LABORATORY INVESTIGATION

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Hypothermia Effect on Apoptotic Neuronal Death in Traumatic Brain Injury Model

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Objective : Many researchers believe that the hypothermia shows neuro-protective effect on brain injury. To understand the molecular mechanism of the hypothermic treatment, this study investigated its effects on the expression of cell death or survival related proteins such as p53, Bcl-2 and Bax in the rat traumatic brain injury(TBI) model.
Methods : Twenty rats (Spraque Dawley, 200~250g) were subjected to the brain injury of moderate severity (2.4~2.6atm) using the fluid percussion injury device and five rats were received only same surgery as controls. During 30minutes after the brain injury, the hypothermia group was maintained the body temperature around 34°C while the control group were maintained that of 36°C. Five rats in each group were sacrificed 12h or 24h after brain injury and their brain sections was analyzed for physical damages by H-E stains and the extent of apoptosis by TUNEL assay and immunohistochemical stains. The tissue damage after TBI was mainly observed in the ipsilateral cortex and partly in the hippocampus.
Results : Apoptosis was observed by TUNEL assay and the Bax protein was detected in both sample which harvested 12h and 24h after TBI. In the hypothermia treatment group, tissue damage and apoptosis were reduced in HE stains and TUNEL assay. In hypothermia treatment group rat shows more expression of the Bcl-2 protein and shows less expression of the Bax protein, at both 12h and 24h after TBI.
Conclusion : These results show that the hypothermia treatment is an effective treatment after TBI, by reducing the apoptotic process. Therefore, it could be suggested that hypothermia has a high therapeutic value for treating tissue damages after TBI.

KEY WORDS : Traumatic brain injury · Apoptosis · TUNEL assay · Bax · Bcl-2.

Introduction

The mechanisms of neuronal cell death after traumatic brain injury(TBI) result from the apoptosis and necrosis[4,6]. Apoptosis occurs when cytochrome c activates caspase-3. Both of the cytochrome c and the activity of caspase-3 are increased at 6, 12, and 24hours after the brain injury[20]. The expression of bcl-2, an apoptosis-inhibiting gene, was increased in cerebral cortex, hippocampus, and dentate gyrus at 6 and 24hours, and was even increased in cortex at 72hour after the brain injury[4-6]. The cytochrome c secretion from mitochondria may be dependent on free radical in necrosis rather than apoptosis[11,15]. The changes in the expression of Bax and bcl-2 are considered to be major factors related to neuronal cell death in TBI. The increase in the expression of p53, reported as a tumor suppressor gene, is found in the injured area in TBI, indicating the role of p53 in the apoptosis in TBI[4,5,17,21]. The increase of Bax, apoptosis-associated protein, in TBI has already been reported[9]. Such increase in Bax was inhibited by magnesium ion while was sharply increased by bcl-2 as cell protection activity. In the other words, magnesium ion might affect on apoptosis-associated proteins to inhibit apoptosis[14,18,21]. Hypothermia was proved to have the neuro-protective effect by inhibiting the inflammatory response in trauma and stroke in animal model[8]. In this study, the authors tried to observe whether apoptosis was associated with cell death after TBI and to identify the mechanism of cell protection by hypothermia in traumatic brain injury model.

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Materials and Methods

Animal model

In this study, 30 Sprague-Dawley white rats with 200~250g in weight were selected to be anesthetized by ketamin/xylazine (70/6mg/kg, im, respectively). We made a 3mm diameter of burr hole on the right parietal region (the middle of bregma and lambda, 4mm from lateral position), and fixed female Luer-Lock, then close the wound. After 24hours, we induce moderate brain injury (2.4~2.6 atmospheric pressure, atm) by the fluid percussion injury device (HPD-1700, Dragonfly Research & Development, Silver Spring, MD, USA). The experimental groups were classified into the normothermia groups (36~37°C) and the hypothermia groups (33~34°C). The rats in the control group were maintained at 36~37°C in rectal temperature (thermometer : BAT-12, Harvard Bioscience Inc, MA, USA) for 30minutes. The hypothermia group, the temperature was maintained at 33~34°C for 30minutes by putting ice on the side and using fans.

Histomorphological examination

The brain tissues were fixed by 10% of neutral formalin, embedded by paraffin to be made as slices with 3μm in thickness. The slices were stained by common hematoxylin-eosin to be compared of the morphologic changes of the tissues.

TUNEL stain

Apoptosis was confirmed by *in situ* terminal transferase d-UTP nick-end labeling (TUNEL) by using the DNA Fragmentation Detection Kit (QIA33, Oncogen, Intergen, NY, USA). The nonspecific response in paraffin embedded tissue slices was prevented by using the proteinase K (20μg/ml, Sigma Chemical, St. Louis, MO, USA). To remove the endogenous peroxidase, we apply the 3% of hydrogen peroxide solution for 5minutes, and treated with 1X TdT equilibration buffer for 30minutes, then using the TdT labeling reaction mixture including TdT enzyme (terminal deoxynucleotidyl transferase), biotin-dNTP and unlabeled dNTP at 37°C, lavel the apoptosised cells. Then, to stop the reactions, we apply the stop buffer, and react with the avidin-biotin complex. And then we observed with optical microscope after colored by NovaRED (Vector Laboratories, CA, USA).

Western blot assay

The brain tissues in TBI were separated into cerebral cortex and hippocampus and then quenched by liquid nitrogen. After lysis buffer including protease inhibitors (1mM DTT, 10μg leupeptin, 10μg trypsin inhibitor, 2μg aprotonin, 1mM PMSF, 1μg pepstatin) was poured, the tissues were smashed of cell membranes at 4°C by ultrasound system. And centrifuged at 4°C, 12,000g for 30minutes to isolate the supernatant. The isolated protein samples were quantified by Bradford assay. The 50μg of sample protein obtained from the above-mentioned process was heated at 100°C for 10minutes, and was isolated of the protein by electrophoresis at 10% SDS-PAGE. The protein-shifted membranes were processed by blocking buffer (5% skim milk in TTBS-T buffer) for an hour to inhibit the nonspecific bond with antibodies. Then, the membranes were responded with anti-Bax and anti-bcl-2 (Santa cruz, CA, USA; 1 : 2000), polyclonal antibodies, at room temperature for two hours. we wash with TTBS-T, and apply the secondary antibody (anti-goat IgG-HRP, Santa cruz; 1 : 5000) at room temperature for two hours. Finally, we treat the membranes with ECL (Amersham, Aylesbury, U.K.), and analyze of the degree of expression through the X-ray film (Kodak, Rochester, NY, USA).

Immunohistochemical evaluation

After PBS was perfused through heat in order to remove the whole blood in brain, the brain tissues were fixed by the perfusion of 4% paraformaldehyde. The tissues were soaked sufficiently by 30% sucrose, and embedded by paraffin. Then the tissue were made to be slices with 3μm in thickness. The slices were responded with 3% hydrogen peroxide solution for five minutes to remove endogenous peroxidase, and then were responded with serum-free protein to be inhibited from nonspecific antigen-antibody reaction. After then, we apply the primary antibodies (Bax and bcl-2, Santa cruz, CA, USA; 1 : 100), and the secondary antibody and avidinbiotin complex (Vector Laboratories, Burlingame, CA, USA). After colored by NovaRED kit (Vector Laboratories Burlingame, CA USA), observed by microsc-

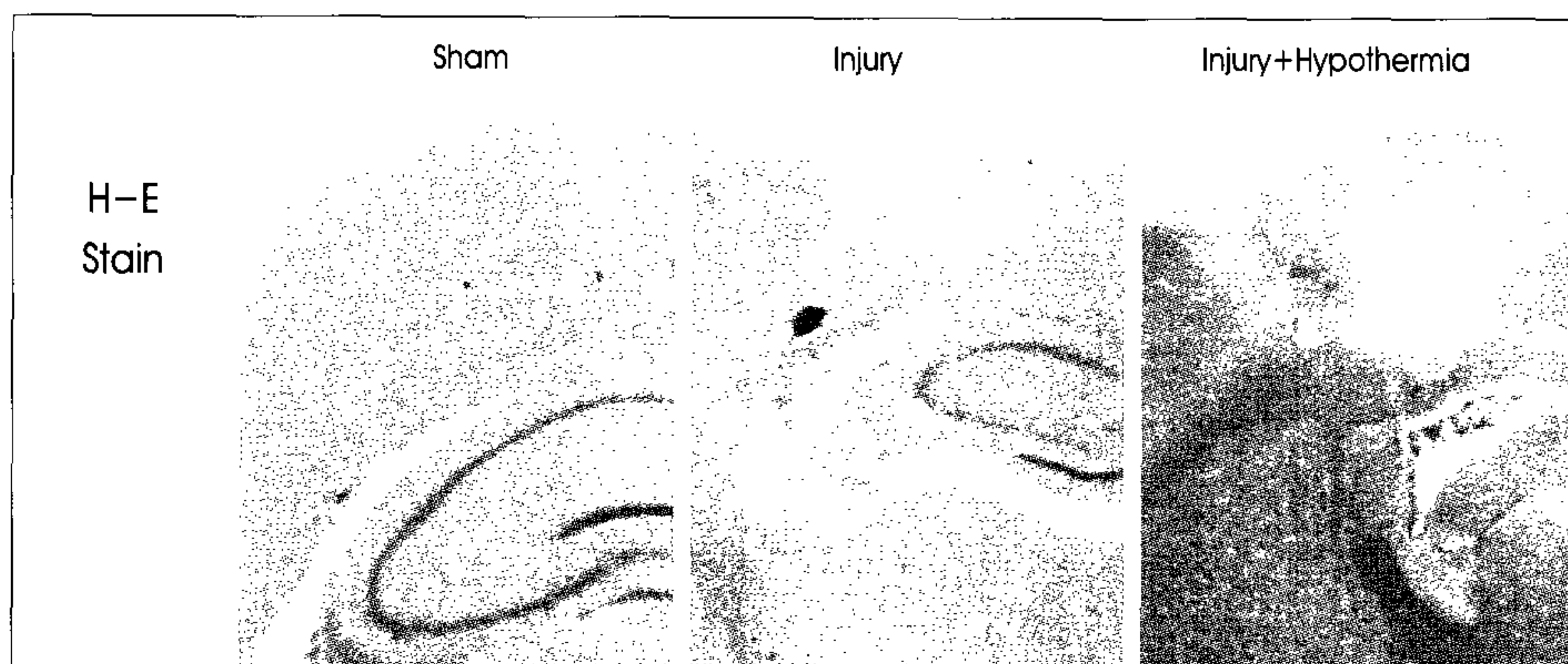

Fig. 1. Effect of hypothermia on the apoptosis after traumatic brain injury. The ipsilateral cortex of the rat brain was stained for morphological study 24h after-injury (X10).

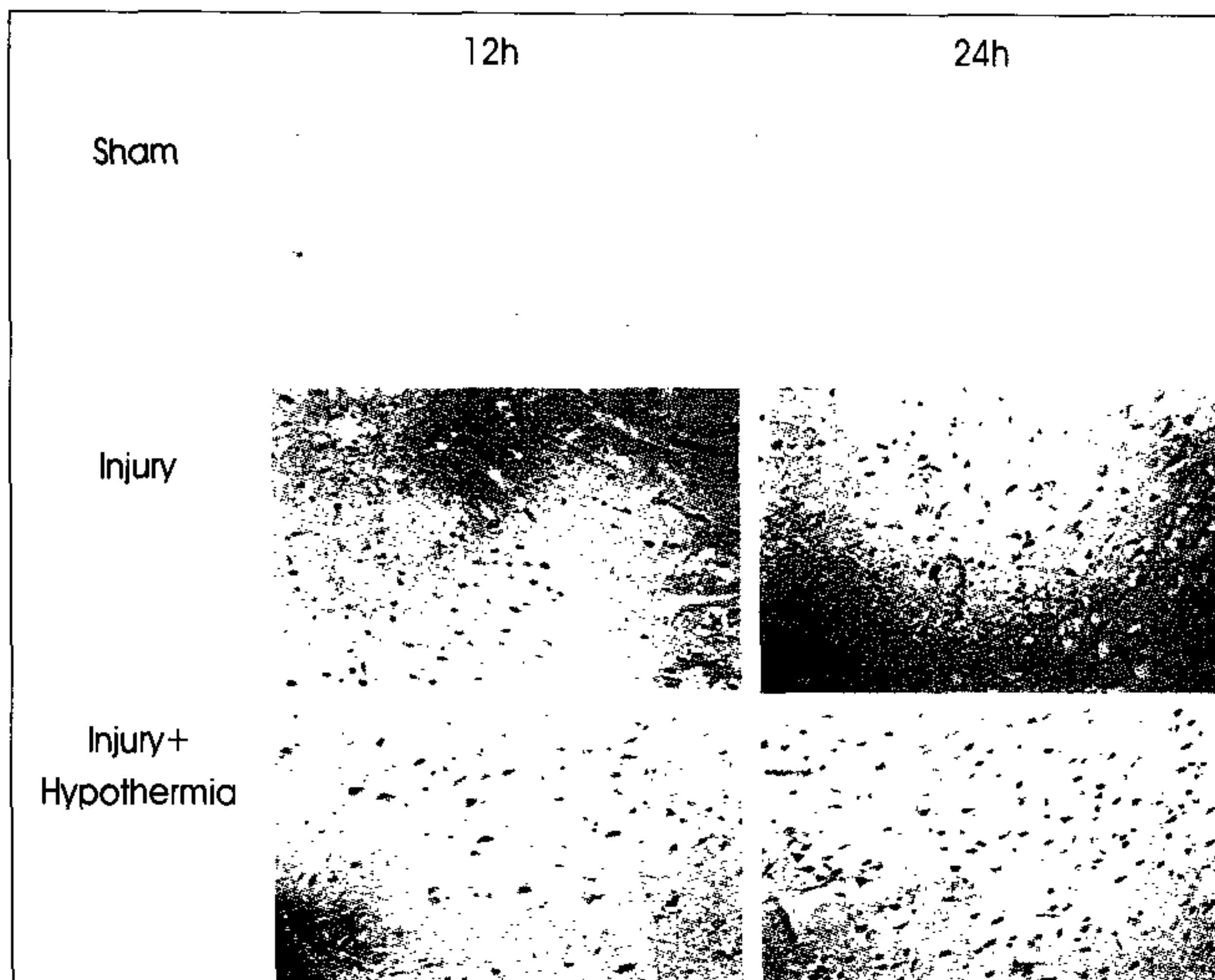

Fig. 2. Effect of hypothermia on apoptosis after traumatic brain injury. The ipsilateral cortices from the rats of the injury, the hypothermia after injury and the sham control were TUNEL stained after 12h and 24h after-injury (X200).

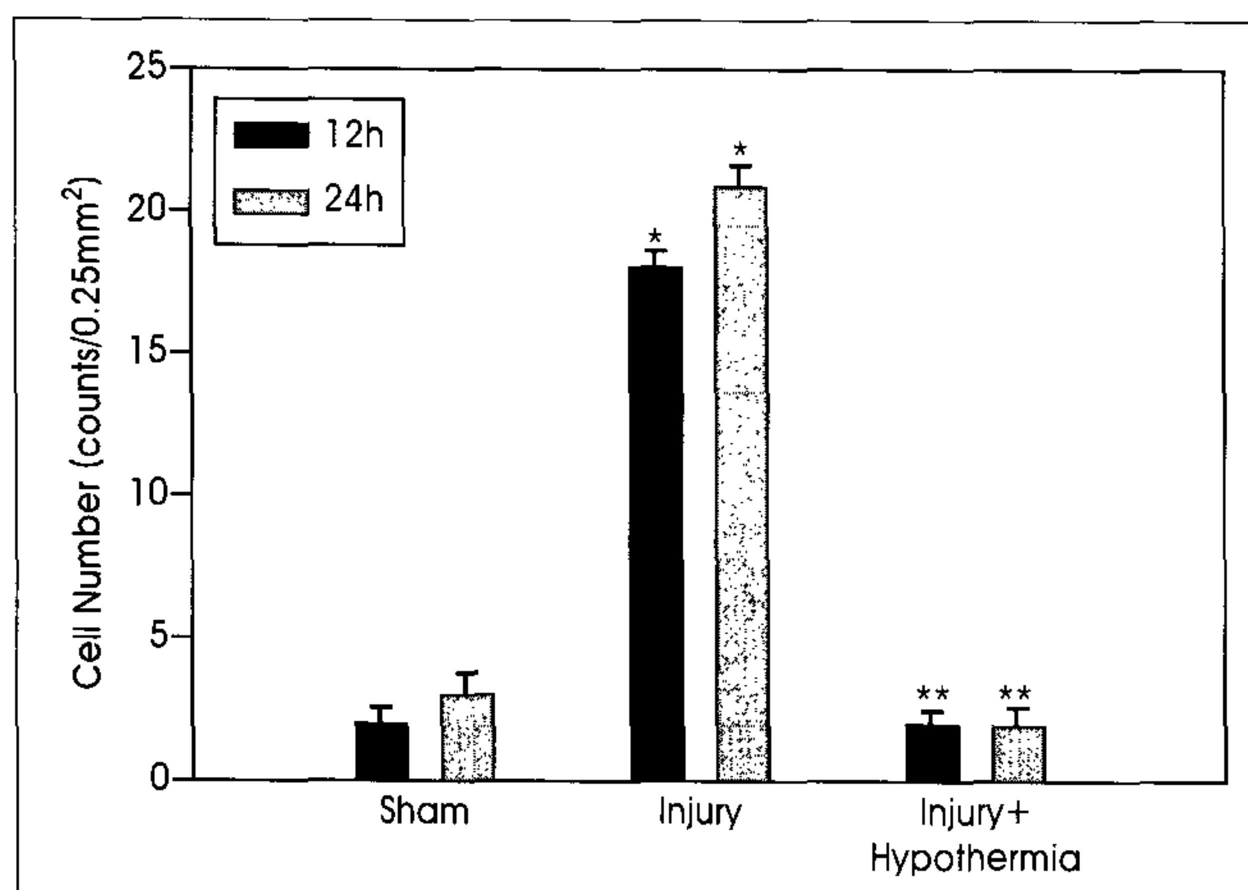

Fig. 3. Effect of hypothermia on the apoptotic cell number 12hr or 24hr after traumatic brain injury. (*$p<0.05$ vs. sham, **$p<0.05$ vs. injury). Data represent means ± S.D. (X400).

ope. Stained by hematoxylin, and dehydrate the specimens. Count the number of the cells showing immune reaction to apoptosis-positive cells, Bax, and bcl-2 was measured. The numbers of the cells in five selected areas ($0.25mm^2$) at a regular distance in each slice were measured.

Statistical analysis

The control and the normothermia group, and the nomothermia and the hypothermia group were compared by using of Kruskal-wallis test. Statistical significance was defined as the p value less than 0.05.

Results

The effect of hypothermia on apoptosis

The damaged brain tissue after the TBI in the hypothermic

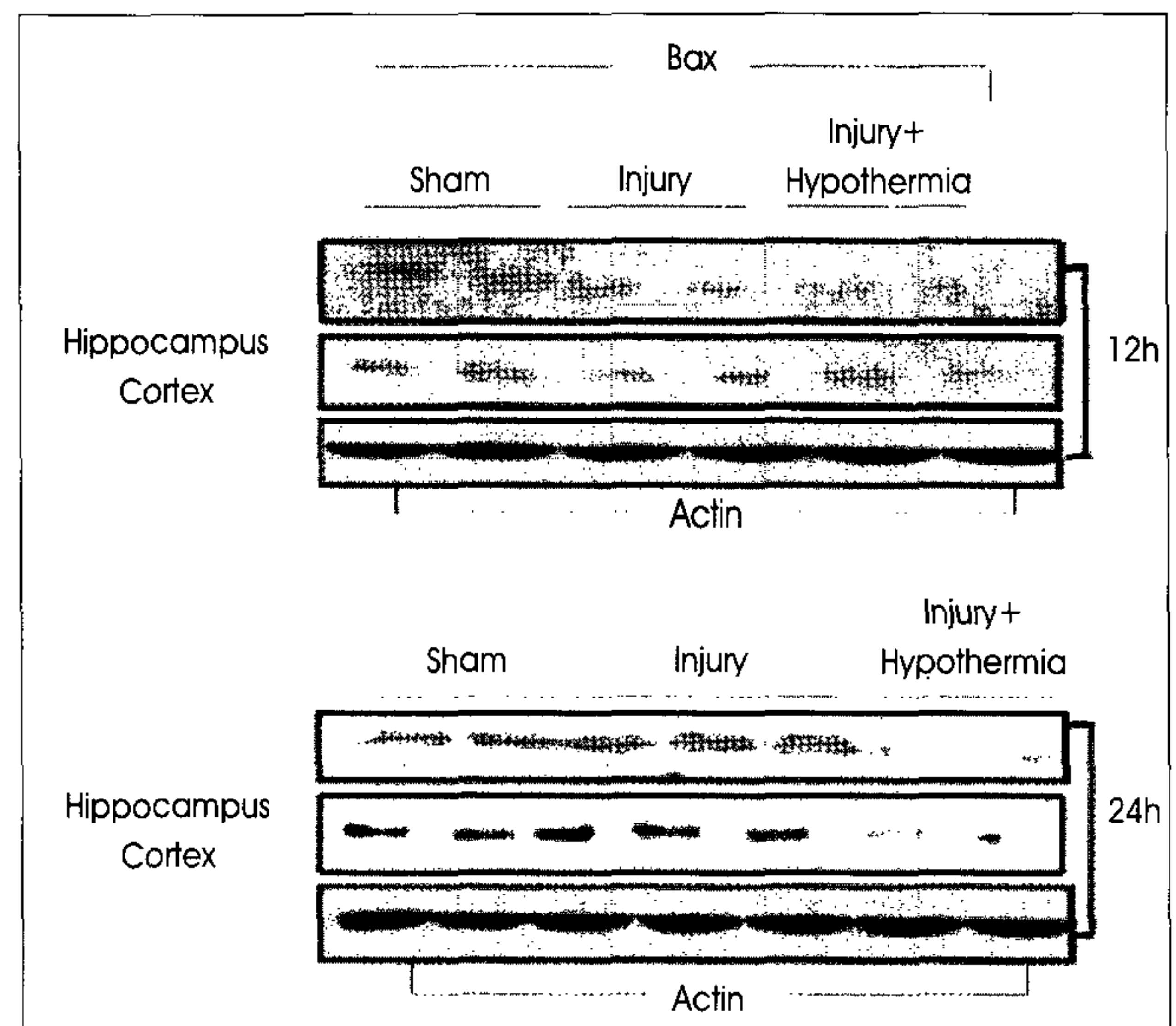

Fig. 4. Western blot analysis of Bax in the ipsilateral cortex and hippocampus of rat brain 12h and 24h after-injury. Expression of actin served as an internal standard.

treatment group was less damaged compared with non-treated one (Fig. 1). Also, in the cell death evaluation through the TUNEL stain shows the cell death was considerably reduced more in the hypothermia group than in the normal temperature group on the 12 and the 24hours after the TBI (Fig. 2). In particular, the number of dead cells on the 12th hour of TBI in the hypothrmic temperature group was less than that of the normothermia group (Fig. 3).

The effect of hypothermia on the expressions of protein associated with cell death and survival (Bax and bcl-2) in TBI model

The quantitative changes in Bax and bcl-2

The expressions of Bax and bcl-2, were observed in hippocampus and cerebral cortex by western blot assay. The Bax in the hippocampus on the 12hour after TBI in the normothermia group was not more significantly increased when compared to the control group, while the protein was significantly reduced in the hypothermia group compared to the control group. However, after 24hour of the injury, Bax expression was more remarkably increased in the hippocampus in the normothrmia group when compared to the control group, while its expression was considerably reduced in the hypothermia group (Fig. 4). No apparent changes in Bax in the cerebral cortex on the 12hour of TBI in the three groups, while on the 24hour, its expression was more slightly increased in the normothermia group than in the control group but was reduced in the hypothermia group (Fig. 4). When the expression of bcl-2 protein on the 12 and 24hours after TBI was observed in hippocampus, the control group and the normothermia group showed no changes while the hypothermia group showed the increase in its

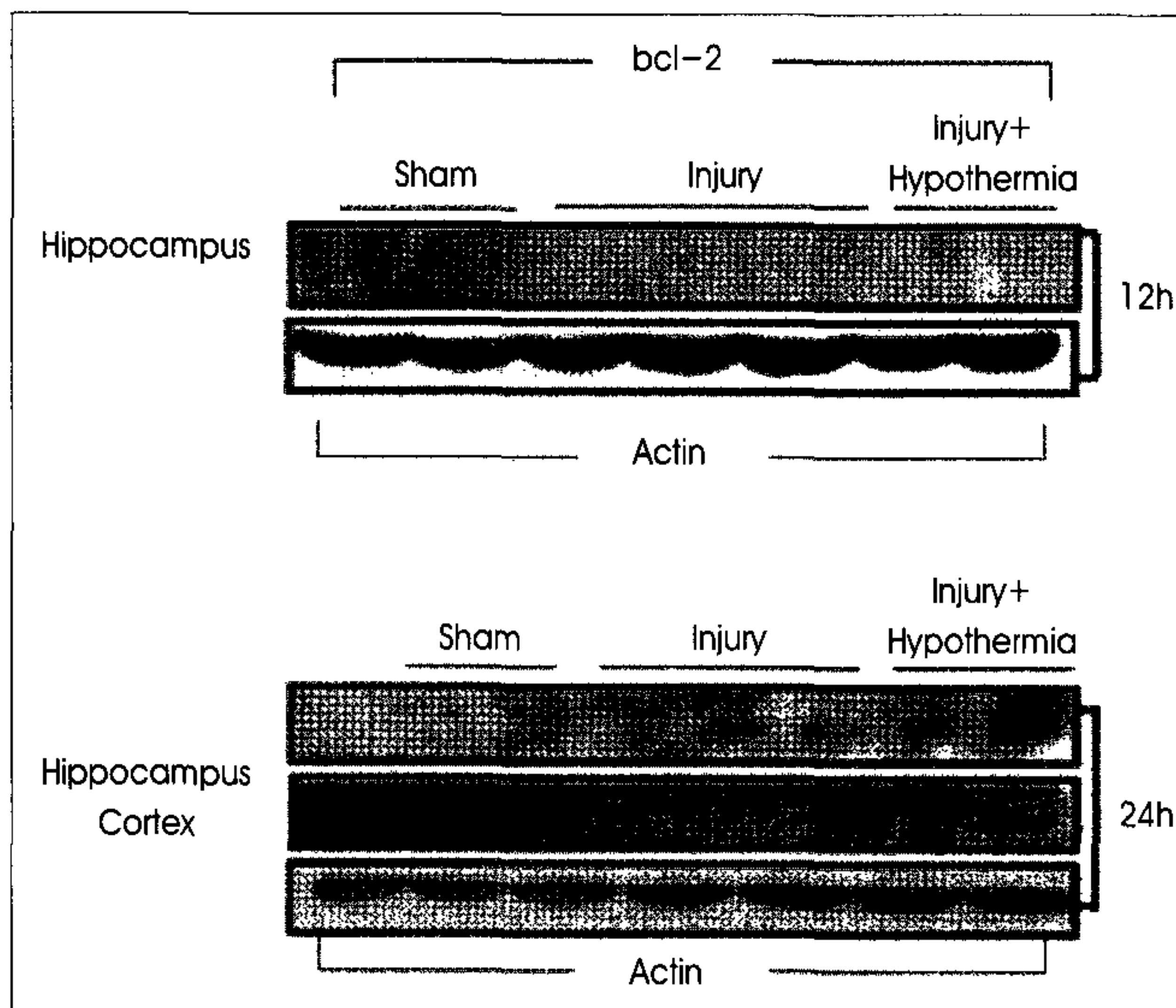

Fig. 5. Western blot analysis of bcl-2 in the ipsilateral cortex and hippocampus of rat brain 12h and 24h after-injury. Expression of actin served as an internal standard.

expression when compared to the control group (Fig. 5).

The expression of bcl-2 was not observed in cerebral cortex on the 12hour, while was remarkable in the area on the 24hour in the normothermia group and the hypothermia group. In particular, bcl-2 expression was increased more in the hypothermia group than in the normothermia group (Fig. 5).

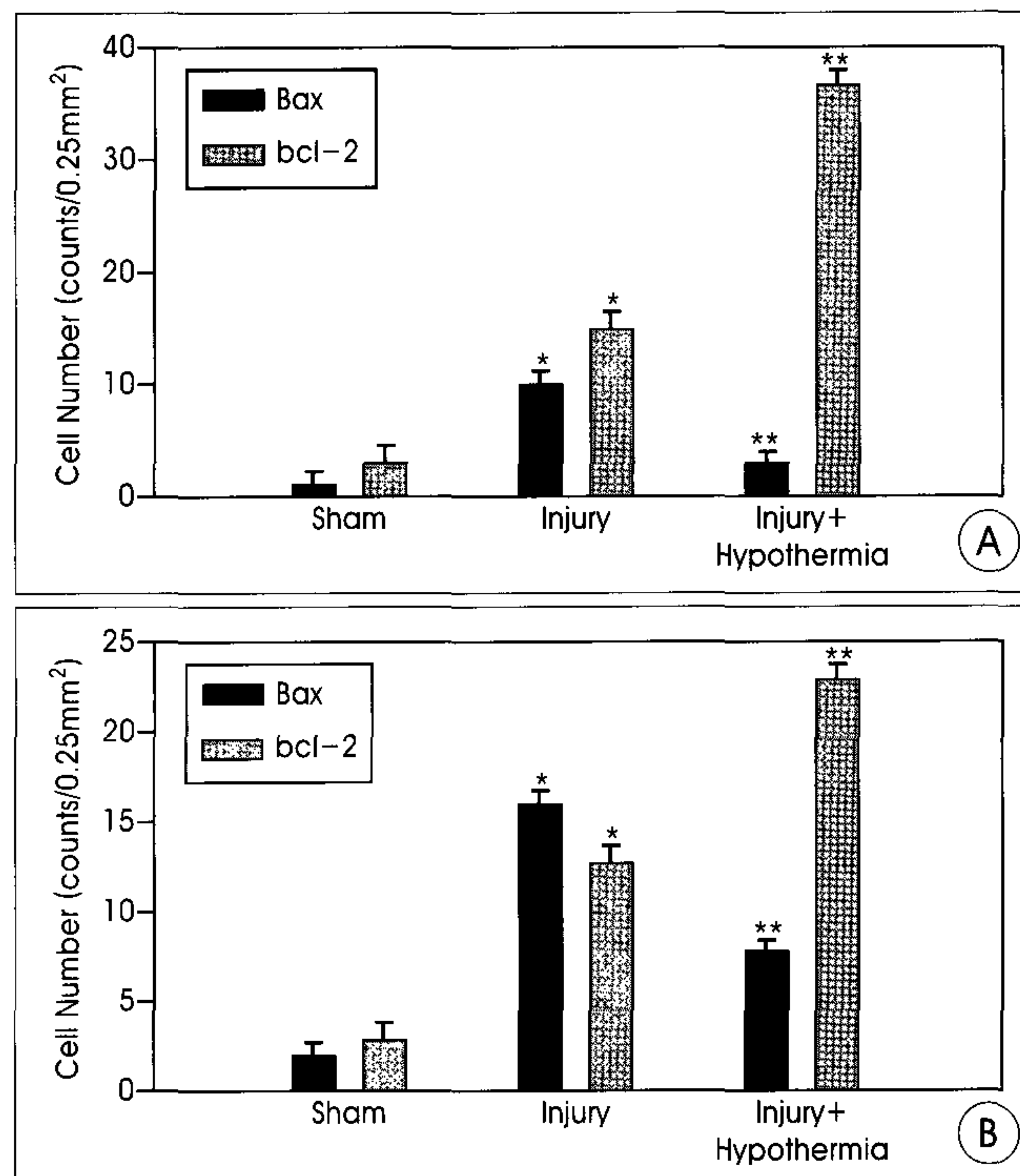

Fig. 7. Effect of hypothermia on the Bax and bcl-2 expressing cell number after traumatic brain injury. The level of Bax and bcl-2 expression were also presented 12h(A) and 24h(B) after-injury ($*p<0.05$ vs. sham, $**p<0.05$ vs. injury). Data represent means ± S.D.

The changes in the immune reactivities of Bax and bcl-2

The expression of Bax was observed in the cerebral cortex on the 12 and 24hours after TBI. The immune reactivity of Bax on the 12hour was apparently increased in the normothermia group than in the control group, while the reactivity was decreased in the hypothermia group (Fig. 6A). The immune reactivity of Bax on the 24hour was significantly increased in the normothermia group but was decreased in the hypothermia group (Fig. 6B). Meanwhile, according to the results of the observation of the immune reactivity of bcl-2 in the cerebral cortex on the 12 and 24hours after TBI, bcl-2 immunoreactive cells were increased more in the normothermia group and the hypothermia group than in the control group, and particularly, the cells were more remarkably increased in the hypothermia group than in the normothermia group (Fig. 6A, B). In the comparison of the changes in the number of Bax and bcl-2 immunoreactive cells after TBI, the Bax expression was significantly increased in the normothermia group on the 12 and 24hours but was significantly decreased in the hypothermia group (Fig. 7). Such results indicate that hypothermia reduces the expression of Bax after TBI. On the contrary, the number of bcl-2 immunoreactive cells in the hypothermia group on the

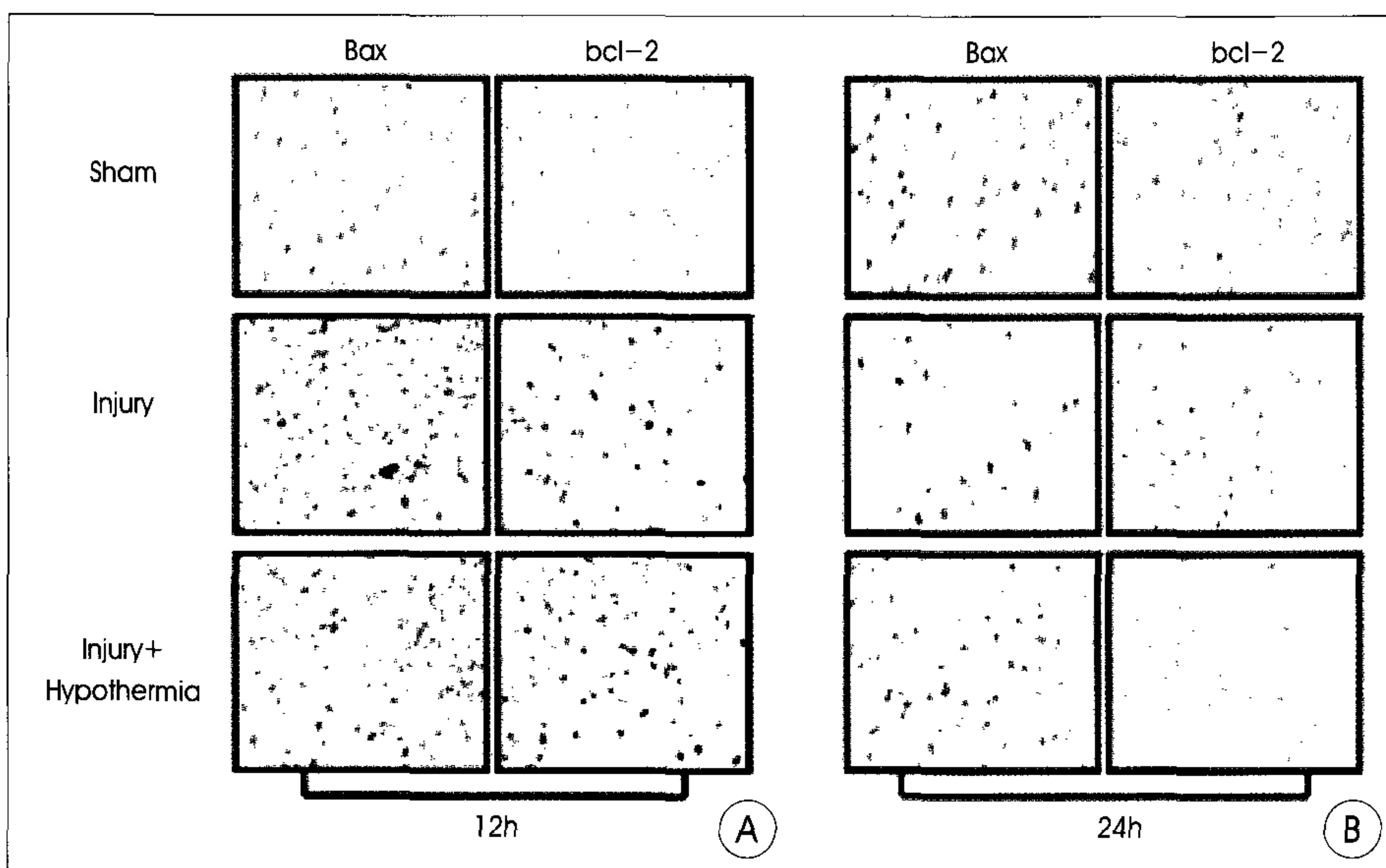

Fig. 6. Effect of hypothermia on the Bax and bcl-2 after traumatic brain injury (X200). The ipsilateral cortex of the rats of injury, the hypothermia after injury and the sham control were TUNEL stained for apoptosis 12h(A) and 24h(B) after-injury. The levels of Bax and bcl-2 expressions were examined with specific antibodies.

12hour after TBI was increased by 2.5 folds of that of the normothermia group, while the number on the 24hour in the former was increased by 1.7 folds of that of the latter (Fig. 7). Such results indicate that hypothermia increases bcl-2 expression associated with cell survival and affects the survival in TBI. It can be said that such expressions of Bax and bcl-2 have significance with the immunohistological expression. Therefore, it is indicated that hypothermia affects protein expression in TBI, despite the differences in the extent by tissues.

Discussion

Pathologic process in traumatic brain injury can be divided into immediate injury and secondary injury[4,10,13,16]. The secondary brain injury may be induced by ischemic/hypoxic and exitotoxic amino acid injuries which result from increased intracranial pressure. Namely, in brain injury cerebral blood flow decreased but brain metabolism increased[2]. The main goal of the treatment in traumatic brain injury is to reduce the degree of secondary injury, and various methods have been developed and tried. Magnesium ion is considered important to inhibit glutamate secretion, N-methyl-D-aspartate receptor activity, calcium channel, lipid peroxidation, free radical generation, and edema to reduce secondary injury in TBI[1,9,16].

Many researchers are studying on the magnesium ion as a neuroprotective agent, but the mechanism has not been clarified. However, recent studies reported that magnesium ion inhibits the apoptosis in TBI, such inhibition affects apoptosis-associated proteins, and then Bax is reduced and bcl-2 is increased. Such results indicate that apoptosis may be the one of the main pathogenesis in TBI, and the inhibition of apoptosis is related with the cell protection by magnesium ion[9]. Meanwhile, hypothermia as well as the use of magnesium ion is reported to be effective in the treatment of TBI. Zhu et al. reported that hypothermia remarkably inhibited the amount of brain infarct in hippocampal CA1 and the neuronal cell damage shown in hypoxia-ischemia model and were associated with apoptosis inhibition[22]. Hamann et al. reported that hypothermia maintained microvascular integrity and inhibited the activities of matrix metalloproteinases (MMP), and tissue-type plasminogen activator and urokinase-type plasminogen activator, plasminogen activators, to reduce cerebral hemorrhage[12]. The effect of hypothermia is not always equal in the ischemic model of white rats : its effect is varied based on strains and Long-Evans strain and spontaneously hypertensive rat strain showed different effect by hypothermia. Therefore, various factors should be considered in experimental models[19]. In this study, the authors observed the effect of hypothermia on apoptosis through the expressions of Bax and bcl-2, apoptosis-associated proteins, in white rats with TBI. When apoptosis was confirmed by TUNEL stain, apoptosis was remarkably increased in the normothermia group but the number of cells of apoptosis was considerably reduced in the hypothermia group. Raghupathi et al. reported that the bcl-2 expression was considerably reduced in the hippocampal CA3 and occipital cortex on the 2hour after brain injury while the Bax expression was slightly reduced[18]. In the western blot analysis, while Bax and bcl-2 were sharply reduced, the ratio of Bax and bcl-2 was remarkably increased from 2hour and that was maintained till the 7th day. Moreover in the cortex, the Bax expression was increased by 25% on the 24hour after brain injury when compared to the control group, and was recovered to be similar to that of the control group on the 7th day. Bax mRNA was increased in the cell membranes in damaged cortex, while showing no difference in the hippocampal CA3. Many researchers perform experiments on the optimal temperature for obtaining maximum effect from hypothermia. Mild hypothermia (33 or 34°C) protected cells in brain injury[3]. The maximum effect of cell protection in hypothermia was obtained at 30°C, and the mortality at the temperature (9.1%) was considerably lower than that at 33°C (41%) and that at 36°C (37.5%)[10]. In this study, mild hypothermia (33 or 34°C) was used, and therefore, further experiments in which hypothermia at 30°C are necessary for comparative analysis[7]. Time should be critically considered in the application of hypothermia in TBI. Hypothermia (30~32°C) applied within 60minutes after TBI reduces edema formation or promotes neurologic recovery, but hypothermia applied after 90minites after TBI is reported to have no effect[2,22]. Some of young pigs showed secondary increase in intracranial pressure in TBI while the expressions of DNA fragmentation and β-amyloid precursor by TUBEL stain were increased and the expression of microtubule-associated protein 2, a factor for axonal injury, was reduced[2,8,11,22]. Hypothermia could not apparently inhibit axonal injury in TBI, but decreased the expressions of β-APP and DNA fragmentation shown by ICP and increased the expression of MAP-2[2]. In this study, the authors observed apoptosis, the expression of apoptosis-associated proteins, and the inhibition of apoptosis by hypothermia in TBI, but more detailed classification of experimental groups and more various analytic methods would be accompanied in further studies.

Conclusion

The results of this study indicate that apoptosis and the expressions of apoptosis-associated proteins are related with traumatic brain injury. Also hypothermia may serve as

a therapeutic modality by inhibiting the apoptotic neuronal cell dealth.

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References

1. Bareyre FM, Saatman KE, Helfaer MA, Sinson G, Weisser JD, Brown AL, et al : Alterations in ionized and total blood magnesium after experimental traumatic brain injury : relationship to neurobehavioral outcome and neuroprotective efficacy of magnesium chloride. **J Neurochem 73** : 271-280, 1999
2. Brodhun M, Fritz H, Walter B, Reinhart K, Zwiener U, Bauer R, et al : Immunomorphological sequelae of severe brain injury induced by fluid-percussion in juvenile pigs-effects of mild hypothermia. **Acta Neuropathol 101** : 424-434, 2001
3. Busto R, Globus MY, Dietrich WD : Effect of mild hypothermia on ischemia induced release of neurotransmitters and free fatty acids in rat brain. **Stroke 20** : 904-910, 1989
4. Chang UK, Choe WJ, Kim KJ, Chung CK, Kim HJ : The apoptosis and expression of p53, Bcl-2 in graded contusion injury of rat spinal cord. **J Korean Neurosurg Soc 36** : 317-323, 2004
5. Chung C, Park SW, Kim YB, Hwang SN, Kim MK, Kwon JT, et al : Apoptosis and the expression of p53, Bcl-2 in very delayed focal cerebral infarction and penumbra. **J Korean Neurosurg Soc 27** : 855-864, 1998
6. Clark RSB, Chen J, Watkins SC, Kochanek PM, Chen M, Stetler RA, et al : Apoptosis suppressor gene bcl-2 expression after traumatic brain injury in rats. **J Neurosci 17** : 9172-9182, 1997
7. Clifton GL, Jiang JY, Lyeth BG : Marked protection by moderate hypothermia after experimental traumatic brain injury. **J Neurotrauma 10** : 57-64, 1993
8. Deng H, Han HS, Cheng D, Sun GH, Yenari MA : Mild hypothermia inhibits inflammation after experimental stroke and brain inflammation. **Stroke 34** : 2495-2501, 2003
9. Feldman Z, Gurevitch B, Artru Aa, Oppenheim A, Shohami E, Reichenthal E, et al : Effect of magnesium given 1h after head trauma on brain edema and neurological outcome. **J Neurosurg 85** : 131-137, 1996
10. Fritz HG, Bauer R : Secondary injuries in brain trauma : effects hypothermia. **J Neurosurg Anesthesiol 16** : 43-52, 2004
11. Globus MY, Alonso O, Dietrich WD, Busto R, Ginsberg MD : Glutamate release and free radical production following brain injury : effects of posttraumatic hypothermia. **J Neurochem 65** : 1704-1711, 1995
12. Hamann GF, Burggraf D, Martens HK, Liebetrau M, Jager G, Wunderlich N, et al : Mild to moderate hypothermia prevents microvascular basal lamina antigen loss in experimental focal cerebral ischemia. **Stroke 35** : 764-769, 2004
13. Kaya SS, Mahmood A, Li Y, Yavuz E, Goksel M, Chopp M : Apoptosis and expression of p53 response proteins and cyclin D1 after cortical impact in rat brain. **Brain Res 818** : 23-33, 1999
14. Kim CH, Cheong JH, Bak KH, Kim JM, Ko Y, Oh SJ : Effect of Bcl-2 expression and telomerase activity on apoptosis of the brain tumor. **J Korean Neurosurg Soc 32** : 35-41, 2002
15. Lewen A, Fujimura M, Sugawara T, Matz P, Copin JC, Chan PH : Oxidative stress dependent release of mitochondrial cytochrome c after traumatic brain injury. **J Cereb Blood Flow Metab 21** : 914-920, 2001
16. McIntosh TK, Vink R, Nobel L, Yamakami I, Soares H, Faden AI : Traumatic brain injury in the rat characterization of a lateral fluid percussion model. **Neuroscience 28** : 233-244, 1989
17. Napieralski JA, Raghupathi R, McIntosh TK : The tumor-suppressor gene, p53, is induced in injured brain regions following experimental traumatic brain injury. **Brain Res Mol Brain Res 71** : 78-86, 1999
18. Raghupathi R, Strauss KI, Zhang C, Krajewski S, Reed JC, Mcintosh TK : Temporal alterations in cellular Bax : bcl 2 ratio following traumatic brain injury in the rat. **J Neurotrauma 20** : 421-431, 2003
19. Ren Y, Hashimoto M, Pulsinelli WA, Nowak TS : Hypothermic protection in rat focal ischemia models : strain differences and relevance to "reperfusion injury". **J Cereb Blood Flow Metab 24** : 42-53, 2003
20. Sullivan PG, Keller JN, Bussen WL, Scheff SW : Cytochrome c release and caspase activation after traumatic brain injury. **Brain Res 949** : 88-96, 2002
21. Yi HJ, Kim CH, Bak KH, Kim JM, Ko Y, Oh SJ : p53 and Bcl-2 expression and apoptosis in human brain tumor. **J Korean Neurosurg Soc 31** : 357-363, 2002
22. Zhu C, Wahg X, Cheng X, Qiu L, Xu F, Simbruner G, et al : Postischemic hypothermia induced tissue protection and diminished apoptosis after neonatal cerebral hypoxia ischemia. **Brain Res 996** : 67-75, 2004