/Ref-1, is a multifunctional protein in the DNA base excision repair pathway, which is responsible for repairing the apurinic/apyrimidinic site in DNA.[7,31,32,33] When cells become committed to apoptosis, presumably due to excessive DNA damage, APE/Ref-1 expression is down-regulated.[32] A decrease in APE/Ref-1 expression may result in apoptotic cellular death.[34] In contrast, over expre ssion of APE/Ref-1 could protect cells from the toxicity of oxidizing agents.[35] In a present study, APE/Ref-1 immunoreactivity was markedly diminished in the severely damaged ischemic core, and was not co-localized with ISNT positive cells. Of particular note, is that the increase in APE/Ref-1 immunoreactivity was evident in those brain tissues which were preconditioned with hypoperfusion for 4 weeks, which implicated the presence of cellular defense responses to ischemia.

Chronic cerebral hypoperfusion may induce or facilitate the adaptive

growth of preexisting collateral arteries (arteriogenesis) and new capillary formation (angiogenesis).[36,37,38] An occlusion of a large artery causes a change in the pressure gradient through interconnecting arteries, which finally results in the redistribution of the blood flow. In those rats underwent BCAL, hemispheres are supplied from the vertebrobasilar arterial system through the posterior communicating artery. Arteriogenesis was induced by the occlusion of one common carotid in combination with the bilateral vertebral arteries (3-vessel occlusion) in the Sprague-Dawley rats.[37]

Both arteriogenesis and angiogenesis require an active process of vascular remodeling. The MMPs, which constitute a family of zinc-dependent proteolytic enzymes that degrade most extracellular matrix molecules, play an important role in the pathologic process, as in the adaptive process, of vascular remodeling by degrading the vascular extracellular matrix. Among the diffe- rent MMP families, MMP-2 and MMP-9, which belong to a gelatinase family and digest vascular basal lamina by degrading laminin and type IV collagen, are known to be present in increased quantities in ischemic brain tissues.[39,40,41,42] In this study, the changes of MMP-2 and MMP-9 in the brain tissues of those animals that underwent chronic cerebral hypoperfusion were examined. MMP-2 was upregulated in those brains which had suffered from chronic cerebral hypoperfusion, but no overt ischemic tissue damage was observed. The exact role of this increased MMP-2 level in chronically hypoperfused tissues is uncertain. However, it has been suggested that MMP-2 may play a role in angiogenesis degradation of the vascular basement mem- brane, thus facilitating endothelial migration, proliferation, and capillary tube formation.[23,39,40]

In contrast to MMP-2, the MMP-9 activity in the brain was not

changed by chronic cerebral hypoperfusion. The MMP-9 has been reported to increase during the early phase of ischemia[41,42] and mediate cellular injury as well as brain edema and hemorrhagic transformation following cerebral ischemia.[43,44,45,46] MMP-9 may be detrimental to ischemic brain tissues, because the infarction size can be reduced by an MMP-9 inhibitor.[45] In the present study, when focal and severe ischemia was induced by MCAO/R, an increase in MMP-9 activities was observed in both groups with or without chronic cerebral hypoperfusion, as the reperfusion time increased. However, the degree was lower in those subjects that were subjected to chronic cerebral hypoperfusion than in those subjects that did not undergo chronic cerebral hypoperfusion, which reflected the less severe ischemic damage in those subjects that were chronic cerebral hypoperfusion.

V. CONCLUSION

Although many strokes occur in brain tissues which have been in a chronically hypoperfused state due to a significant arteriosclerotic stenosis or the occlusion of a large supplying artery, the exact cellular and vascular responses in these conditions are unknown. In the present study, we investigated the effect of chronic cerebral hypoperfusion induced by BCAL on the acute and severe ischemic injury caused by MCAO/R. The results can be summarized as follows.

1. The number of positive cells in the *in situ* nick translation study, which was taken as an indication of cellular injury, was significantly reduced in those subjects that were subjected to chronic cerebral hypoperfusion

2. There is evidence that the reduction in cell damage observed in hypoperfused tissues is coupled with a known mechanism of cellular defense. Immunoreactivity for APE/Ref-1, which plays a role in the DNA repair pathway, was markedly increased in the brain tissues which were subjected to chronic cerebral hypoperfusion.

3. Indirect evidence of extracellular matrix remodeling, which might be associated with adaptive arteriogenesis or angiogenesis, was observed in the form of increased MMP-2 activity in the hypoperfused brain.

The findings of this study provided experimental evidence that chronic sublethal cerebral hypoperfusion provided protection against subsequent severe

ischemic insults, which may account for the occurrence of less severe stroke in patients with atherosclerosis than in those with cardioembolism. The present study stimulates the development of specific animal models that can represent each stroke mechanism observed in humans. Future studies on cellular and molecular responses specific to each mechanism would substantially contribute to the development of a custom-tailored therapeutic approach to treating and preventing human stroke.

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국문요약

자발성 고혈압 쥐에서 만성 뇌 혈류저하가 중대뇌동맥 폐색 유발성 세포 손상에 미치는 영향

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뇌경색의 발생 기전으로는 동맥경화증, 심인성 색전증, 혈액 역동학적 장애 등 그 원인이 다양하다. 동맥경화증에 기인한 뇌경색인 경우 심인성 색전증에 의한 뇌경색 보다 뇌경색의 크기와 신경학적 결손이 적다. 또한 치사 직전의 손상을 받은 대부분의 기관들이 그 이후에 따라오는 극심한 손상을 일으키는 환경의 변화에 대해서 내성을 갖는 현상이 실험적으로 관찰되었다. 이제까지 자발성 고혈압 쥐에서 경동맥을 결찰하여 만성 뇌혈류 저하의 동물 모델을 유도한 많은 연구가 있었다. 본 실험에서는 만성 뇌혈류 저하가 허혈성 뇌손상에 대한 내성을 제공할 것이라고 가정하였다. 이를 검증하기 위해 두 가지 잘 알려진 쥐 실험 모델인 양측 경동맥 결찰 모델과 중대뇌동맥 폐색 모델을 함께 사용하였다. 자발성 고혈압 쥐에서 양측 경동맥 결찰을 하거나, 양측 경동맥 결찰을 하지 않고 수술 과정만 동일하게 시행한 동물 군을 4주동안 사육한 후 중대뇌동맥을 폐색하여 국소 뇌허혈을 유도 하였다. 뇌 조직을 파라핀 조직과 냉동 조직을 위해 처리하였고, *in situ* nick translation study와 apurinic/apyrimidinic endonuclease/redox factor-1 (APE/Ref-1)과 matirx metalloproteinase (MMP)-9을 관찰하기 위한 면역 조직화학 염색을 시행하였다. MMP-9와 MMP-2의 정량 분석을 위해 zymography을 시행하였다. *In situ* nick translation study에서 양성 세포 수가 만성 뇌 혈류 저하가 없었던 경우보다 만성 뇌 혈류 저하 상태에서 중대뇌동맥

이 폐색된 경우에 더 적음을 알 수 있었다. APE/ Ref-1 활성도는 만성 뇌 혈류저하 후의 뇌조직에서 더 증가하였다. 만성 뇌혈류 저하에 대한 적응 현상 중에 하나인 신생혈관 형성과 연관되어 있을 가능성이 높은 MMP-2의 증가를 만성 뇌혈류 저하 후의 뇌조직에서 관찰하였다. 본 실험에서는 만성 뇌혈류 저하 후의 뇌조직에서 국소적인 뇌혈관 폐색에 대한 허혈 내성이 나타남을 밝혔고, 그 기전으로 DNA 복구 활동성 증가와 세포 외 기질의 재구성을 통해 신생혈관 형성이 연관될 가능성이 높음을 제시하였다.

핵심되는 말: 만성 뇌혈류 저하, 중대뇌동맥 폐색, 허혈 내성