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# Effects of Selective Cyclooxygenase-2 Inhibitor NS-398 Pretreatment on the Rat Spinal Cord after Contusion Injury

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**Background :** Secondary spinal cord injury (SCI) that follows an initial mechanical insult can exacerbate the overall damage, limit the restorative processes and eventually lead to an increased neurological deficit. We hypothesized that selective inhibition of cyclooxygenase-2 (COX-2) may decrease the delayed cell death, and so this will contribute to decreased level of the secondary injury. **Methods :** The dorsal surface of the cord at the T9 level was subjected to weight drop impact using a 10 g rod. To block COX-2 activation, a selective COX-2 inhibitor (NS-398) was administered (5 mg/kg, i.p.) 15 min prior to SCI. The COX-1, COX-2, Caspase-3 and $PGE_2$ expressions were measured by real time quantitative RT-PCR and fluorescence immunostaining. **Results :** Many activated caspase-3 positive cells were observed at 6 h and they increased until 72 h after SCI. The expression of COX-2 peaked at 6 h after SCI, while the COX-1 expression was unaffected. The principal cells that showed a COX-2 expression were the neurons and microglia. Pretreatment with NS-398 caused a significant decrease in the expression of prostaglandin E2 and activated caspase-3 positive cells after SCI. **Conclusion :** These data suggest that COX-2 is one of the main factors related with the pathologic deficits from secondary SCI.

**Key Words :** Spinal cord injury; Cyclooxygenase 2 inhibitors; NS-398; Cell death

Spinal cord injury (SCI) is thought to significantly affect morbidity and mortality after traumatic injury. Injury to the spinal cord results in long lasting or permanent neurological deficits of both the motor and sensory systems. The pathologic mechanisms of SCI comprise the primary injury and the secondary injury. The initial mechanical insult causes the primary injury, which gives rise to parenchymal hemorrhage, interruption of the blood flow and disruption of the central gray matter due to its soft consistency and great vascularity. Thus, primary injury is observed in the acute phase after trauma. However, for a large number of patients with SCI, secondary injury is known to further provoke and extend the damaged area and so increase the long-term morbidity.[1] The mechanisms of the secondary injury consist of ischemia, hemorrhage, edema, excitotoxicity, calcium-mediated secondary injury, fluid electrolyte disturbances, immunologic injury, disturbances in mitochondrial function, apoptosis and other miscellaneous processes.[2,3]

Tissue damage and oxidative stress activate phospholipase A2, which hydrolyzes the membrane phospholipids into arachidonic acid.[4] Cyclooxygenase (COX) is the enzyme that plays the main role in the rate-limiting step in the metabolism of free arachidonic acid to prostaglandin (PG). It is reported that an increase in eicosanoid synthesis brings about damage of the central nervous system (CNS) as well as that the SCI causes damage via various pathologic processes.[5] The two isoforms of COX, i.e., COX-1 and COX-2, are 90% similar in their amino acid sequence and they are 60% structurally homologous.[6] In most tissues outside the CNS, COX-1 is constitutively expressed and its level remains

fairly constant, whereas COX-2 is upregulated as an immediate-early gene in response to injury.[6] However, in the brain and spinal cord, both COX-1 and COX-2 are constitutively expressed.[7] The induction of COX-2 was shown to enhance the eicosanoid synthesis that occurs after cerebral ischemic insult,[8] excitotoxin injection[9] and SCI.[10] Accordingly, it is well established that COX-2 is an important component of the post-traumatic inflammatory cascade during various types of CNS damage, but its role in the pathophysiologic mechanisms is still unknown.

Despite the generally agreed assumption that such trauma-induced spinal cord cell death represents the form of necrosis,[11] recent evidence suggests that cell loss in many neurological disorders, including SCI, may partly reflect programmed cell death that results in apoptosis.[12,13] The existence of apoptotic cell death after SCI was reported both in human patients and experimental models.[13-15] Apoptosis in neurons and glial cells contributes to delayed cell loss after SCI, which clearly has a negative effect on the recovery phase after SCI. The mechanism of apoptosis that occurs in the nervous system is much less understood compared to the apoptosis of a non-nervous tissue origin. In apoptotic cell death, intracellular proteolysis is affected by the activation of caspases that cleave critical substrates. All caspases are able to cleave the substrates that are essential for cell preservation. Especially, the activation of caspase-3 is a critical downstream event in many forms of programmed cell death,[16] and it has been shown that activation of procaspase-3 along with substrate cleavage occurs after SCI.[15] A study conducted by Citron *et al*.[17] showed that cell death in the spinal cord occured after post-translational activation of caspases, which at least included caspase-3, and this supports that the cellular localization of activated caspase-3 correlates with apoptotic cell death.

Although apoptotic cell death may have been involved in the cell loss after spinal cord injury, the precise mechanisms are unknown. Here, we postulated that apoptotic cell death is related to the delayed cell loss following spinal cord injury and that COX-2 induction is a related factor. We studied the delayed cell death after SCI by analyzing the patterns of active caspase-3 immunoreactivity and also by measuring the level of COX-2 expression via real-time quantitative RT-PCR in the spinal cords of Sprague-Dawley rats. The results that followed were compared with those of spinal cord injured rats that were treated with N-(2-cyclohexyloxy-4-nitrophenyl)-methane-sulfonamine (NS-398), the selective inhibitor of COX-2. In addition, we observed the expression of PGE2, which is generally regarded as a suitable marker of COX-2 activity.

## MATERIALS AND METHODS

### Animal model of SCI

Adult male Sprague-Dawley rats (200-225 grams each, n=60, Dae-Han Laboratory Animal Research Center Co. Ltd., Eumseong, Korea) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and then they were immobilized in a stereotaxic instrument (Stoelting, Wood Dale, Illinois, USA). The surgical field was shaved and a longitudinal incision was made to expose the thoracolumbar segments; a laminectomy was performed at the T9-T10 level, exposing the cord underneath without disrupting the dura. After the spinous processes of T8 and T11 were clamped to stabilize the spine, the exposed dorsal surface of the cord at the T9 level was subjected to impact from a dropped weight with using a 10 g rod (2 mm in diameter) dropped at a height of 5 cm through a guide tube. For the sham controls (n=24), the same surgical procedure was followed, except that the weight was not dropped. After the injury or sham surgery, the muscle, fascia and skin were sutured. The postsurgery survival times following spinal cord injury were 6, 24, and 72 h.

### Drug delivery

The selective COX-2 inhibitor N-(2-cyclohexyloxy-4-nitrophenyl)-methane- sulfonamine (NS-398, Cayman Chemical, Ann Arbor, USA) was dissolved in 0.5 mL of 1:1 DMSO/saline v/v (Sigma, St. Louis, USA) such that the final dose delivered was 5 mg/kg NS-398. Fifteen minutes prior to injury, the animals received intraperitoneal injections of either NS-398 or DMSO/saline v/v vehicle (n=5 at each time point).

### Histopathology

For the histopathologic examination, the rats were anesthetized with an overdose of chloral hydrate and they were perfused intracardially with 0.9% cold saline followed by 4% paraformaldehyde in PBS (pH 7.4). For performing histological evaluation, a 20 mm cord segment centered at the injury site was removed from the vertebral canal and placed in the same fixative overnight. The cord segment was dissected at 2 mm intervals and 10 pieces of cord tissues were finally embedded in paraffin. Serial 10 $\mu$m cross-sections were cut and then stained with hematoxylin-eosin.

### Measurement of COX-1 and COX-2 mRNA by real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR)

At 6, 24, and 72 h after the contusion injuries, the spinal cord segments, 8 mm in length and, centered on the injury site, were removed from both the control and SCI rats (n=5 at each time point). The removed spinal cords were divided into the rostral and caudal parts (sectioned at the epicenter, 4 mm in length each), and the COX-1 and COX-2 mRNA expressions were analyzed using real-time quantitative RT-PCR. The total RNA was isolated from the frozen specimens by the acid guanidinium thiocyanate-phenol-chloroform extraction method with using TRIzol (Life Technologies, Rockville, Maryland, USA). The cDNA synthesis was carried out with using TaqMan Reverse Transcription reagent (Applied Biosystems, California, USA) in a 20 $\mu$L reaction volume that contained 4 $\mu$g total RNA, 1×RT-PCR buffer, 500 $\mu$M dNTPs, 2.5 $\mu$M random hexamers, 0.8 U of RNase inhibitor and 25 U of MultiScribe reverse transcriptase. The real-time PCR reaction was performed by using the ABI PRISM 7000 sequence detection system (Applied Biosystems) and SYBR green PCR master mix. The amplifications were carried out in a 96 well plate in a 25 $\mu$L reaction volume that contained 12.5 $\mu$L of 2×SYBR Green PCR Master Mix (Applied Biosystems), 0.04 $\mu$M each of forward and reverse primers and cDNA. The oligonucleotide primer pairs for COX-1, COX-2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as follows: COX-1 sense primer: 5′-AGCGGCCCATGACTCTCA-3′, antisense primer: 5′-CTGCACCCAAACACCAAGGT-3′, COX-2 sense primer: 5′-CCTTGAAGACGGACTTGCTCAC-3′, antisense primer: 5′-TCTCTCTGCTCTGGTCAATGGATCTG-3′, GAPDH sense primer: 5′-TGCCAAGTATGATGACATCAAGAAG-3′, antisense primer: 5′-AGCCCAGGATGCCCTTTAGT-3′. The thermal profile for the PCR was 2 min at 50℃, 10 min at 95℃ followed by 40 cycles of 15 s at 95℃ and 1 min at 60℃. All the experiments were performed in duplicate. The copy numbers of the cDNA for COX-1 and COX-2 were standardized to those of GAPDH for the same sample. We used the $2^{-\Delta\Delta CT}$ method[18] to calculate the relative expression level of the target gene.

### Immunofluorescent staining of activated caspase-3, COX-2 and $PGE_2$

The tissue sections were dewaxed in xylene and rehydrated through a graded series of alcohol solutions. Antigen retrieval was carried out by heating the samples for 10 min under pressure in a microwave oven in sodium citrate buffer (pH 6.0), and then incubating them with blocking solution that contained normal goat serum. Each of the tissue sections was incubated with antibodies to caspase-3 (polyclonal, Cell Signaling Technology, Beverly, USA) at a dilution of 1:50, COX-2 (polyclonal, Cayman Chemical, Ann Arbor, USA) at a dilution of 1:300, and PGE2 (polyclonal, Assay Design, Ann Arbor, USA) at a dilution of 1:500. The reaction was stopped by rinsing in TBS and then incubation was done with goat anti-rabbit AlexFluor 488 secondary antibody (Molecular Probe, Leiden, Netherlands) for the caspase-3 staining, and incubation was done with goat anti-rabbit AlexFluor 568 secondary antibody (Molecular Probe, Leiden, Netherlands) for the COX-2 and $PGE_2$ staining.

Double-labeling studies were performed to identify the cells showing the expression of COX-2 after SCI. For the first immunofluorescent staining, the tissue sections were incubated with anti-COX-2 antibody as described above, followed by a second round of immunofluorescent staining with neuronal and glial cell antibody markers that were reactive for microglia (monoclonal, ED-1, Serotec, Oxford, UK), oligodendrocytes [mouse-anti-oligodendrocyte monoclonal antibody (Oligo mAb), Chemicon, Temecula, USA], neurons (monoclonal, synaptophysin, DAKO, Glostrup, Denmark) and astrocytes (monoclonal, GFAP, Molecular Probe). The antibodies for the representative neuronal and glial cell markers were diluted 1:150 for ED-1, 1:10,000 for Oligo mAb, 1:50 for synaptophysin, and 1:100 for GFAP in TBS that contained 0.1% bovine serum albumin. After reaction with ED-1, Oligo mAb, synaptophysin, and GFAP, the tissue sections were incubated with goat anti-mouse AlexFluor 488 secondary antibody.

### Statistical analysis of the cells with an activated caspase-3 expression

The numbers of caspase-3 immunoreactive cells were counted under fluorescence microscopy (×200, 30 fields) at ten axial levels. Statistical analysis of caspase-3 immunostaining was done by Student's T-test and the ANOVA test: p values $<0.05$ were considered significant.

## RESULTS

### Histopathology

The spinal cords from the sham-operated controls showed

tightly packed cells in the gray matter with clearly delineated gray-white junctions. Six hours after a weight drop insult (a 10 g rod 2 mm in diameter that fell 5 cm), an area of injury was visibly apparent, which was defined by the loss of neurons and the petechiae in the gray matter of the epicenter. The white matter of the epicenter revealed focal hemorrhage, edema and axonal degeneration. The cross sectional slices of the spinal cord 2 mm apart from the epicenter, both rostrally and caudally, showed extravasation of red blood cells and edema without the prominent loss of neurons. Neutrophilic infiltration and activation of microglia and astrocytes were noted. Twenty-four hours after injury, the lesion area extended about 2 to 4 mm apart from the epicenter, and a well-defined cyst was formed at the epicenter. By 72 h, a well-defined cyst was formed and there was the accumulation of numerous macrophages. The features of spinal cord destruction became less severe as the distance from the epicenter increased. The spinal sections taken 4 mm or more apart from the epicenter showed relatively preserved histology without any prominent inflammatory infiltration or cell loss. In general, the results in the NS-398-treated and vehicle-treated groups showed no remarkable change until 72 h after SCI.

### Expression of COX-1 and COX-2 mRNA

The change in the COX/GAPDH ratio after rat spinal cord contusion injury was accessed by performing real-time quantitative RT-PCR, and this revealed that the relative amount of COX-2 mRNA peaked at 6 h after SCI, and it returned to a control level at 72 h. The spinal cords of the sham-operated animals showed a basal level of COX-2 activity (Fig. 1A). However, the level of COX-1 mRNA revealed no remarkable change during the time course, and the amount of COX-1 mRNA did not show a noticeable difference between the sham operated group and the injured group (Fig. 1B).

### Immunoreactivity of COX-2

In the control rats, several neurons revealed weak immunoreactivity for COX-2 protein. Six hours after SCI, an increased expression of COX-2 protein was observed throughout the spinal cord, especially at the segment nearest to the epicenter in which the neuronal loss is not prominent, and the strong immunoreactivity for COX-2 was sustained until 24 h after SCI. At this time point, many of the neurons and glial cells revealed COX-2 immunoreactivity in their cytoplasm (Fig. 2). Many of the neurons, macrophages and activated microglia revealed strong COX-2 immunoreactivity (Fig. 2A to F). Only a few astrocytes and oligodendrocytes in the white matter showed a COX-2 expression (Fig. 2G to L). At 72 h after SCI, there was decreased immunoreactivity for COX-2.

### Immunoreactivity of $PGE_2$

The cross-sectional slices of the spinal cord taken 4 mm rostral and caudal from the injury epicenter showed an increased $PGE_2$ expression compared to the control animal at 6 h following spinal contusion injury (Fig. 3A). The expression of $PGE_2$ was significantly decreased at the same spinal level with NS-398 pretreatment (Fig. 3B).

### Immunofluorescent staining analysis of activated caspase-3

In the sham operated control, there was no activated caspase-3 positive profile in the spinal cord. On the contrary, many profiles appeared (total score of 8 levels: 934±68.3) 6 h after SCI (Fig. 4A, B) and there was no significant difference between the levels [2 mm rostral from the epicenter (R1): 127±26.0, 4 mm rostral (R2): 120±30.6, 6 mm rostral (R3): 111±19.9, 8 mm rostral (R4): 113±20.p, 2 mm caudal (C1): 91±8.0, 4 mm caudal (C2):

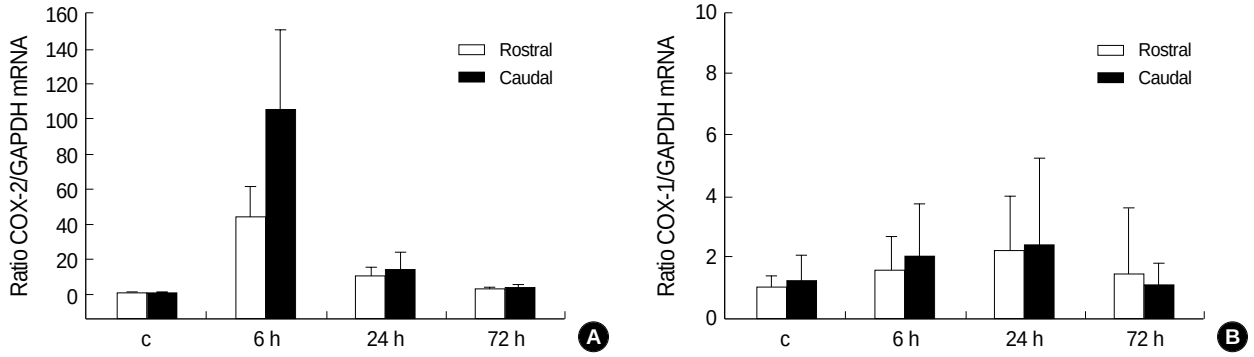


Fig. 1. COX-2 and COX-1 mRNA expression in spinal cord after contusion injury. Real time quantitative RT-PCR was used to demonstrate up-regulation of COX-2 (A). Expression of COX-1 mRNA is not significantly changed after SCI (B).

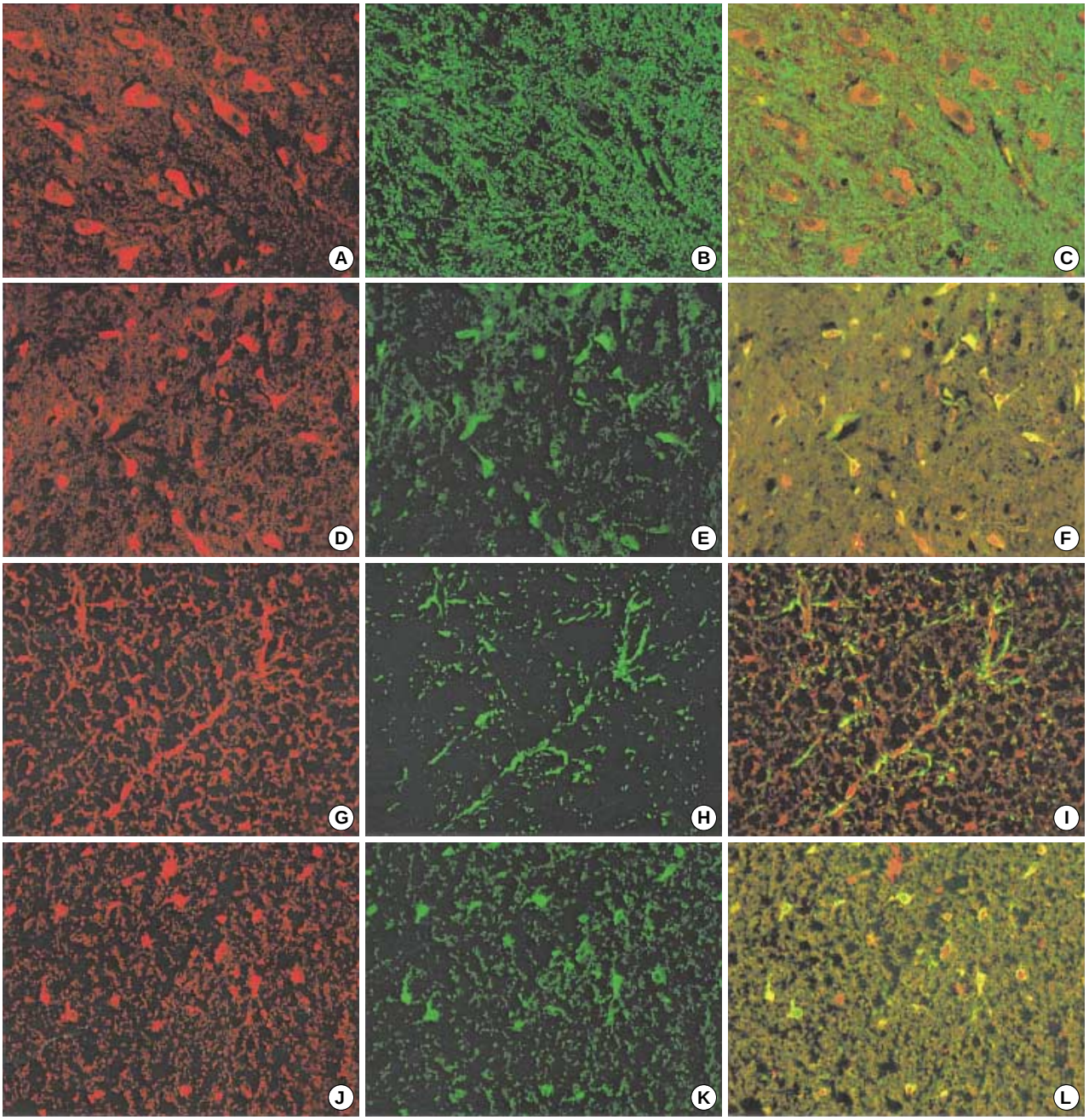


Fig. 2. Double labeling of COX-2 and spinal cord cell markers in rat spinal cord 6 h after contusion injury. Four pictures in left lane (red colored, A, D, G, J) for COX-2, four pictures in middle lane for cell markers (green colored), synaptophysin (B), ED1 (E), GFAP (H), and monoclonal antibody for oligodendrocyte (K). Merged figures in right lane (C, F, I, L) showing bright yellow to scarlet colored cells with expression of COX-2 and their cell markers (×200).

168±31.7, 6 mm caudal (C3): 109±5.6, 8 mm caudal (C4): 96±10.7]. The total score of the 4 levels of the rostral side (470±25.1) and that of the caudal side (464±45.7) was not significantly different. The activated caspase-3 positive profiles in the gray matter (793±12.8) were significantly more than those in the white matter (141±70.2, Fig. 5B). At 72 h after cord contusion injury, the total score (1,032±49.7) of the positive cells in the 8 levels was increased more than that of the 6 h group, but the difference was not statistically significant and the score of each level (R1: 107±48.6, R2: 133±4.2, R3: 141±61.1, R4: 120±22.4, C1: 143±39.9, C2: 131±35.27, C3: 127±20.7, C4: 129±32.5) was not significantly different. In this group,

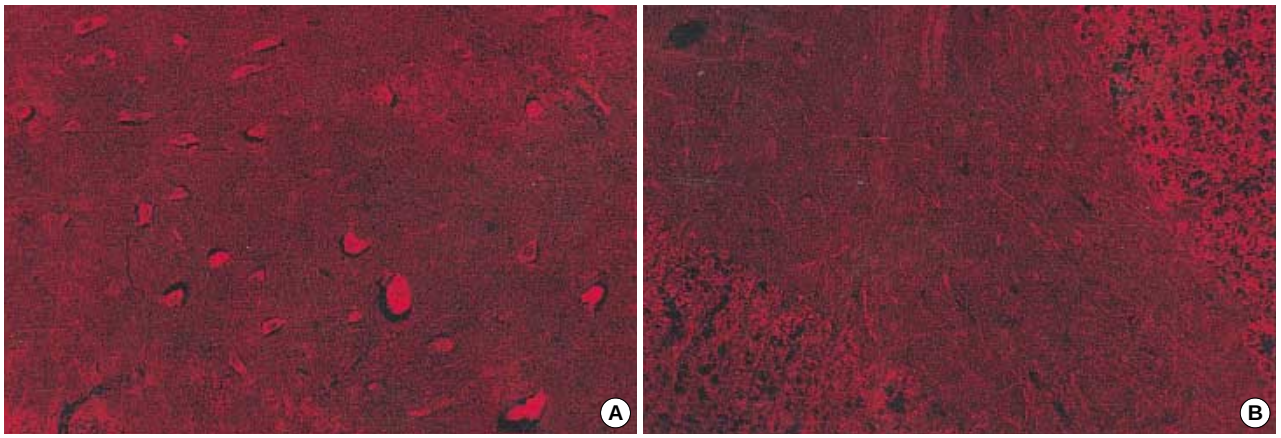


Fig. 3. Immunostaining of PGE2 in rat spinal cord 6 h after contusion injury. After saline/DMSO vehicle pretreatment, strong PGE2 immunoreactivity is noted both in the gray and white matter (A), on contrary COX-2 selective inhibitor, NS-398, pretreatment markedly reduce the intensity of PGE2 immunoreactivity in the gray and white matter (B) ( × 100).

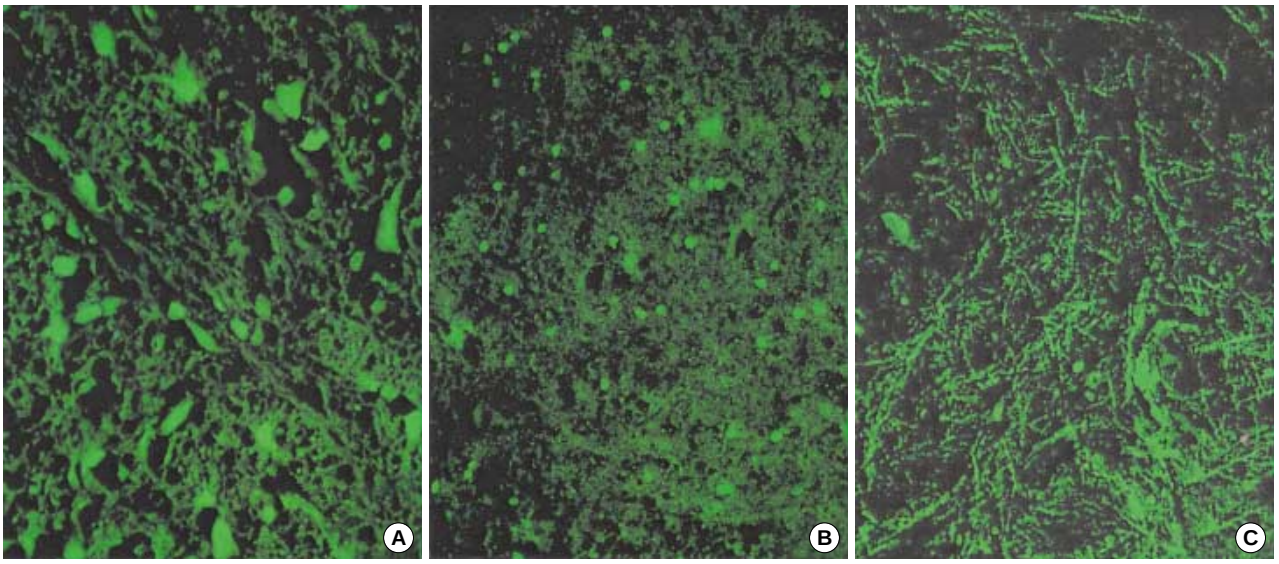


Fig. 4. Immunostaining of activated caspase-3 in rat spinal cord 6 h after contusion injury. After saline/DMSO vehicle pretreatment, strong activated caspase-3 immunoreactivity is noted both in neurons and glial cells (A, × 200; B, × 100). Pretreatment of COX-2 selective inhibitor, NS-398, reveals marked decrease in the activated caspase-3 positive profiles both in neurons and glial cells. (C, × 100)

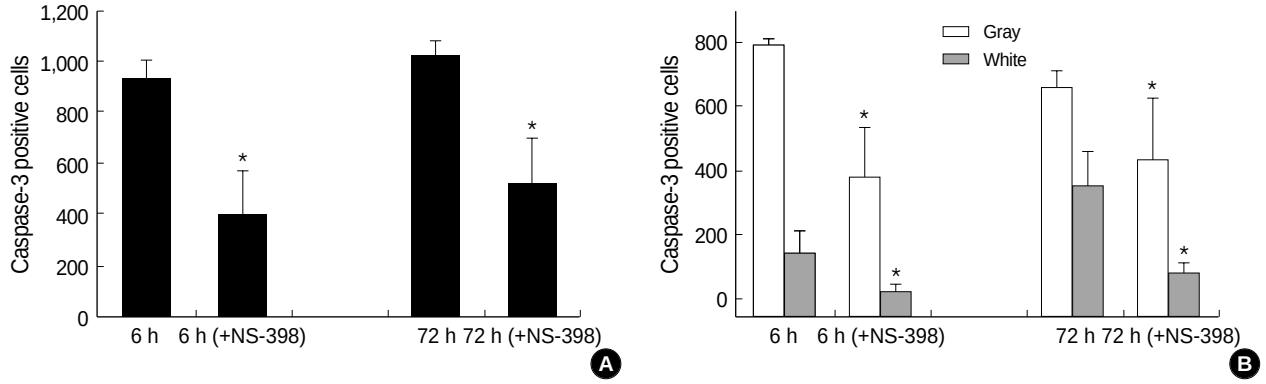


Fig. 5. The score of activated caspase-3 immunoreactive cells with saline/DMSO vehicle and NS-398 pretreatment in rat spinal cord 6 h and 72 h after contusion injury. With NS-398 pretreatment, both total score (A) and the score of gray and white matter (B) are profoundly decreased. Asterisks indicate values significantly different from controls ($p<0.05$).

the positive cells in the gray matter (661±49) were more abundant than in the white matter (358±99, Fig. 5B).

After NS-398 treatment, the score of the positive cells was significantly decreased ($p<0.05$) both at 6 h (total score of 8 levels: 406±165.3, R1: 48±25.4, R2: 86±29.5, R3: 62±15.7, R4: 70±12.2, C1: 27±36.1, C2: 47±57.0, C3: 43±25.9, C4: 31±16.8, Fig. 4C) and 72 h (total score of 8 levels, 523±173.8. R1: 93±44.5, R2: 68±39.6, R3: 67±27.5, R4: 70±13.1, C1: 61±12.7, C2: 99±6.7, C: 73±15.9, C4: 44±12.2) after SCI (Fig. 5A). With the NS-398 treatment, the score of the activated caspase-3 positive profiles was significantly decreased both in the gray matter (380±151.3 at 6 h after SCI and 438±182 at 72 h after SCI) and in the white matter (27±20.1 at 6 h after SCI and 85±21 at 72 h after SCI, Fig. 5B). A 52% decrease at 6 h and a 43% decrease at 72 h after SCI in gray matter and a 82% decrease at 6 h and a 76% decrease 72 h after SCI in white matter compared with the experimental groups with SCI only without NS-398 treatment (Fig. 5B).

## DISCUSSION

Recent evidence has provided insight into the role of the inflammatory mediators after SCI[15,19], and eicosanoids have been implicated in the pathogenesis of spinal cord injury. Up-regulation of neuronal COX-2 and elevation of $PGE_2$ have been reported to occur after SCI.[20] Takadera *et al*.[21] have recently demonstrated that $PGE_2$, one of the major products of COX-2, induces apoptosis in rat cortical cells, and this suggests a potential role of COX-2 in the mechanisms of delayed neuronal death in the pathological biology of the CNS. However, whether COX-2 and its reaction products are directly related to the induction of apoptosis in neuronal cells is unclear.

In this study, we demonstrated the rapid up-regulation of caspase-3 at 6 h after SCI in rats and the increased positive activated caspase-3 profile until 72 h after SCI. The number of activated caspase-3 positive cells was counted to evaluate the pattern of cells with apoptosis. The positive profiles were scattered as wide as 8 mm from the epicenter to both the rostral and caudal directions, and the difference of the scores between the two directions was insignificant. It has been reported that the pattern of the activated caspase-3 positive profiles is consistent with the distribution of apoptosis.[17,22] Since apoptosis occurs after SCI, it involves caspase activation, which leads to cleavage of proteins that are important for maintaining the healthy status of cells.[13,15] Apoptosis of spinal cord cells following contusion injury was not confined to the immediate impact site, nor was it confined in time to the immediate postinjury period. There was a burst of neuronal and glial apoptosis in the gray and white matter at the lesion site within approximately 24 h.

We have also demonstrated that spinal cord contusion injury increases the expression of COX-2 mRNA and protein after SCI, while this inconsequentially changed the expression of COX-1. Such an increase in the COX-2 expression in the spinal cord peaked at 6 h postinjury and it persisted until 24 h. Many of the spinal cord cells, predominantly the neurons, activated microglia and macrophages, expressed COX-2 protein after contusion injury and only a few astrocytes and oligodendroglia in the white matter revealed COX-2 immunoreactivity. However, the control animal showed a weaker intensity of the COX-2 immunolabelled neurons than did the injured animals. Beiche *et al*.[7] already reported on the expression of COX-1 and COX-2 in spinal cord under the basal condition, and the results of our study demonstrated that COX-2, and not COX-1, was rapidly up-regulated after spinal cord contusion injury. It has recently been indicated that there is upregulation of the expression of COX-2 in the central nervous system in Alzheimer's disease,[23] amyotrophic lateral sclerosis,[24] experimental brain trauma[25] and trimethyltin-induced hippocampal degeneration.[22]

In the present study, a selective COX-2 inhibitor, namely NS-398, served as a pharmacological tool to determine whether COX-2 was involved in both the apoptotic cell death and the cell loss within the spinal cord cell after the spinal cord contusion injury. This study demonstrated that inhibition of COX-2 that followed a single administration of NS-398 before SCI was an effective means to decrease apoptosis, as well as this causing a significant decrease in the activated caspase-3 positive cells., and these results were consistent with previously reported experimental research.[13] Recent evidence has shown that COX-2 inhibitors protect against neurodegeneration in experimental models of various kinds of CNS pathologies.[15,25-27] The results presented here strongly suggest that COX-2 is one of the important factors related with the observed neuronal loss after spinal cord contusion injury. COX-2 is the inducible form of COX and its expression is induced following tissue damage, excitatory amino acid release and ischemia in neurons, and then activated macrophages enter the spinal parenchyma through the perivascular spaces after trauma.[27] COX-2 synthesizes such prostanoids as prostaglandins and also thromboxane from arachidonic acid, and the superoxide anion is released as a by-product of the reaction.[28] The basis for the possible contribution of COX-2 to neuronal vulnerability and death seems to be through the production of the superoxide anion, and this

has potent damaging effects on lipids, proteins and DNA.[28] With regard to this, COX-2 plays an important intermediary role in contributing to the extended excitotoxicity and lipid peroxidation, which is the result of apoptotic cell death in SCI.

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