# Effects of mild hypothermia on the regulation of metalloproteinase expression in a focal model of transient cerebral ischemia

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**BK 21 Project for Medical Science,**
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# Effects of mild hypothermia on the regulation of metalloproteinase expression in a focal model of transient cerebral ischemia

**Directed by Professor Jong Eun Lee**

**The Master's Thesis submitted to BK 21 Project for Medical Science, the Graduated School of Yonsei University in partial fulfillment of the requirements for the degree of Master of Medical Science**

***Yoon Yone Jung***

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# This certifies that the Master's Thesis of Yone Jung Yoon is approved.

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**The Graduate School**
**Yonsei University**

**June 2002**

# Acknolwedgments

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# Effects of mild hypothermia on the regulation of metalloproteinase expression in a focal model of transient cerebral ischemia

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*(Directed by Professor Jong Eun Lee)*

Mild hypothermia is a robust neuroprotectant, but the mechanisms ha ve not been elucidated well. One aspect of this protection has been associated with the prevention of blood brain barrier (BBB) disruption. Matrix metalloproteinase (MMPs) can degrade the extracellular matrix and is significantly increased after ischemia. In this study, we defined what is the protective mechanism of hypothermia in ischemic injury and what relationship is there between hypothermia and MMPs expression. We also examined whether BBB preservation from mild hypothermia is due to alterations in MMP expression and we showed how MMPs can regulate apoptosis proteins in the hypothermic condition. We made animal model using the middle cerebral artery occlusion for 2 hrs. Animals were maintained either at normal brain temperature or 33℃ after MCA occlusion. Brains were lasted 2hrs, 6hrs and 24hrs post MCA occlusion and harvested each reperfusion times. Expression of MMP-9 protein showed increase in the ischemic cortex compared to the contralateral side. The expressions of MMP-2 and MMP-9 were reduced in the hypothermic brains at 6 hrs and 24 hrs after ischemic injury. Neurons and astrocytes expressed the MMP-2 and MMP-9 in the

normothermic brain after ischemic injury, however, the MMP-2 and MMP-9 immunoreactivities were decreased in the neurons of the hypothermic brain. Fas levels were decreased in the hypothermic brain after ischemic injury, however levels of membrane-bounded Fas-ligand in the hypothermic brains were expressed more than in the normerthermic brains after ischemic injury. In neuroprotection of mild hypothermia attenuates BBB disruption by decreasing of MMPs' expression and suppressing of MMPs' activity. Moreover, mild hypothermia inhibited the expression of Fas and reduced the soluble Fas-ligands, possibly due to the lower levels of MMPs need to degrade it, in the brain after ischemic injury.

Key words: Ischemic injury, Hypothermia, Neuroprotection, Apoptosis, Matrix metalloproteinase.

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## Ⅰ. Introduction

Stroke remains one of the major causes of death and disability throughout the world[1]. Cerebral ischemia can divide two types of the global cerebral ischemia and focal cerebral ischemia[2]. Global cerebral ischemia involves the entire brain and occurs during cardiac arrest. Focal cerebral ischemia affects restricted brain regions. Whereas the hemodynamic, cellular, molecular changes initiated by cerebral ischemia have been better defined, the mechanisms leading to brain damage are beginning to be clarified[3].

Occlusion of one of the major cerebral arteries, produces an immediate reduction in cerebral blood flow (CBF) to the areas of the brain supplied by that particular artery. If the arterial occlusion is released, CBF returns to the ischemic territory. Initially there is a phase of increased flow followed by a long period of reduced flow[4]. When blood flow decreases approximately 50%, a decrease or block of protein synthesis is one of the first biochemical changes to occur after focal cerebral ischemia. A decrease in ATP is not the signal for a block in protein synthesis because ATP does not decrease until flow falls to 20% of the normal level. Ribosomal protein synthesis appears to be the sensitive step that responds to this

reduced blood flow, occurring because of in inactivation of initiation factor 2 (eIF2), guanine nucleotide exchange factor (eIF-2-GTP complex factor), and eukaryotic elongation factor (eEF-2). Glutamate-dependent phosphorylation of eEF-2 provides a direct link between ischemia-induced increases of extracellular glutamate and ischemia-induced inhibition of protein synthesis. Phosphorylation of eIF-2 by protein kinase R also provides a control point in protein synthesis that is sensitive to oxygen and/or adenosine monophosphate[5]. Protein synthesis continues in cells that survive in an infarct. In some infarcts, some or all of the blood vessels survive. When this occurs, protein synthesis can continue within blood vessels. For example, heat shock protein 70 (HSP70) continues to be expressed in blood vessels in an infarct [6] as well as iNOS, eNOS[7] and many other genes. Importantly, there may be expression of cell adhesion molecules, cytokines, and chemokines by vascular cells within infarcts and at the margins of infarcts. Finally, inflammatory cells inside of infarcts, including neutrophils and macrophages, mount a specific genomic response to the dying and dead neurons and glia[8].

In cerebral ischemia study, it is known that hypothermia is highly effective in reducing injury. Deep to moderate hypothermia (20 to 30℃) has been found to be useful clinically following head trauma and during surgical procedures that disrupt the cerebral circulation[9]. Mild hypothermia (~33℃), while less protective than deep hypothermia, has gained considerable interest recently because it provides significant neuroprotection with far fewer complications[10]. The effect of hypothermia on clinical ischemic stroke has not yet been examined in large clinical trials. In animal models of global ischemia, mild hypothermia can reduce infarct size and the extent of ischemic neuronal damage in non-infarcted regions[11]. For focal

ischemia, mild hypothermia can reduce the size of the necrotic region and the extent of induction of apoptosis in non-necrotic regions. It can also help to prevent neuronal and endothelial damage associated with reperfusion injury, if it is initiated during or after ischemia[12,13,14]. Lowering the body temperature 3-5 degrees slows anaerobic metabolism, high-energy phosphate depletion reduced CBF preservation BBB. Blood brain barrier integrity protects the neuronal microenvironment[15]. During cerebral ischemia-reperfusion, endothelial basal lamina dissolution of BBB starts and BBB is disrupted by proteolytic enzyme.

In animal study, matrix metalloproteinase (MMPs) break down the extracellular matrix (ECM) in reperfusion injury, and in other neuroinflammatory conditions[16]. BBB break down occur in a biphasic pattern during reperfusion injury[17]. MMPs are a family of Zn-dependent endopeptidase, which have the specialized function in degrading the extracellular matrix (ECM) components[18, 19]. MMPs are synthesized in preproenzyme form and most of them are secreted from cells as proenzymes and activated in the extracellular compartment. The expression and activity of MMPs are tightly regulated. Specific proteins known as the tissue inhibitors of metalloproteinases (TIMPs) are the physiological regulators of these enzymes. Currently, more then 20 MMPs are described, which are involved in normal and pathological processes connected with remodeling and destruction of the ECM. The matrix metalloproteinases (MMPs) include MMP-7 (matrilysin), MMP-3, -10, -11, -13 (stromelysins), MMP-14 (membrane MMP), and MMP-2 and MMP-9 (gelatinase A and B, respectively)[20]. MMP-2 and MMP-9 have been the subject of recent studies because they attack type IV collagen, laminin, and fibronectin, the major components of the basal lamina around cerebral blood vessels. MMP-2 is expressed

constitutively in the brain and may play a role in ischemia[21]. MMP-9 is the 92kD type IV collagenase. After ischemia, proMMP-9 is induced in the core within 2 hrs with enzymatic activity and mRNA induction being detected by 4 hrs[22]. Induction of MMP-9 mRNA could be mediated by a NF-kB site in the MMP-9 promoter[23]. Activation of MMP-9 correlates with blood-brain barrier break down[24] and in at least one study correlated with areas of hemorrhagic conversion after focal ischemia[25]. MMPs may be important for producing increases of blood brain barrier permeability and brain edema after stroke[26]. MMPs may also promote tissue invasion of neutrophils and macrophages, and contribute to hemorrhages that result after reperfusion of ischemic tissue[27, 28].

Mild hypothermia is a robust neuroprotectant, but the mechanisms have not been well elucidated. One aspect of this protection has been associated with the prevention of blood brain barrier (BBB) disruption. MMPs degrade the extracellular matrix and are significantly increased after ischemia. Among other functions, MMPs cleave the receptor mediated, apoptotic protein Fas.

In this study, we defined the protective mechanism of hypothermia in ischemic injury and regulation of MMPs expression by hypothermia in ischemic injury. We examined whether BBB preservation from mild h ypothermia is due to alterations in MMP expression and showed how MMPs can regulate apoptotic proteins in the hypothermic condition.

# Ⅱ. Methods and Materials

## 1. Animals

Forty male Sprague-Dawley rats weighing between 220g and 250g from Samtako (Osan, Korea) were used for this study. The animals were anesthetized with 4% chloral hydrate. We applied whole body cooling and warming in the range 33-38℃ (rectal temperature) to 4% chloral hydrate (0.01 ml/g)-anesthetized rats and monitored for rectal temperature. Brian temperature was reduced to 33℃ by placing the animal on a cooling blanket and spraying ethanol onto the body and to 38℃ by placing the animal in a hot pack. Temperatures were monitored during conditions of ischemic normothermia and mild hypothermia as described subsequently.

## 2. Ischemia model

The animal was placed in a heating / cooling blanket to maintain body temperature between 38℃ and 33℃. Ischemia was induced using an occluding intraluminal suture. A cervical midline incision was made and the left carotid artery and branches were isolated. The common carotid artery (CCA), external carotid artery (ECA), and pterygopalatine artery (PPA) were identified. An uncoated 30 ㎜ long segment of 3-0 nylon monofilament suture with the tip rounded by a flame is inserted into the stump of the CCA and advanced under direct visualization into the ICA approximately 19-20 mm from the bifurcation in order to occlude the ostium of the MCA. A temporary aneurysm clip was also placed on the CCA. Animals were either kept normerthermic (normothermia, rectal temperature = 37-38℃) or rendered mildly

hypothermic (hypothermia, rectal temperature = 33℃) with in 15-20 min after MCA occlusion and maintained for 2 hrs. After 2 hrs of ischemia, the aneurysm clip and suture were removed and the surgical incisions closed. The animal was allowed to recover, and then transported to the intensive care unit. And rats were killed at 0 hr, 4 hrs, and 22 hrs postischemic period.


### 3. Measurement of infarct volume

The area of cerebral infarction was quantified in Nissl stained brain. Part of the brains was fixed overnight in a buffered neutral formalin (10% volume/ volume) solution, embedded in paraffin, and deparaffinized by the standard procedure. Serial paraffin-embedded tissue sections were subjected to Nissl staining using *cresyl echt* violet solution (Sigma, St. Louis, MO, USA). Brain tissue sections throughout the deparaffin process were incubated for 3 or 5 min at room temperature with *cresyl echt* violette solution, rinsed in D.W for 2 times. The stained slide was undergone dehydration steps, incubated for 2 min with xylen-balsam mixtures, washed with absolute ethanol for 2 times, and mounted with synthetic resin.

The area of infarction of each section was determined with a computerized image analysis system, Optimas program (Optimas Co., WA, USA).

## 4. Protein extraction

Animal were anesthetized using 4% chloral hydrate and decapitated 0 hr, 4 hrs and 22 hrs after 2 hrs MCA occlusion. The cerebral cortex, striatum and penumbra parts were dissected for extraction of cellular proteins (Figure), treated with 10x lysis buffer (1x PBS, 1% nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitors-PMSF, Aprotinin and Sodium orthovandate) and isolated protein used homogenizer (Dramel, Racine, WI, USA).

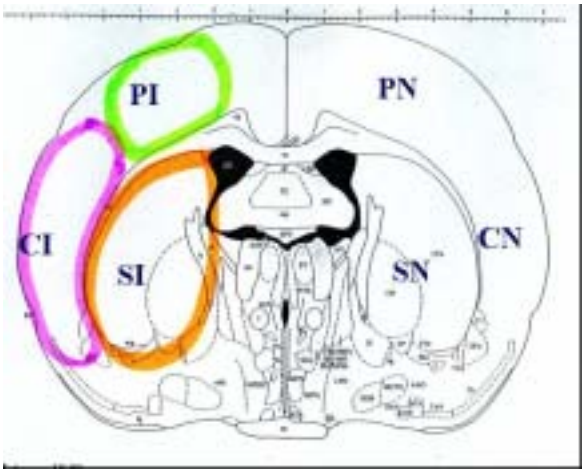



Figure. Scheme of sampling for experiment. SI: ischemic core of striatum, CI: ischemic core of cortex, PI: ischemic penumbra of cortex, SN: contralateral side of ischemic striatum, CN: contalateral side of ischemic cortex, PN: contralateral side of penumbral cortex.

## 5. Gelatin zymography

Samples of cerebral cortex, penumbra, and striatum from each conditions were subjected to detergent extraction and purification for gelatinase activity and gel zymography following the method described by Zhang and Gottschall.[28] Briefly, samples were homogenized in lysis buffer containg detergents. All regents were

from Sigma (St. Louis, MO, USA). An aliquot of the homogenate was centrifuged and the supernatant was incubated with gelatin-Sepharose 4B (Amersham Parmacia Biotech., Uppsala, Sweden)(50 μℓ) for 1h at 4℃. After washing, the Sepharose-pellet was incubated with 150 μℓ of elution buffer containing 10% dimethyl sulfoxide for 30 min at 4℃.

Protien concentrations were determined by the bicinchonic acid method (BCA kit) (Pierce, Rockford, IL, USA). Samples were mixed with an equal volume of 4x sample buffer (0.4 mol/L Tris, pH 6.8, 5% sodium dodecyl sulfate[SDS], 20% glycerol, 0.05% bromophenol blue) and loaded onto a 10% SDS polyacrylamide gel electrophoresis gels containing 0.1% gelatin (Sigma, St. Louis, MO, USA) After running, gels were incubated with renaturation buffer (40% Triton X-100, 50 mM Tris-HCl, 100 mM NaCl) two times for 30 min at room temperature, and incubated for over 18 hrs in the reaction buffer (50 mM Tris-HCl, 10 mM $CaCl_2$) at 38℃ with gentle agitation. The gels were stained with coomassie blue (0.5% coomassie R-250, 30% methanol, 10% acetic acid) for 1 hrs and destained in washing solution (30% methanol, 10% acetic acid).

White bands on the blue background indicated zones of digestion corresponding to the presence of different proMMPs and activated MMPs on the basis of their molecular weight.

### 6. Western blot analysis

For immnuoblot analysis of MMP-2, MMP-9, Fas, Fas-ligand and caspase-8 in the hypothermic and normothermic brain after ischemic injury, total tissue extracts were prepared in the different times after ischemic injury by the protein extract method.

In all cases, equal amounts of protein were subjected to electrophoresis in 12.5% and 15% polyacrylamide gels, and transferred to nitrocellulose membranes (Millipore, Bedford, MA, USA). After 2 hrs of blocking, the membranes were incubated with an optimal dilution of primary antibodies against MMP-2 (rabbit polycronal antibody: AB19015)(Chemicon, Temecula, CA, USA), MMP-9 (rabbit polyclonal antibody: AB19016)(Chemicon, Temecula, CA, USA), Fas (rabbit polyclonal antibody, C-20)(Snata Cruz Biotech. Inc., CA, USA), Fas-ligand (rabbit polyclonal antibody, N-29)(Santa Cruz Biotech. Inc., CA, USA) and Caspase-8 (rabbit polyclonal antibody, AAP-103)(Stress gene, Victoria, BC, Canada) in, Tris-buffered saline containing with 0.05% Tween 20 at 4℃ overnight. After washing, the membranes were further incubated with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (Amersham Parmarcia Biotech., Uppsala, Sweden) and developed using the Enhanced Chemiluminsecence (ECL) system (Amersham Parmarcia Biotech., Uppsala, Sweden). The membranes were reprobed with monoclonal anti-ß-actin antibody and developed as above. Fold proteins expression differences were determined by gel-scope, EGA (BioBud, Seoul, Korea).

### 7. Immunohistochemical staining

Immunohistochemistry was performed in 12 rats after reperfusion for 0 hr, 4 hrs, and 22hrs. Rats were anesthetized with 4% chloral hydrate and brains were moved and made paraffin block. For immunohistochemical staining, paraffin blocked tissue sections were deparaffin processed, rinsed, blocked, and incubated overnight at 4℃ with the primary antibodies (MMP-9, MMP-2, Fas and Fas-

ligand). Diaminobenzidine (DAB) (DAKO Co., Carpinteria, CA, USA) staining was done by incubation the sections for 10min with biotinylated goat anti-rabbit IgG. Sections were rinsed, incubated with DAB, distilled water and 0.3% hydrogen peroxide/ methanol. Slides were dehydrated through alcohols and xylene and mounted in permount. Immunofluorescent staining for neuron-specific nuclear protein (NeuN, CA92590) (Chemicon, Temeala, CA, USA) MMP-2, MMP-9, and phospholyated porcine glial fibrillary acid protein (GFAP, MS1376)(Neomarker, San Fransico, CA, USA) was performed using paraffin block sections prepared from brains 0 hr, 4 hrs, and 22 hrs after ischemia-reperfusion. The procedures for double-label immunofloursence staining [MMP-9 and NeuN, MMP-9 and GFAP, MMP-2 and NeuN, MMP-2 and GFAP] were the same as previous described Chen and Cao[29, 30].

## 8. Reverse transcription (RT)- PCR

Total RNA was extracted from brain tissue using the Trizol reagent (Gibco BRL, Gaithersburg, MD, USA) according to the manufacturer's instructions. mRNA was used as the template for the synthesis of single stranded cDNA using 1 U/㎕ Moloney murine leukemia virus reverse transcrpitase (M-MLV reverse trnscriptase) (Promega Co., Madison, WI, USA) in reaction mixtures containing 2.5 pM oligo (dT)primer, 0.5 mM dNTP, and 1 U/㎕ tibonuclease inhibitor (Takara Shuzo Co., Japan) in total volumn of 20 ㎕. Reverse transcription was performed denaturation at 37℃ for 2 hrs and inactivation at 75℃ for 15 min, and 4℃ for 5 min using by Programmable thermal controller (MJ Research Inc., Wetertown, MA, USA)

Polymerase chain reaction(PCR) was performed by using reaction mixtures containing 1.25 mM $MgCl_2$, 250 nM sense and antisense primer, and 2.5 U/100μℓ t*aq* DNA polymerase (Takara Shuzo Co.. Japan) in a thermocycler.(MJ Research Inc., Watertown, MA, USA). Amplification was performed as followed; 5 mins at 94℃ for denaturaion, 1 min at 47℃ for annealing, and 1 min at 72℃ for extention. followed by 49 cycles of 70℃ for 30 secs, 50℃ for 1 min, and 72℃ for 1 min 30 secs.

The PCR primers were:

| | |
|---|---|
| MMP-9 (Z27231) | sense 5'-AAATGTGGGTGTACACAGGC-3'<br>antisense 5'-TTCACCCGGTTGTGGAAACT-3' |
| MMP-2 (M84324) | sense 5'-CTATTCTGTCAGCACTTTGG-3'<br>antisense 5'-CAGACTTTGGTTCTCCAACTT-3' |
| Fas | sense 5'-AGCCTTGCACACGAACCAGCA-3'<br>antisense 5'-AGCTTGACACGCACCAGTCTTC-3' |
| Fas-ligand | sense 5'-GCTCAAGGTCCATCCCTCTGGA-3'<br>antisense 5'-TAGCTGACCTGTTGGACCTTGT-3'. |

PCR products were electrophorised on an agarose gel and visualized by ethidium bromide staining. Location of the products was determined by using a 100 bp ladder as a standard size marker. Gels were photographed over ultraviolet light.

## 9. Northern blot

Total RNA was extracted from brain tissue using the Trizol reagent (Gibco BRL, Gaithersburg, MD, USA) according to the manufacturer's instructions. Equal amounts of total RNA (10 μg/lane) were fractionated on 1% agarose containing 0.6 M formaldehyde gels and blotted on a nylon membrane (Hybond-

$N^+$)(Amersham Pharmacia Biotech., Uppasala, Sweden) by using the traditional capillary system. RNA was prehybridized at 42 ℃ in 5x saline sodium citrate (SSC), 50% formamide, 5x Dehardt's solution and 0.1% SDS containing 0.1 mg/ml of denatured salmon testis DNA. Hybridization was carried out at 42 ℃ with a $^{32}$P-labeled cDNA probe. The cDNAs were labeled using a random primer labeling kit (Takara Shuzo Co., Japan) according to the manufacturer's instructions. After hybridization, filters were washed three times in 2 x SSC/0.1% SDS for 15 min at room temperature and then in 0.1 x SSC/ 0.1% SDS for 30 min at 60 ℃. For normalization of the brain RNA samples, mouse glyceroaldehyde-3-phosphate dehydrogenase (GAPDH; GenBank accession no. M32599) cDNA was used as a template for the probe. The Kodak X-ray films were exposed with an intensifying screen at -80 ℃.

### 10. Statistical analysis.

All data are presented as mean ± SD. Standard statistical tests were used to determine differences between groups using SigmaStat (Jandel Co, San Rafael, CA, USA). The Student's t-test was used in the case of continuous data (infarct size), and Mann-Whitney for non-continuous data (semi-quantitative scores). A level of $P < 0.05$ was considered statistically significant.

# Ⅲ. Results

## 1. Percentage area of infarct brain after ischemic area in normothermia and hypothermia.

The protective effects of preconditioning ischemia against ischemic injury; Nissl stainging was used to examine the survival of nerve cells. Normal neuronal cells showed round and pale stained nuclei in crecyl violet staining. The shrunken cells after ischemia were counted as dead cell (Figure 1A). As shown figure 1B, the infarct volume was significantly smaller in the hypothermia group than in the normorthermia group. The MRI data were corroborated the results seen on Nissl staining (Data not shown). Cerebral blood flow (CBF) and blood brain barrier (BBB) disruption were reduced in the hypothermia treated as measured by the amount of gadolinium contrast extravasation on T1W scans, was not seen at 2hrs, but was present by 24hrs (Data not shown).

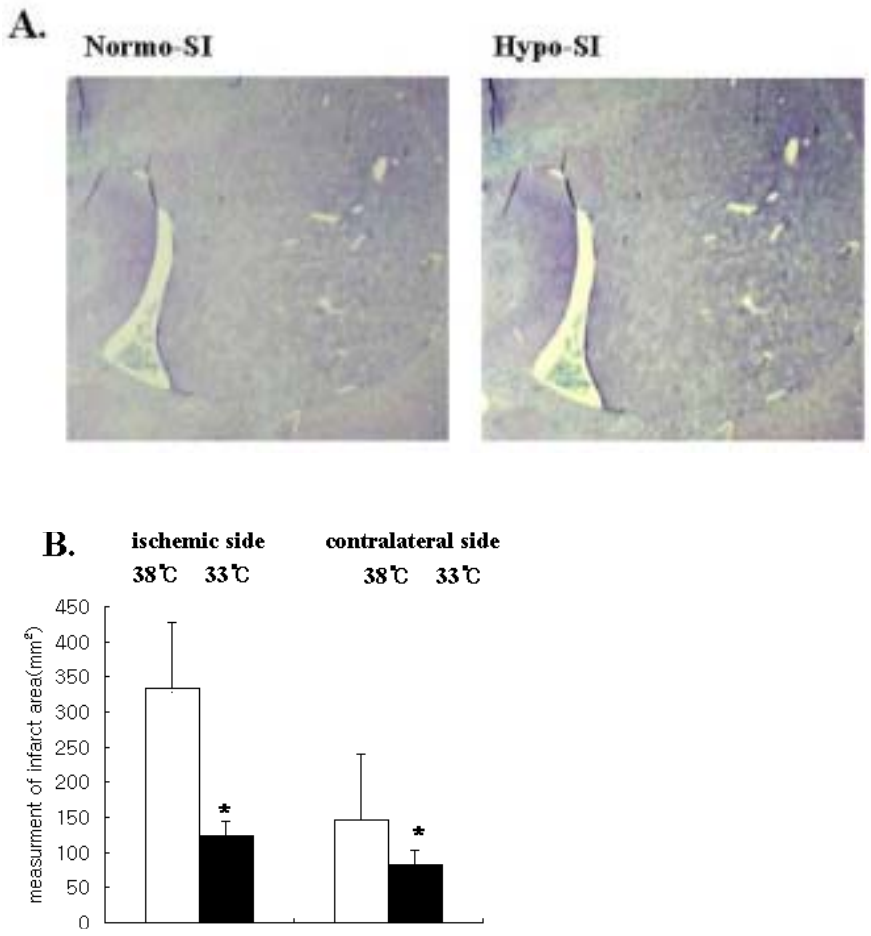



Figure 1. Measurement of infarct area for the hemisphere at 24 hrs postischemia in normothermic and mild hypothermia animals with 2hrs MCA occlusion. (A) Nissl staining of focal cerebral ischemia under conditions of mild hypothermia (coronal plane) and normothermia. Nissl stained tissue images show that the ischemic lesion is reduced in size at all time points compared to normothermia.(B) Graph representing infarct area observed by Optimas from hypothermia and normothermia. ($P<0.01$)

## 2. Hypothermia decrease MMP-2 expression in the postischemic brain.

In the ischemic injured brain, the levels of MMPs affected by the hypothermia were determined with Western blot with MMP-2 and MMP-9 antibodies. The expression of MMP-2 was reduced in the hypothermic cortex, striatum compared to the normothermic brain at 2 hrs, 6 hrs and 24 hrs after ischemic injury. In the penumbral area, the level of MMP-2 was decreased at 6 hrs after ischemic injury with hypothermia, but it was recovered after 22 hrs (Figure 2A and B).

To confirm the expression of MMP-2 in a cellular level, immunohistochemical staining was performed with tissues using MMP-2 antibody in the normothermic and hypothermic brains after ischemic injury. Neurons and astrocytes expressed MMP-2 protein in the normothermic and hypothermic brains (Figure 3A and B). The number of MMP-2 positive neurons was decreased in the hypothermic brain tissues after ischemic injury. However, the number of MMP-2 immuno-positive astrocytes was not changed in the hypothermic brains (Figure 3B).

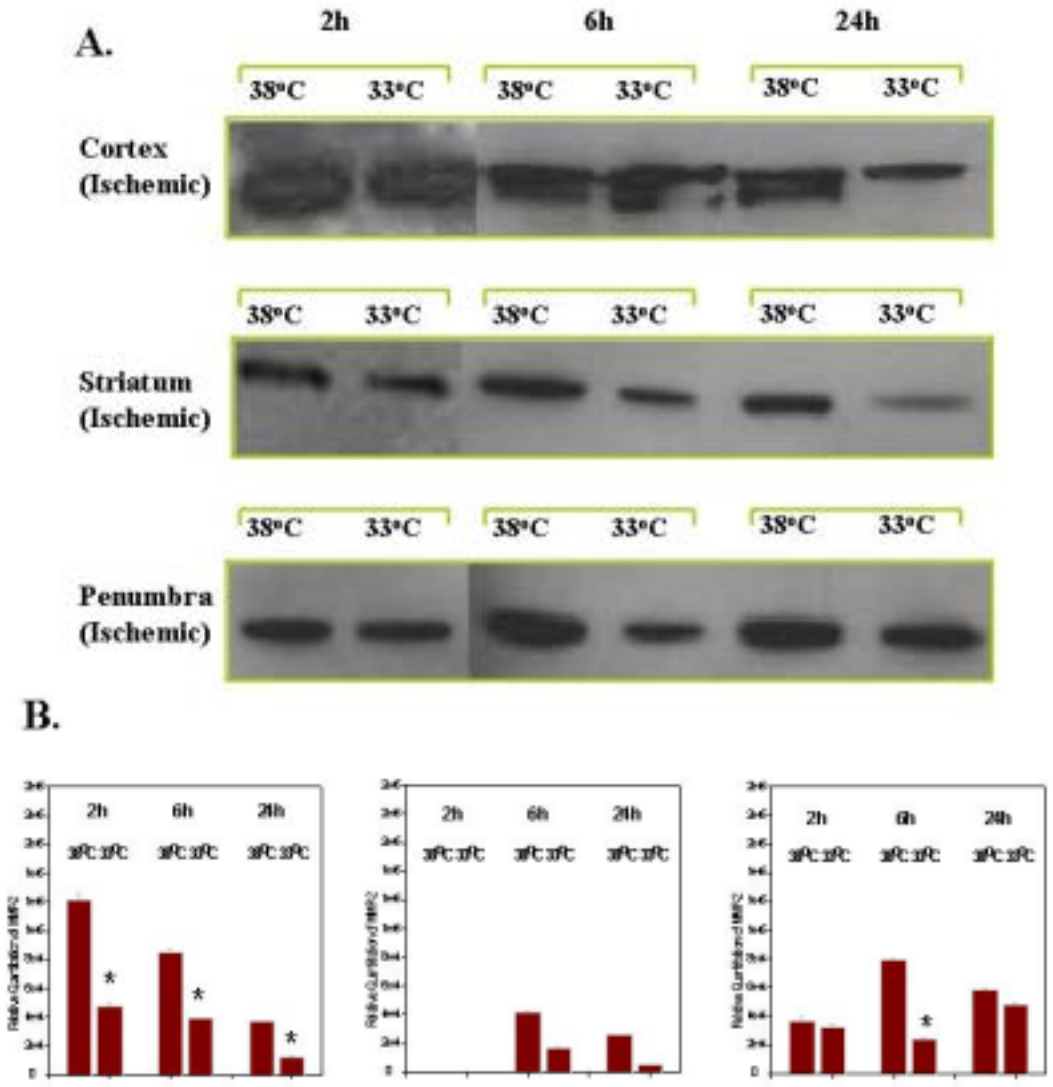



Figure 2. Expression of MMP-2 protein in the hypothermic and normothermic brain after ischemic injury (A) Expression of MMP-2 was reduced at 6 hrs in the hypothermia. (B) Representative western blotting analysis of MMP-2 extract from ischemic injured brain under hypothermia and normothermia. In the brain part – cerebral cortex, penumbra, and striatum, MMP-2 expression reduced under hypothermic condition ($P<0.05$).

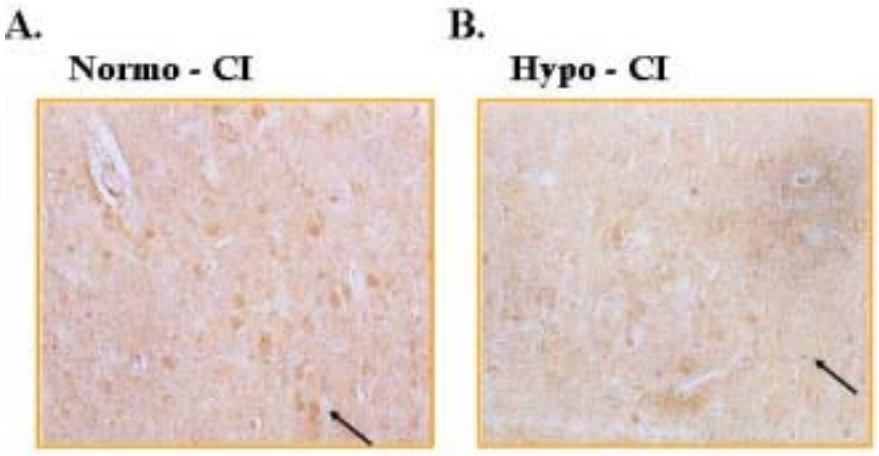



Figure 3. MMP-2 immunoreactive cells in the hypothermic and normothermic brain after 22 hrs ischemic injury. MMP-2 positive cells increase in the normothermia. Arrows show neurons cells immunostained with MMP-2 antibodies.

### 3. Regulation of MMP-9 expression by hypothermia.

Western blots of MMP-9 showed increases in the ischemic cortex compared to the contralateral side (Figure 4A). Among brains treated with mild hypothermia, MMP-9 was remarkably reduced in the ischemic cortex compared to normothermic brains at 6 hrs and 24 hrs (Figure 4B). In the immunostaining data, the number of MMP-9 positive cells, especially neurons were reduced in the hypothermic brain (Figure 5).

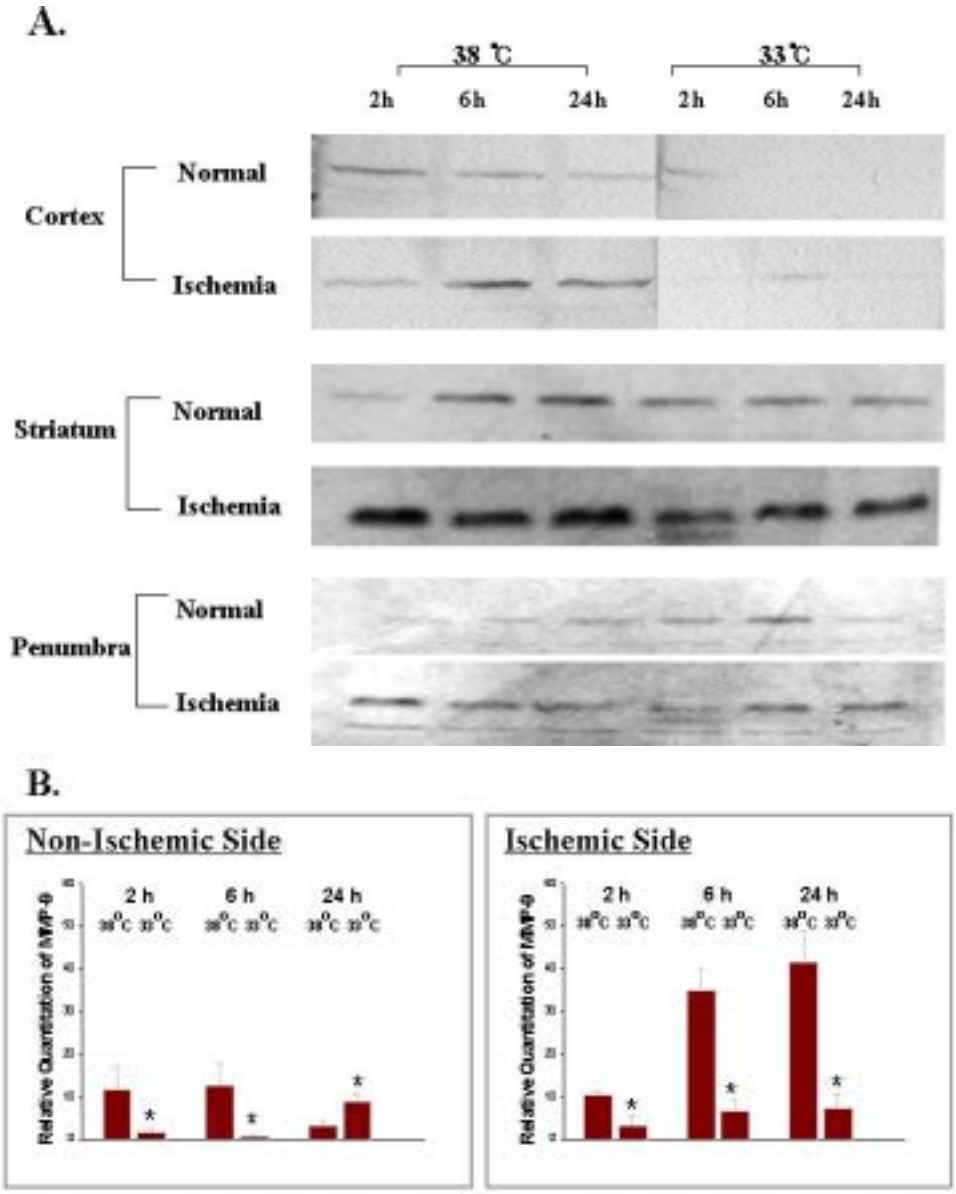

A.
38 ℃
33℃
2h
6h
24h
2h
6h
24h
Cortex
Normal
Ischemia
Striatum
Normal
Ischemia
Penumbra
Normal
Ischemia
B.
Non-Ischemic Side
Ischemic Side
2 h
6 h
24 h
Relative Quantitation of MMP-9

Figure 4. Expression of MMP-9 protein in nonischemic and ischemic side of brain under hypothermia and normothermia (A) MMP-9 expression showed increases in the ischemic cortex compared to the contralateral side. Among brains treated with mild hypothermia, the level of MMP-9 was markedly reduced in the ischemic cortex compared to normothermic brains at 6 hrs and 24 hrs. (B) Representative western blotting analysis of MMP-9. The level of MMP-9 was reduced at 6 hrs under hypothermic condition.
($P<0.05$)

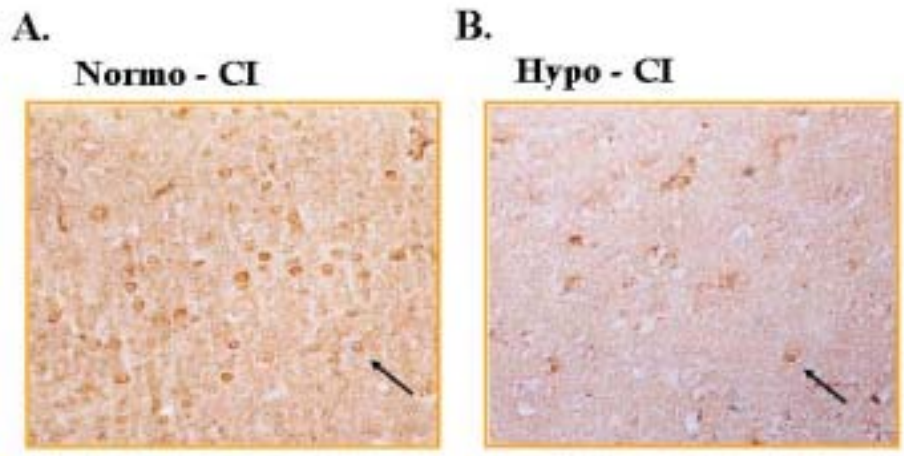



Figure 5. MMP-9 immunoreactive cells in the hypothermic and normothermic brain after 22 hrs ischemic injury. Reactive nerve cells (arrows) in the cerebral cortex are stained with MMP-9 antibody. In the normothermic cortex part, MMP-9 expression is markedly increased than in the hypothermia cortex part.

4. **Hypothermia suppresses MMP-2 and MMP-9 production in the postischemic brain.**

To determine whether hypothermic treatments affect the activity of MMPs, I performed zymography for MMP-2 and MMP-9. After ischemic injury, the activity of MMP-2 (72 kDa) and MMP-9 (92 kDa) was decreased at 6 hrs and 12 hrs in the hypothermic brains (Figure 6).

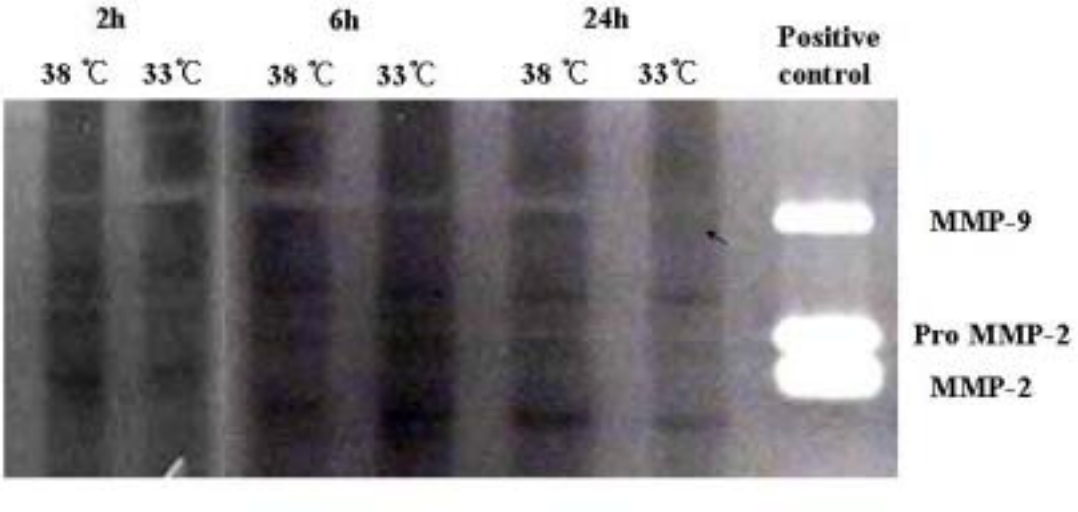



Figure 6. Zymographic analysis of MMP-2 and MMP-9 activities in the hypothermic and normothermic brain after ischemic injury. Tissue extracts were analyzed for stroke-induced MMP activities over a time course of 2 hrs, 6 hrs and 24 hrs with the use of SDS-PAGE zymograms containing gelatin. Active MMP-9 (molecular weight [MW]=87 kD) and MMP-2 (MW=67 kD) as well as the Latent "pro" forms (MW=92 kD and 72 kD, respectively) were detected in the gelatin-containing zymogram shown here. MMP-9 activity was decreased at 24 hrs in the hypothermia.

5. **Hypothermia increases TIMP-2 release from the postischemic brain.**

To investigate that tissue inhibitor matrix metalloproteinase protein-2 (TIMP-2) which regulates MMP-2 activity, the Western blot analysis for TIMP-2 was performed. The expression of TIMPs was lower in hypothermic brains than normothermic brains, however they were increased during reperfusion time in hypothermic brains (Figure 7).

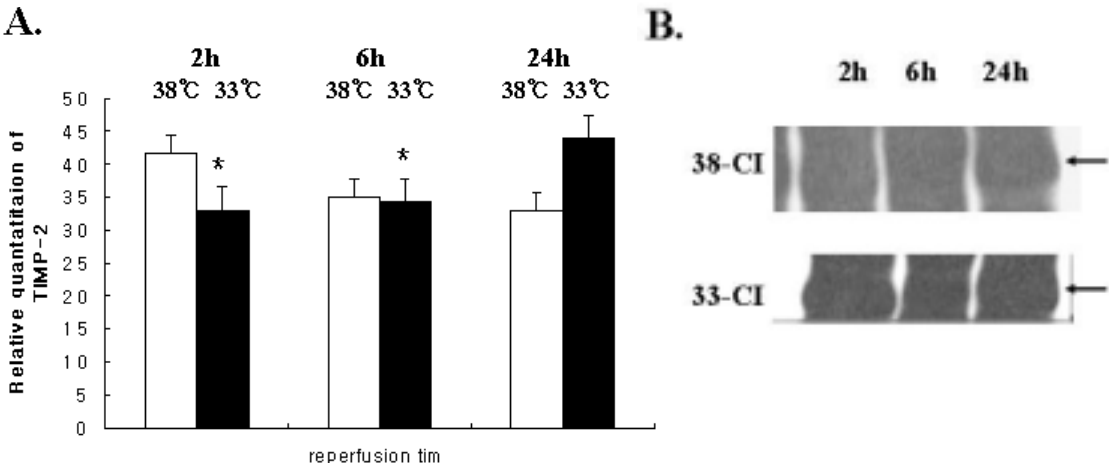


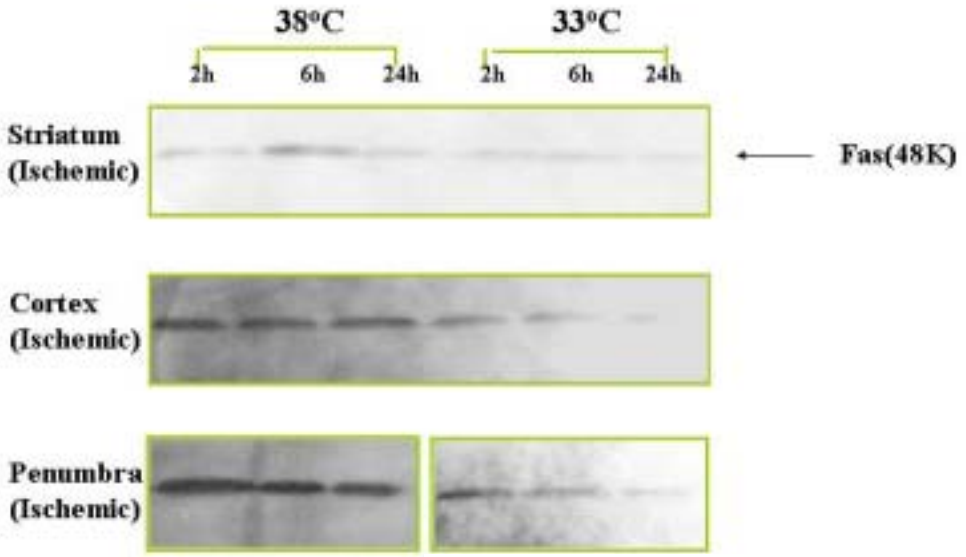

Figure 7. Western blot analysis of TIMP-2 protien in the hypothermic and normothermic brain after ischemic injury. We detected TIMP-2 in the injured cortex part. TIMP-2 was increased at 24 hrs in the hypothermia. TIMP-2 in the normorthermic brain was decreased at 6 hrs ($P<0.05$).

### 6. Expression and function of Fas and membrane bounded Fas-ligand in normothermic and hypothermic brain after ischemic injury.

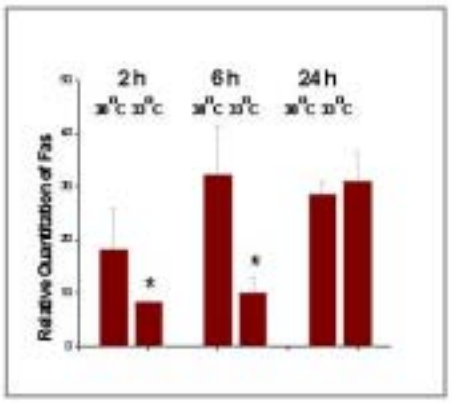
To define the protective mechanism of hypothermia, the suppression of MMPs, we investigated, may be involved in the signaling of apoptotic proteins, such as Fas or Fas-ligand protein. We detected the levels of apoptotic proteins, Fas and Fas-ligand, in the normothermic and hypothermic brains after ischemic injury from the Western blot. Our results also showed that the expression of Fas protein was reduced in the ischemic injured brain treated with hypothermia (Figure 8). Moreover, the inhibition of Fas and Fas-ligand interactions by hypothermia increased the levels of membrane-bounded Fas-ligand (Figure 9) and it may contribute to the neuroprotective effects of hypothermia for the ischemic tolerance.

It suggests that the suppression of MMPs by hypothermia was involved in the Fas and Fas-ligand interactions and thus inhibited procaspase-8 shifting to caspase 8. We showed that the levels of pro-caspase 8 in the hypothermic brains were higher than normothemic brains (Figure 10). And the expression of caspase 8 was reduced at 6hrs and 24hrs under hypothermia condition.

A.


38°C
33°C
2h
6h
24h
2h
6h
24h
Striatum
(Ischemic)
Fas(48K)
Cortex
(Ischemic)
Penumbra
(Ischemic)

B.


2h
6h
24h
Relative Quantization of Fas

C.

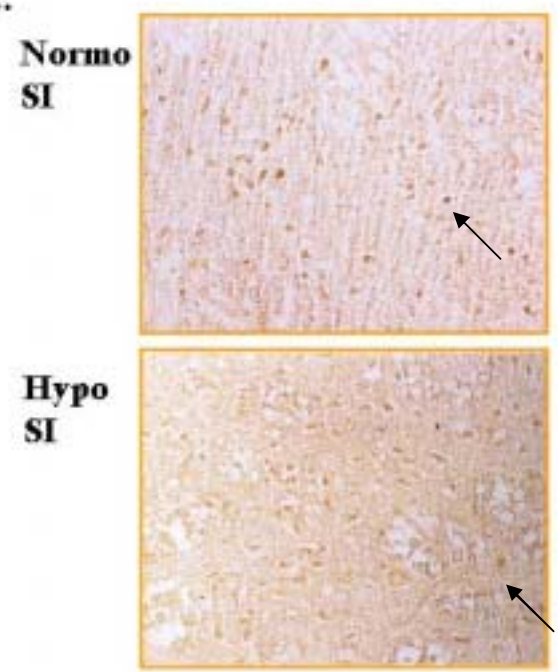

Normo
SI
Hypo
SI

Figure 8. Expression of Fas protein in the hypothermic and normothermic brain after ischemic injury Western blot using Fas antibody. (A) The expression of Fas was decreased cortex, penumbra and striatum at 2 hrs and 6 hrs but increased at 24 hrs in the hypothermia. (B) Representative western blotting of Fas. ($P<0.05$) (C) Immunoreactivitiy of Fas. Positive cells of Fas were decreased in the hypothermic striatum part.

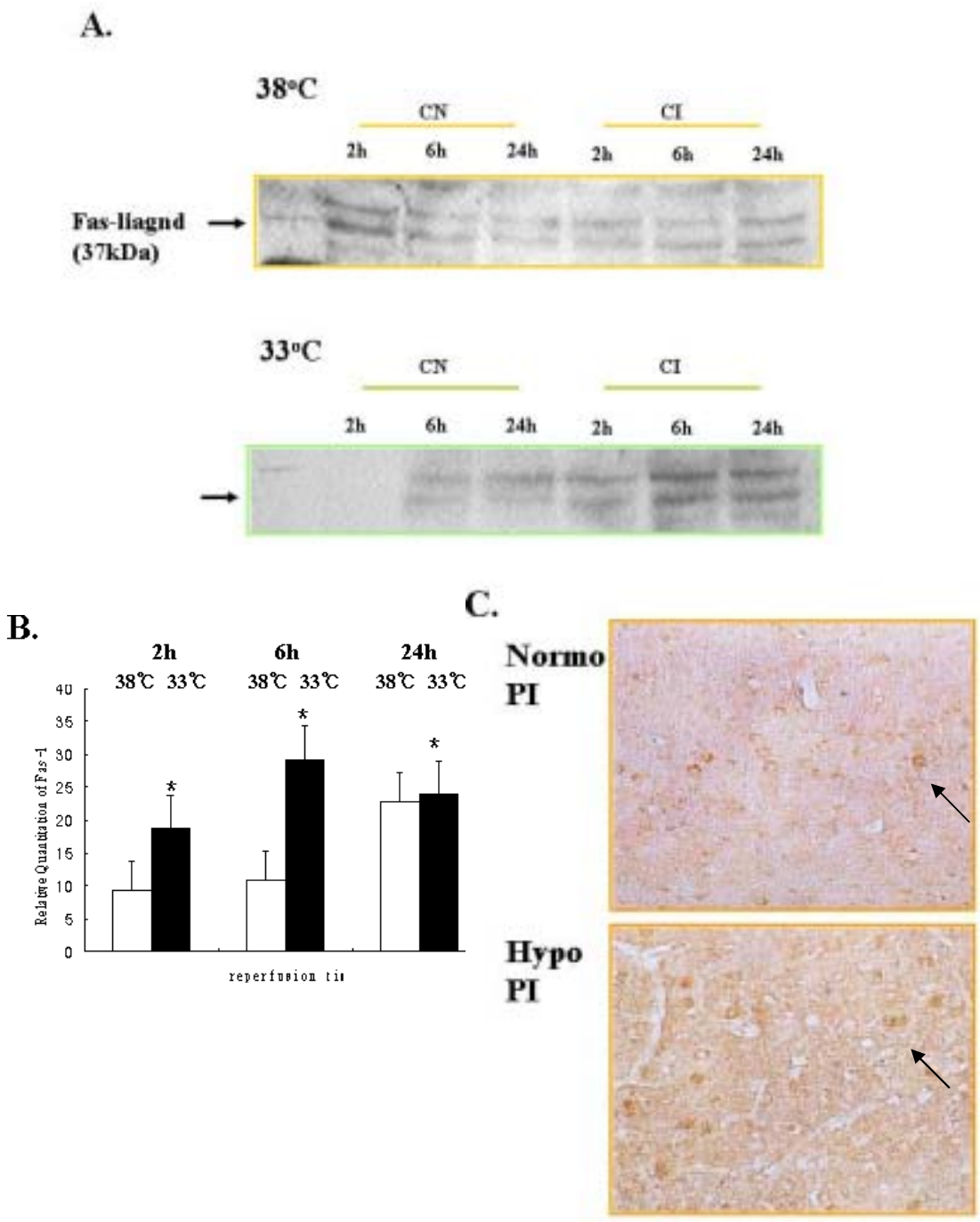

A.
38°C
CN
CI
2h
6h
24h
2h
6h
24h
Fas-liagnd
(37kDa)
33°C
CN
CI
2h
6h
24h
2h
6h
24h
B.
2h
6h
24h
38℃ 33℃
38℃ 33℃
38℃ 33℃
Relative Quantitation of Fas-l
40
35
30
25
20
15
10
5
0
*
*
*
reperfusion ti
C.
Normo
PI
Hypo
PI

Figure 9. Expression of membrane –bounded Fas-ligand in the hypothermic and normothermic brain after ischemic injury. (A) Western blot using Fas-ligand antibody. Fas-ligand expression increased at 6hrs and 24hrs in the cortex treated hypothermia but reduced at 6hrs in the normothermia. (B) Graph shows that quantative of western blotting of Fas-liagnd. (C) Immunostaining of Fas-ligand. Fas-ligand decreased in the normothermia.

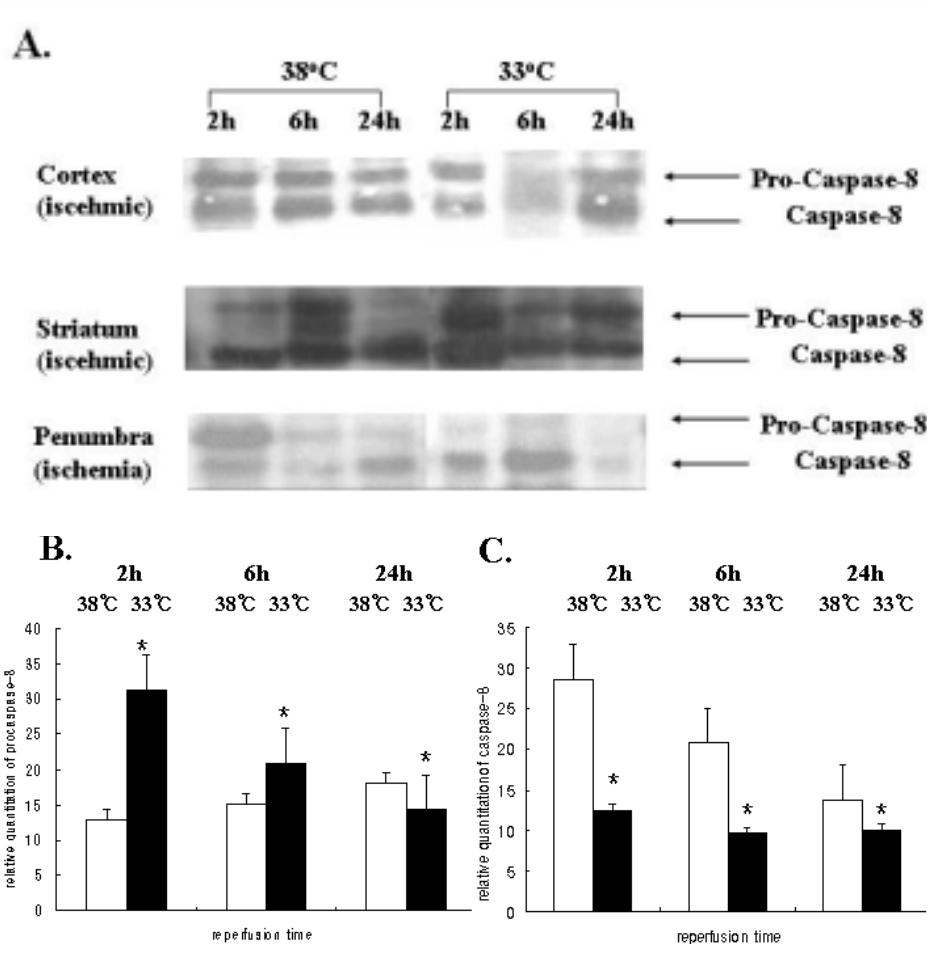



Figure 10. Expression of caspase-8 in the hypothermic and normothermic brain after ischemic injury. (A) Procaspase-8 was reduced at 2hrs in the normothermic striatum part. Caspase-8 has no change in the hypothermia, and markedly was decreased as reperfusion time in the nornothermia. (B) Representative of expression of procaspase-8. The amounts of procaspase-8 expressed highly in the hypothermia at 2 hrs. ($P<0.05$) (C) Representative of expression of active form of caspase-8.

### 7. mRNA expression of MMP-2, MMP-9 in normothermic and hypothermic brain after ischemia.

We showed that the mRNA expressions of MMP-2, MMP-9 were regulated by hypothermia in the quantitative RT-PCR and Northern blot data. The mRNA levels of MMP-2 and MMP-9 were decreased at 6 hrs and 24 hrs in the hypothermic brain (Figure 11). Therefore, Northern blot data verified that MMP-2 and MMP-9 mRNA were down-regulated by lowering of body temperature. In the hypothermic condition, the mRNA expression of MMP-2 and MMP-9 were reduced (Figure 12A and B).

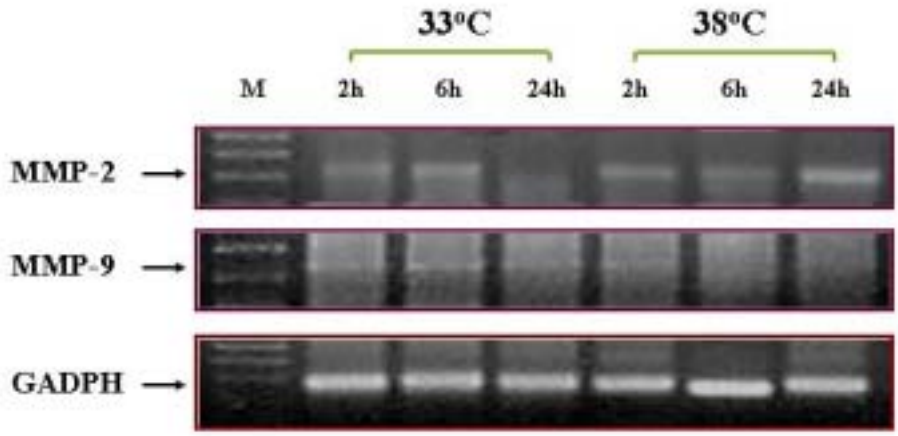



Figure 11. Expression of MMP-2 and MMP-9 mRNA in the hypothermic using Quantitative RT-PCR. MMP-2 mRNA were reduced at 24 hrs in the ischemic injured cortex. MMP-9 mRNA at 6 hrs and 24 hrs was reduced in the hypothermia.

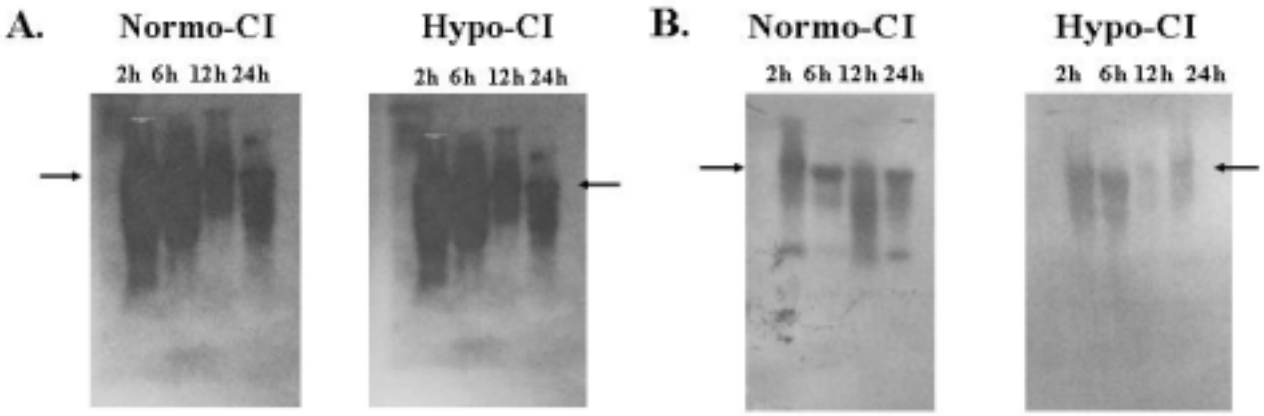



Figure 12. Northern analysis of MMP-2 and MMP-9 mRNA in the hypothermic and normothermic brain after ischemic injury. (A) MMP-2 was no change in the all cortex part (Arrows show the MMP-2 mRNA). (B) But MMP-9 reduced in the ischemic part and in the hypothermia part (Arrows show the MMP-9 mRNA).

# IV. DISCUSSION

Ischemic stroke caused by transient or permanent reduction of cerebral blood flow is a leading cause of death and disability in human. Although the solubilization of clots with tissue plasminogen activator or urokinase within a brief time span after stroke onset can be used for the treatment of victims, neuroprotection is another possible approach. However, clinical studies on the efficacy of candidate drugs for neuroprotection, such as glutamate receptor anatagonist, sodium channel blockers, calcium channel blockers, and free radical scavengers, have not been successful[31].

The more clinical relevant issue of hypothermia in a practical use depends on the effectiveness when it applied during the delayed intraischemic or postischemic period. Delayed intraischemic hypothermia is usually effective when it is initiated relatively soon after the onset of a focal ischemia[32].

Mild hypothermia has been shown to be an effective treatment against cerebral ischemic injury by numerous groups, but the mechanisms have not been elucidated well. Lowered body temperatures are thought to preserve metabolic stores and associated with decreased accumulation of excitatory amino acids, generally. In this study, we also showed that hypothermia significantly reduced infarct volume at 6 hrs after ischemic injury.

Another aspect of this protection has been associated with the prevention of blood brain barrier (BBB) disruption. Following ischemia, there is a loss of microvascular integrity manifested by major alterations in vascular permeability and the vascular basal lamina, a constituent of the extracellular matrix. Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes that

can degrade the extracellular matrix and are significantly increased after ischemia. Among other functions, MMPs cleave the receptor mediated, apoptotic protein Fas in NK cells.

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that have been associated with tumor cell invasion and metastasis due to their ability to hydrolyze a variety of extracellular matrix proteins. MMP 2 and MMP-9 among MMPs are thought to degrade a major structural protein in basement membrane called type Ⅳ collagen, and thus contributed to the disruption of blood brain barrier containing lamina and type Ⅳ collagen[33].

Reperfusion causes a loss of basal lamina macromolecules, BBB opening, and an increased risk of hemorrhage associated with delayed release of MMP-9. Rogenberg[34] showed the marked increase of MMP-2 and MMP-9 after both permanents and transient ischemia.

In animal studies, MMPs increase was significantly only after 12 hrs after ischemia, revealing a discrepancy between MMPs induction and the first signs of BBB alterations. In our study, the protein level of MMP-2 and MMP-9 increase after ischemic injury and reperfusion time. In addition to the rise in MMP-2 and MMP-9 reduced in the hypothermia condition, a second, more marked decrease occurred at 6 hrs and 24 hrs. The expressions of MMP-2 and MMP-9 in the hypothermia reduced more than in the normothermia. The protein level of MMPs is regulated by hypothermia. Yuiko[35] showed the enzyme activity of MMP-2 and MMP-9 was induced after cerebral ischemia. We may suggest that hypothermia can modulate MMP-2 and MMP-9 activities. This is also supported by my studies that detected MMP-2 and MMP-9

activities in ischemic injured brain. The induction of MMP-2 and MMP-9 after ischemic injury was significantly reduced in hypothermic brains after ischemic injury.

MMPs are expressed and activated during the process of disruption, and the activated MMPs vary depending on the ischemic injury and reperfusion time[36, 37]. In our study, MMP-2 and MMP-9 mRNA were expressed in the normothermia more than in the hypothermia and remarkably reduced at 24 hrs in the hypothermia.

Neurons and astrocytes expressed MMP-2 and MMP-9 proteins in the normothermic and hypothermic brains. The number of MMP-2 and MMP-9 positive neurons were decreased in the hypothermic brain tissues after ischemic injury. However, MMP-2 and MMP-9 immunoreactive astrocytes were not changed in the hypothermic brains. Astrocytes express the matrix metalloproteinase-2 (gelatinase A) and MMP-9 (gelatinase B). Interleukin-1 and lipopolysaccaride stimulate the production of MMP-2, MMP-9 and MMP-3 in astrocytes. Microglia constitutively express low levels of a 94 kDa gelatinase (gelatinase B) activity. However, the cellular source or the function of MMPs, or how their expression is regulated in brain is not known.

MMPs are also regulated by their inhibitors. Specific proteins known as the tissue inhibitors of Metalloproteinase (TIMPs) are the physiological regulators of MMPs. The TIMP family comprises 4 members (TIMP1-4) with high gene and protein homology[38]. TIMP-2 forms a trimolecular complex on the surface of the cell with MT1-MMP and proMMP-2, and regulates the formation and levels of concentration of mature MMP-2[39]. Our results show that TIMP-2 was regulated by body temperatures. The levels of TIMP-2 increased at 24 hrs in the hypothermic brain and decreased markedly at 6 hrs in the normorthermic brain. Hypothermia also

affected TIMP-2 expression associated with MMP-2 and formation of the complex of TIMP-2 and proMMP-2.

Because hypothermia related with synthesis of amino acid and enzyme activity, TIMP-2 increased the hypothermia treated brain.

The regulation of MMP-2 and MMP-9 by hypothermia plays an important role in the inhibition of apoptosis. Apoptosis is an important physiological cell death mechanism to eliminate damaged, whereas the regulated apoptosis has been implicated in many human diseases such as cancer, neurodegenerative diseases, and ischemic disease[40]. Recent evidence has shown that atherogenic factors such as oxidative stress, inflammatory cytokines, and hypoxia induced endothelial cell apoptosis. MMPs degrade ECM consistituents and other proteins Fas like membrane linked apoptotic proteins 21[41] after ischemic injury. Fas-ligand (Fas-L; also called CD95 ligand or APO-1 ligand)[42] is a cytokine that mediated apoptosis by binding to its receptor, Fas (also called CD95 or APO-1). Fas-ligand is mainly expressed on NK cells, but recently expressed on neuronal cells. Fas-L expresses constitutively endothelial cell (EC). The soluble Fas-ligand is released from EC[43]. Fas-L, a member of the TNF family, is rapidly cleaved off from the membrane by metalloproteinase to become a soluble form (sFas-L) and soluble Fas-L acts as Fas death signal[44].

The protein level of Fas was increased in normothermic brains, but reduced in hypothermic brains. Thus, soluble Fas-L as acts death signal associated with Fas death pathway, is regulated by MMPs activity that is down-regulated by hypothermia. Therefore, to confirm that MMPs regulated by hypothermia may down-regulate caspase-8 associated with Fas death signal pathway, the level of caspas-8 was detected by Western blot from normothermic and hypothermic brains. The levels of caspase-8 were increased at 24

hrs in the normorthermic striatum, and the levels of procaspase-8 were decreased in the penumbra part of normorthrrmic brains at 6 hrs and 24 hrs. The result shows that hypothermia inhibits procaspase-8 shifting to caspase-8.

There are a lot of evidences showing that mild hypothermia protects animals in laboratory their brains from the ischemic injury. We can suggest the one of neuroprotective mechanisms of hypothermia in this paper. This study indicates that hypothermia exerts a neuroprotective effect by reducing MMP-2 and MMP-9 and the regulation of MMPs affect many down streams of cell signaling. The hypothermia can regulate TIMP-2 associated with MMPs and the amounts of soluble Fas-L cleaved by MMPs.

# Ⅳ. CONCLUSION

In this study, We defined what relationship is there between hypothermia and MMPs expression in order to understand the protective mechanism of hypothermia in ischemic injury. We also exa mined whether BBB preservation from mild hypothermia is due to al terations in MMPs expression and how MMPs can regulate apoptosis proteins in the hypothermic condition.

We are achieved the results as follows;

1. The expression of MMP-2 and MMP-9 protein levels was decreased in the hypothermic brains after ischemic injury.
2. The activity of MMP-2 and MMP-9 were suppressed by hypothermia.
3. The levels of TIMP-2 were increased in the hypothermic brains and decreased in the normothermic brain.
4. The mRNA level of MMP-2 and MMP-9 were decreased in the hypothermic brains.
5. Under the hypothermic condition, MMPs may regulate the receptor mediated apoptotic protein, such as Fas. The expression of Fas proteins were decreased, however membrane-bounded Fas-ligands were increased in the hypothermic brain.
6. The levels of pro-caspase 8 assocciated with Fas-death pathway in the hypothermic brains are higher than in the normothermic brain.

Therefore, neuroprotective effect of hypothermia might be associated with regulation of MMPs and Fas signaling. We suggest that the suppression of MMPs by hypothermia was involved in the inactivation of Fas and Fas-ligand death signaling, and inhibition procaspase-8 shifting to caspase 8.

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국문요약

## 뇌 허혈 손상시 신경계의 보호작용과 관계된 저체온법과 MMPs의 효과.

저체온법은 뇌허혈동안 손상을 줄이는데 매우 효과적으로 외과 수술시 유용하게 쓰이고 있다. 국부 뇌허혈에서 경한 저체온법은 괴사가 일어나지 않은 부위에서의 아폽토시스 유도와 괴사가 일어나는 부위를 감소시킬 수 있다. 그리고 저체온법을 뇌허혈 손상 후나 뇌허혈이 일어나는 동안 초기에 시행한다면 재관류로 인해 일어날 수 있는 손상으로부터 신경세포를 보호할 수 있다. 이러한 저체온법의 뇌보호기작에 대한 연구가 많이 진행되고 있지만 아직 뚜렷한 기작에 대해서는 밝혀진 바가 없다. 지금까지 알려져 있는 저체온법에 의한 뇌보호 기작으로 글루코오스와 산소의 소비비율이 낮추어지므로 cerebral metabolic rate가 감소되어 뇌허혈손상과 관계되어 발현되는 유전자를 조절할 수 있다고 생각되어진다.

뇌허혈손상시 뇌혈관 장벽을 파괴함으로 신경세포사멸을 유발하는 물질중에서 matrix metalloproteinase(MMPs)가 있다. 이 MMPs중 Gelatinase A와 B 로 알려진 MMP-2 와 MMP-9은 type Ⅳ collagenease로 혈관의 기저판을 파괴함으로써 뇌혈관 장벽을 열어주어 손상을 증폭시키는 것으로 알려져 있다. 따라서 뇌허혈손상시 뇌혈관 장벽의 파괴를 이끄는 MMPs를 억제함으로써 손상의 정도를 줄일 수 있을 것으로 생각된다. 그러므로 본 연구에서는 경한 저체온법에 의한 MMPs 발현 및 활성 조절 기전을 규명하고자 하였다. 뇌의 중간대뇌동맥을 막음으로써 국부적인 뇌허혈손상 모델을 만들었다. 동물의 체온은 중간대뇌동맥을 막은 후 38℃와 33℃로 각각 유지하고 뇌허혈 손상이후 재관류 0, 4, 22시간에 따라 뇌를 적출하였다. 저체온을 처리한 경우 뇌허혈손상부위가 재관류 22시간에 줄어든 것을 확인 할 수 있었으며 저체온을 유지한 경우가 정상체온보다 MMP-9의 발현정도가 줄어든 것을 확인 할 수 있었다. 저체온을 유지한 뇌의 경우 대뇌피질부

위에서 재관류 4시간과 22시간에서 MMP의 양이 줄어들었다. 그리고 이들의 효소 활성 정도도 저체온에 의해 영향을 받는 것을 확인하였다. 뇌허혈 손상에서 MMPs의 자신의 역할뿐만 아니라 아폽토시스와 관련되어 막에 존재하는 단백질인 Fas와 Fas-ligand의 상호작용에도 영향준다. MMPs의 발현을 억제함으로 MMPs와 관련된 아폽토시스 단백질인 Fas 와 Fas-ligand의 상호작용을 억제하여 신경보호작용을 한다고 생각되어진다. 뇌허혈 손상시 Fas의 단백질 발현정도는 저체온시 증가하였지만 Fas-ligand의 mRAN 발현은 저체온이 정상체온에 비해 줄어들었다. 그러므로 저체온을 처리한 경우 뇌허혈 손상을 받은 부위 중 괴사가 일어나지 않는 부위에서 Fas와 Fas-ligand 상호작용을 억제함으로 아폽토시스를 감소시킬 수 있다고 생각되어진다.

그러므로 뇌허혈 손상시 저체온을 유지하면 뇌혈관 장벽의 파괴를 일으키는 MMPs의 단백질 발현이나 효소활성정도가 억제되게 된다. 뇌허혈 손상시 MMPs에 의해 활성화 되어 아폽토시스를 일으키는 Fas와 Fas-ligand의 상호작용도 저체온에 의해 억제되어 신경보호효과를 얻을 수 있다.

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핵심되는말: 뇌 허혈손상, 저체온법, 신경보호작용, 아폽토시스, Matrix metalloproteinase,