DNA damage and repair system in spinal cord ischemia

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Background and Purpose: **Spinal cord ischemia-reperfusion injury may be initiated by a number of mediators, including reactive oxygen species. Recent studies have shown that human MutY homologue (hMYH), human 8-oxo-7,8-dihydrodeoxyguanine (8-oxoG) glycosylase (hOGG1), and human MutS homologue 2 (hMSH2) are important DNA mismatch repair genes. We hypothesized that ischemia-reperfusion injury in spinal cord causes DNA damage manifested by 8-oxoG production and activates the DNA repair system involving hMYH, hOGG1, and hMSH2.**

Methods: **Spinal cords of rabbits were removed at 1, 3, 6, 24, and 48 hours after 30 minutes of infrarenal aortic occlusion. DNA damage was determined with 8-oxoG staining. The expression and localization of DNA repair enzymes, such as hMYH, hOGG1, and hMSH2, were studied with Western blot analysis and immunohistochemical staining. The level of apoptosis was determined with TUNEL study. Activation of caspase-3, an enzyme induced by cellular injury that leads to apoptosis by degrading cellular structural proteins, was also studied.**

Results: **DNA damage monitored with 8-oxoG level was significantly present from 1 hour to 6 hours after reperfusion in gray matter neurons of ischemic spinal cord. The levels of hMYH, hOGG1, and hMSH2 were markedly increased in gray matter neurons at 6 hours after reperfusion. Caspase-3 was also induced at 6 hours to 24 hours after reperfusion in ischemic spinal cord. However, the peak level of TUNEL reactivity was found at 48 hours after reperfusion in spinal cord neurons.**

Conclusion: **This study has shown, for the first time, the rapid expression of DNA damage-repair processes associated with spinal cord ischemia and subsequent reperfusion. (J Vasc Surg 2003;37:847-58.)**

Paraplegia is a well-recognized and catastrophic complication after operation of the descending thoracic and thoracoabdominal aorta.[1-4] The incidence rate of paraplegia ranges from 0.9% to 40% in operations on the thoracic aorta.[5,6] The inciting cause of spinal cord injury is ischemia, but the metabolic mechanisms leading to full partial or delayed spinal cord dysfunction are not well understood.

Ischemia-reperfusion injury may be initiated by a number of mediators, including reactive oxygen species, calcium ions, and extracellular glutamate. Previous studies reported delayed and selective motor neuron death after transient ischemia.[7-10] The exact mechanism of this phenomenon is not fully understood but has been attributed to postoperative hypotension, embolization, or thrombosis to the anterior spinal artery and occlusion of reimplanted intercostal arteries.[11-14] Research also has indicated other factors as mediators of delayed cell death after central nervous system ischemia, including free radical production, deleterious effects of leukocytes and microglia, and apoptosis.[15-20]

The reactive oxygen species, such as superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (^-OH), play a critical role in the pathogenesis of ischemia-reperfusion injury.[21] Eight DNA lesions are known to be characteristic of DNA damage mediated by free radicals. Among the different oxidative-damage DNA products, 8-oxo-7,8-dihydrodeoxyguanine (8-oxoG) is the most stable and deleterious adduct.[22,23] If the 8-oxoG lesions in DNA are not properly repaired, a high percentage of guanine/cytosine to thymine/adenine transversion will occur through the 8-oxoG/adenine replication intermediate.[24,25] Recent studies showed that human MutY homologue (hMYH),[26] human 8-oxoG glycosylase (hOGG1),[27] and human MutS homologue 2 (hMSH2)[28] protein play important roles in repairing such DNA mismatch injury (Fig 1). Human MYH and hMSH2 can effectively remove adenine misincorporated opposite 8-oxoG or guanine after DNA replication.[29-32] Human OGG1 gene encodes a DNA glycosylase activity catalyzing the excision of the mutagenic lesion 8-oxoG from oxidatively damaged DNA.[33-35] To date, no information exists regarding DNA damage and the 8-oxoG repair system during spinal cord ischemia and reperfusion injury. Therefore, this study was designed to investigate the level of DNA damage (such as 8-oxoG production) and the expression of DNA repair enzymes (such as hMYH, hOGG1, and hMSH2) during spinal cord ischemia and reperfusion. Furthermore, this study also was designed to investigate the relationship of DNA damage and traditional apoptosis in rabbit spinal cord neuronal cells. Moreover, the expression of apoptosis-related gene, caspase-3, in the spinal cord during ischemia-reperfusion injury was also investigated. We hypothesized that ischemia-reperfusion injury in neuronal cells stimulates DNA damage, such as 8-oxoG production, and activates

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Competition of interest: none.

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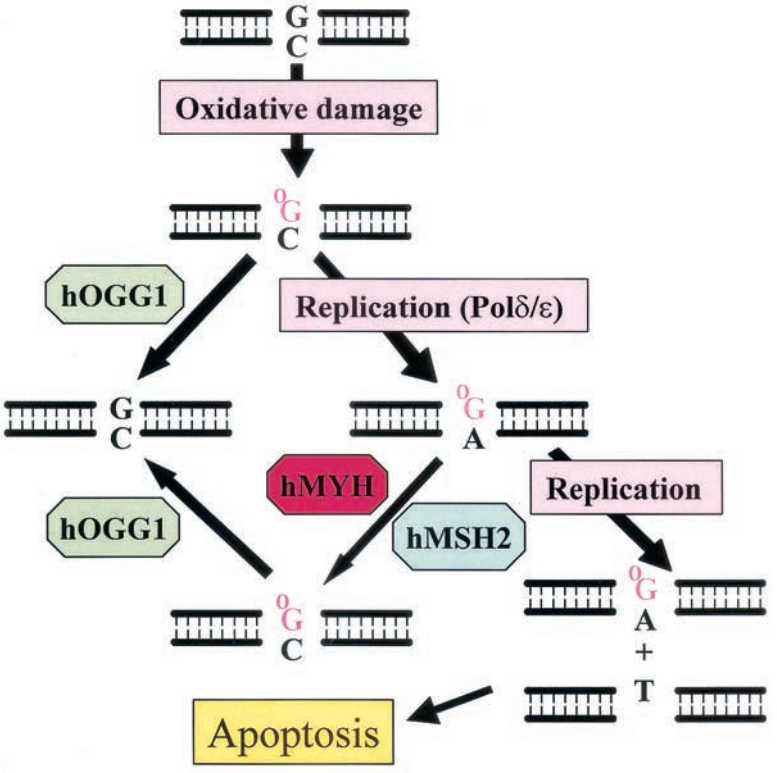

Fig 1. Diagram shows 8-oxoG generation and repair system during oxidative damage. Ischemia-reperfusion injury may be initiated by a number of mediators, including reactive oxygen species. Interactions between DNA and hydroxyl radical produce DNA strand breaks and base modifications, which are frequently assessed with measurement of nucleoside 8-oxoG level. If 8-oxoG lesions in DNA are not properly repaired, high percentage of guanine/cytosine to thymine/adenine transversion will occur through 8-oxoG/adenine replication intermediate. hMYH, hOGG1, and hMSH2 play important roles in repairing DNA mismatch injury. hMYH and hMSH2 can effectively remove adenine misincorporated opposite 8-oxoG or guanine after DNA replication. hOGG1 gene encodes DNA glycosylase activity catalyzing excision of mutagenic lesion 8-oxoG from oxidatively damaged DNA. If these DNA mismatch repair enzymes are not activated enough, then cell apoptosis occurs.

the DNA repair system, such as hMYH, hOGG1, and hMSH2 expression, in spinal cord neurons.

METHODS

Spinal cord ischemia. New Zealand white albino rabbits (Charles River Laboratories, Inc, Wilmington, Mass) weighing 3.5 to 4.5 kg were used in these studies. Rabbits were used because they have a segmental blood supply to the spinal cord. Thus, infrarenal aortic occlusion regularly causes paraplegia. The ischemic challenge of 30 minutes was selected because previous studies have shown that all rabbits were paraplegia on awakening from anesthesia.[36,37] Animal care and experiments complied with the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health.

Animals underwent pretreatment with atropine (0.4 mg/kg, intravenously), and general anesthesia was induced with medazalam (0.05 mg/kg, intravenously), metomidine (0.05 mg/kg), and propofol (2 mg/kg, intravenously). General anesthesia was maintained with continuous infusion of propofol (5 mg/min). After sterile preparation, a flank incision was made and the infrarenal aorta was exposed through a retroperitoneal approach. Spinal cord ischemia was induced with cross clamping of the aorta (T12 level) with a surgical microclamp (Biover vessel clips, Arex, Palaiseau, France) for 30 minutes. Electrocardiographic results were continuously monitored throughout the procedure. Percutaneous arterial oxygen saturation was continuously monitored. Core temperature was supported with a heating lamp throughout the procedure. The aortic clamps were removed after 30 minutes, and the flank was closed in two layers. The bladders of the paraplegic animals were emptied twice daily with Credé's maneuver.

The animals were randomly assigned to be killed at 1, 3, 6, 24, and 48 hours (five rabbits in each group). Animals in the sham operation group were killed at 3 hours (n = 5) and 24 hours (n = 3) after exposure of the aorta without cross clamping of the vessel. No differences were seen in any study parameters between animals with sham opera-

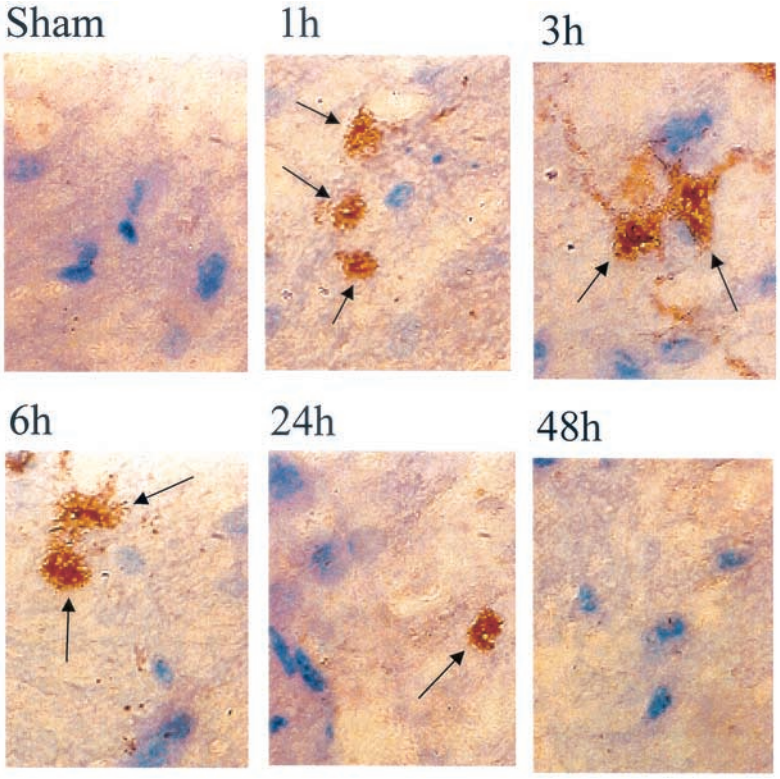

Fig 2. DNA damage, such as 8-oxoG staining, in sham-control and ischemic spinal cord. Positive staining of 8-oxoG *(arrows)* was detected predominantly within intermediate gray matter and ventral horn. 8-oxoG level was markedly increased in ischemic neurons from 1-hour to 6-hour reperfusion period. Peak level of 8-oxoG was found at 6 hours after reperfusion. Furthermore, positive staining of 8-oxoG level was decreased after 48-hour reperfusion period (×1000).

Percentage of positive stained nuclear

	Sham	*1 h*	*3 h*	*6 h*	*24 h*	*48 h*
8-oxoG (%)	0.5 ± 0.05	25 ± 2*	20 ± 3*	16 ± 4*	7 ± 1*	2 ± 0.5
MYH (%)	2.0 ± 0.5	5 ± 1*	11 ± 2*	20 ± 3*	12 ± 2*	4 ± 1
OGG1 (%)	1.0 ± 0.6	3 ± 1	6 ± 2*	16 ± 3*	8 ± 2*	3 ± 1
MSH2 (%)	1.5 ± 0.8	7 ± 2*	12 ± 3*	18 ± 4*	11 ± 3*	5 ± 2
TUNEL (%)	0.5 ± 0.05	0.9 ± 0.1	8 ± 2*	12 ± 2*	15 ± 3*	21 ± 4*

Mean ± standard error.
*$P < .05$ versus sham group.

tions killed at 3 hours and 24 hours. Therefore, we used the 3 hour group for all comparisons. All animals were killed with administration of an intravenous infusion of pentobarbital (100 mg/kg). Before cardiac arrest, the ascending aorta was exposed via median sternotomy, cannulated, and infused with 1 L of saline solution at 4° C. A complete laminectomy was performed from T1 to the sacrum with bone cutters. Segments of high thoracic and lower lumbar spinal cord were removed, frozen, and stored in a −80° C freezer.

The samples for histologic and immunohistochemistry studies were fixed in 4% paraformaldehyde in a 0.1 mol/L concentration of phosphate buffer and then stored at 4° C for 1 week. The tissue samples were cut transversely at about the L3 level and embedded in paraffin.

Determination of DNA damage in spinal cord. The DNA damage level was determined with 8-oxoG staining with monoclonal 8-oxoG antibody. The tissue sections were deparaffined and dehydrated, then covered with 10 μg/mL proteinase K in phosphate-buffered saline solution (PBS), pH 7.4, and incubated for 40 minutes at 37° C. The DNA was denatured with soaking the slides in 4 N HCl for 7 minutes and neutralized with 50 mmol/L Tris base for 5 minutes at room temperature. After two washings with

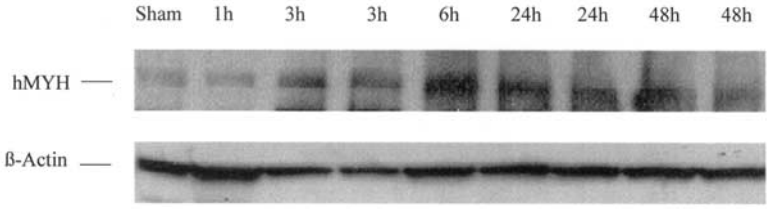

Fig 3. Representative Western blot analysis for hMYH in sham-control and ischemic spinal cord. Immunoreactivity of hMYH was only weakly detected in sham controls. Protein level of hMYH was upregulated as early as 3 hours after reperfusion and remained elevated as late as 1 day. Peak level of hMYH was found at 6 hours after reperfusion. Furthermore, protein level of hMYH was decresed after 48-hour reperfusion period. β-actin was used as internal standard. Each lane is from separate animal. Results are representative of five individual experiments.

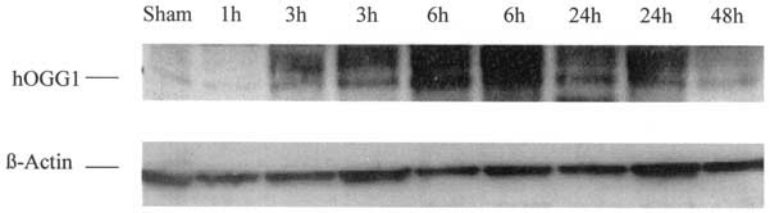

Fig 4. Representative Western blot analysis for hOGG1 in sham-control spinal cord and ischemic spinal tissue. hOGG1 bind was only weakly detected in sham controls. hOGG1 expression was increased at 3 hours after reperfusion and remained elevated as late as 24 hours. Peak level of hOGG1 was found at 6 hours after blood flow restoration. Protein level of hOGG1 became less dense after 48-hour reperfusion period. β-actin was used as internal standard. Each lane is from separate animal. Results are representative of five individual experiments.

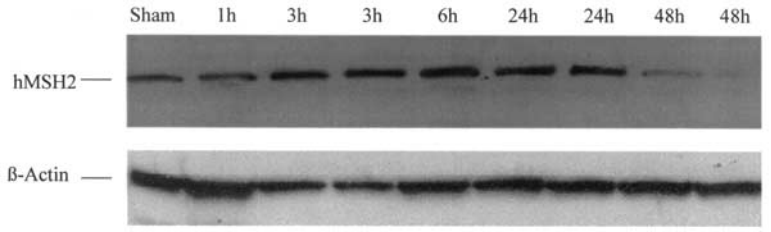

Fig 5. Representative Western blot analysis for hMSH2 in sham-control tissue and ischemic spinal cord. In sham-control spinal cord, immunoreactivity of hMSH2 was only weakly detected. At 3 hours after reperfusion, protein level of hMSH2 was upregulated and remained elevated until 24 hours after reperfusion. Peak level of hMSH2 was found at 6 hours after reperfusion. Furthermore, protein level of hMSH2 was markedly decreased after 48 hours of reperfusion. β-actin was used as internal standard. Each lane is from separate animal. Results are representative of five individual experiments.

PBS, 10% fetal bovine serum was used for 1 hour at room temperature to block nonspecific staining sites. The slides were incubated with 3% hydrogen peroxide solution for 30 minutes at room temperature to block endogenous peroxidase. The slides were incubated with primary anti-8-oxoG monoclonal antibody (Trevigen, Inc, Gaithersburg, Md; diluted 1:50 in 10 mmol/L Tris-HCl, pH 7.5, 10% serum) overnight at 4° C. The slides were rinsed twice with PBS and incubated with secondary antimouse antibody (1:100) conjugated with 20 μg/mL streptavidin-horseradish peroxidase in 1× PBS for 1 hour at room temperature. The slices were stained with diaminobenzamide tetrahydrochloride, and cytoplasm was counterstained with hematoxylin. The percentage of positive staining of 8-oxoG was quantified as following: positive cell/total cell × 100%.

Western blot analysis. Tissue samples (1-g tissue for each sample) were homogenized in a lysis buffer (0.1 mol/L NaCl; 0.01 mol/L Tris-HCl, pH 7.5; 1 mmol/L ethylenediamine tetraacetic acid; and 1 μg/mL aprotinin), and then the homogenates were centrifuged at 7000*g* for 15 minutes at 4° C. Supernatants were used as protein samples. Assays to determine the protein concentration of

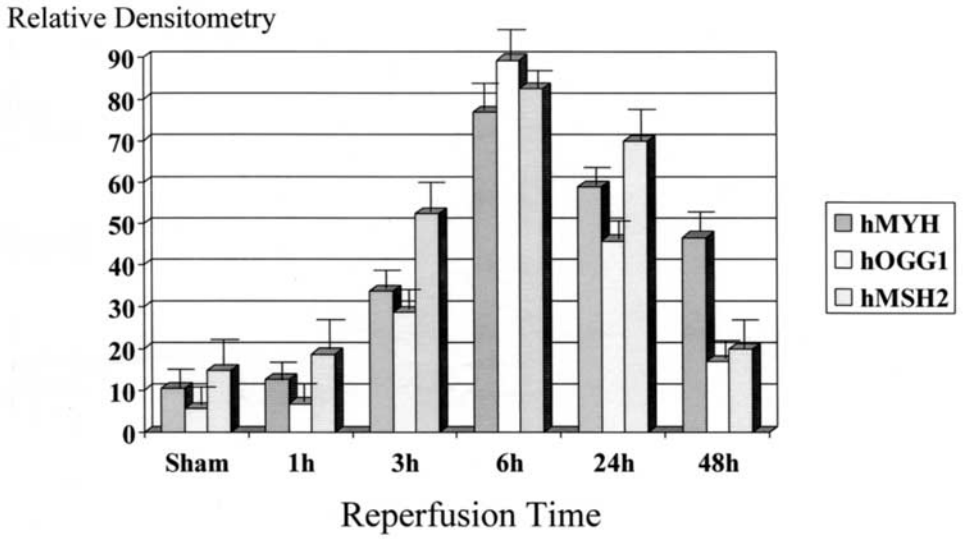

Fig 6. Densitometry comparing hMYH, hOGG1, and hMSH2 expression in tissues taken from sham-control and ischemia spinal cord. Protein levels of hMYH, hOGG1, and hMSH2 were markedly ($P < .05$) increased from 3 hours to 24 hours after reperfusion in spinal cord compared with sham-control tissue. Peak levels of hMYH, hOGG1, and hMSH2 were found at 6 hours after reperfusion period. At 48 hours after reperfusion, levels of those DNA repair enzymes were significantly ($P < .05$) decreased compared with 6 hours after reperfusion period (n = 5 in each time point; mean ± standard error).

the supernatants were subsequently performed with comparison with a known concentration of bovine serum albumin with use of a Bio-Rad protein assay reagent kit (Bio-Rad Laboratories, Hercules, Calif). SDS-PAGE was performed in a 10% polyacrylamide gel with nonreducing conditions. In brief, protein samples were boiled at 100° C in 2.5% SDS and 5% β-mercaptoethanol, and lysates equivalent to 20 μg of protein from each sample were run on the gel for 90 minutes at 20 mA together with a size marker (BenchMark Prestained Protein Ladder, Invitrogen, Carlsbad, Calif). The electophoresis running buffer contained 25 mmol/L Tris base, 250 mmol/L glycine, and 0.1% SDS. Protein on the gel was transferred to a nitrocellulose membrane with a transfer buffer that consisted of 48 mmol/L Tris base, 39 mmol/L glycine, 0.4% SDS, and 20% methanol. After transfer, membrane was placed in 1% powdered milk in PBS to block nonspecific binding. After reacting with the primary and secondary antibodies, the membrane was subjected to the Enhanced Chemiluminescence analysis system from Amersham. Polyclonal hMYH antibody was provided by Dr A. L. Lu. Polyclonal antibodies against hOGG1 and hMSH2 were obtained from Novus Biological (Littleton, Colo). Monoclonal caspase-3 antibody was obtained from BD Transduction Laboratories (San Diago, Calif). Monoclonal antibody against actin (Ab-6, Oncogene Research Products, San Diego, Calif) was used as a control for equal protein loading. Molecular weight for each protein was determined as following: hMYH (62 kDa), hOGG1 (39 kDa), hMSH2 (100 kDa), and caspase-3 (32 kDa). No cross-reacting bounds were seen among hMYH, hOGG1, hMSH2, and caspase-3. To ascertain specific binding of the antibody for the protein, another membrane was stained in a similar way without the primary antibody.

Immunohistochemical staining. To determine the expression and localization of hMYH, hOGG1, and hMSH2, immunohistochemical staining was performed. In brief, tissues were immediately fixed in 10% buffered formalin. After fixation, the tissue was dehydrated and embedded in paraffin. Serial sections were cut at a thickness of 5 μm and mounted on glass slides treated with silica. The slides were incubated at 60° C and deparaffinized with graded concentrations of xylene and ethanol. After samples were deparaffinized, spinal cord sections were rinsed in 0.1 mol/L PBS for 20 minutes and blocked in 10% normal horse serum for 2 hours at room temperature. The sections were incubated with primary antibodies in 10% normal horse serum and 0.3% Triton-X 100 (Sigma, St Louis, Mo) for 20 hours at 4° C. The primary antibodies used were the same as those used for the Western blot analysis described previously, and each dilution was at 1:100 for polyclonal antibodies against hMYH, hOGG1, and hMSH2, respectively. After endogenous peroxidase activity was quenched with exposure of the slides to 0.3% H_2O_2 and 10% methanol for 20 minutes, the slides were washed in PBS and incubated with second antirabbit antibody-horseradish peroxidase conjugate (Santa Cruz Biotechnology, Santa Cruz, Calif) at a dilution of 1:1000. The final reaction was achieved with incubating the sections with freshly prepared reagent containing 3-amino-9-ethylcarbazole (Sigma) dissolved in dimethyl-formamide and sodium acetate. The sections were counterstained with hematoxylin, mounted,

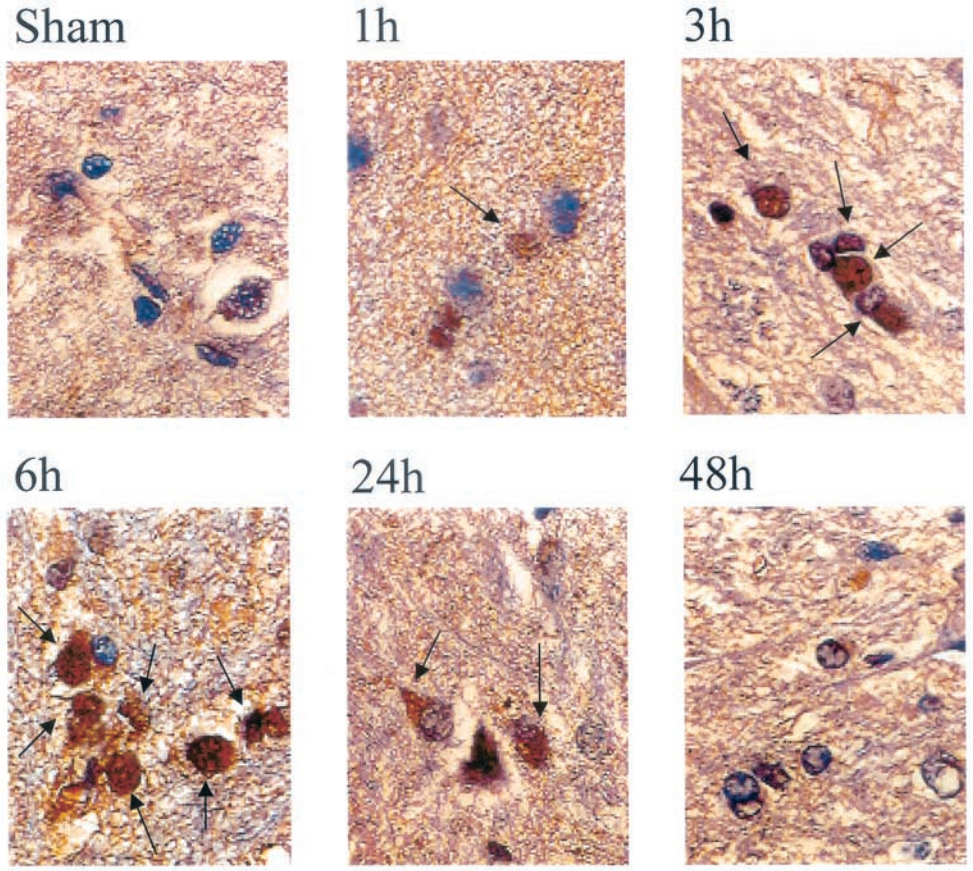

Fig 7. Representative immunohistochemical staining against hMYH in sham spinal cord and at different time points of reperfusion after 30 minutes of ischemia. Positive staining of hMYH was detected in nuclei of ischemic spinal neurons within intermediate and ventral horn area of gray matter at 3 hours to 6 hours after reperfusion. *Arrows* show positive neuron nuclei that express immunoreactive of hMYH. Peak staining density of hMYH-positive neurons was found at 6 hours after reperfusion. At 48 hours after blood flow restoration, positive neurons for hMYH were markedly decreased. Sections without primary antibody revealed no positive staining (data not shown). Results are representative of five individual experiments (×1000).

and reviewed with a Nikon microscope (Image Systems, Inc, Col). Two trained independent observers reviewed these sections (10 sections per slide were analyzed). Sample identities were concealed during investigation, and at least five samples were reviewed per group. The specificity of positive staining was further confirmed with substitution of normal rabbit serum instead of primary antiserum.

In situ detection of DNA fragmentation (TUNEL staining). To detect the DNA fragmentation in situ, nick-end labeling was performed according to our previously reported method with an ApopTag in situ apoptosis detection kit (Oncor, Gaithersburg, MD). Briefly, the nuclei of the tissue sections were stripped of proteins with incubation with 20 μg/mL proteinase K for 10 minutes after deparaffinizing. After being treated with 0.3% hydrogen peroxide (H_2O_2) in distilled water for 5 minutes, the sections were incubated with terminal deoxynucleotidyl transferase buffer (Boehringer Mannheim, Indianapolis, Ind; 30 mmol/L Tris, pH 7.2, 140 mmol/L sodium cacodylate, and 1 mmol/L cobalt chloride) containing terminal deoxynucleotidyl transferase enzyme (0.5 U/mL; Boehringer Mannheim) and biotin-16-dUTP (0.04 mmol/L; Boehringer Mannheim) containing 30 mmol/L cobalt chloride in a humidified chamber at 37° C for 120 minutes. The reaction was terminated with incubating with 300 mmol/L NaCl and 30 mmol/L sodium citrate for 15 minutes at 25° C. After washing with 50 mmol/L Tris-HCl, pH 7.7, sections were stained with diaminobenzadine/H_2O_2 solution. Counterstaining was performed with hematoxylin. After three washes in Tris-HCl, pH 7.7, the sections were dehydrated in ascending ethanol series. After immersion in xylene, the sections were coverslipped with Cytoseal-60 (Richard-Allan Scientific, Kalamazoo, Mich). The positive or negative motor neurons in the TUNEL staining were counted as positive nuclear/total 100 nuclear × 100%. The correlation of apoptosis and 8-oxoG level and hMYH, hOGG1, and hMSH2 expression was determined.

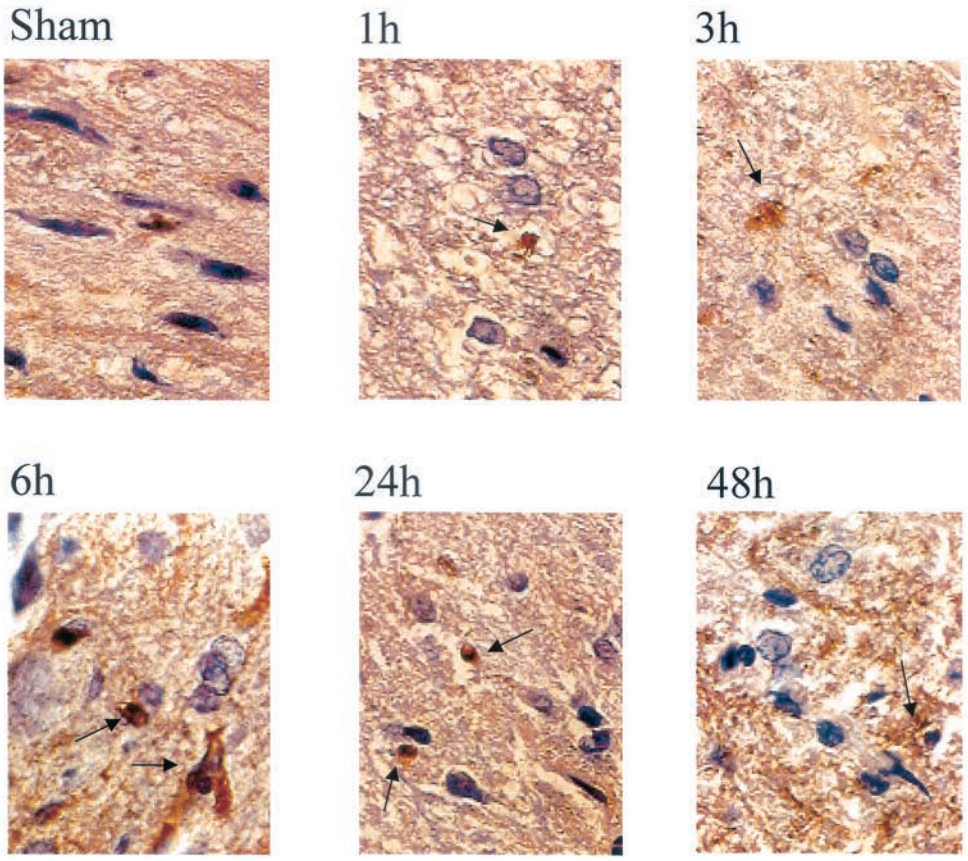

Fig 8. Representative immunohistochemical staining of hOGG1 in sham-control and ischemic spinal cord. hOGG1 expression was detected in ischemic spinal neurons within intermediate and ventral horn area of gray matter at 3 hours to 6 hours after reperfusion *(arrows)*. At 6 hours after reperfusion period, peak staining density of hOGG1 was founded. hOGG1-positive neurons were decreased at 48 hours after reperfusion. Sections without primary antibody revealed no positive staining (data not shown). Results are representative of five individual experiments (×1000).

Statistical analysis. Results of the quantitative studies were expressed as mean ± standard error. Statistical comparisons within each group were performed with analysis of variance for repeated measures following by Fisher least-significant difference test of repeated measures when appropriate, and comparisons between groups were performed with factorial analysis of variance followed by Fisher least-significant difference test of repeated measures. Statistical significance was accepted at a P value of less than .05.

RESULTS

DNA damage in spinal cord ischemia and reperfusion. Photographs of 8-oxoG in spinal cords are shown in Fig 2. No positive staining of 8-oxoG was seen in spinal cord neurons of gray matter in sham-operated tissue. At 1 and 6 hours after reperfusion, 8-oxoG level was markedly increased in ischemic spinal cord. 8-oxoG *(arrows)* was detected predominantly within the intermediate gray matter and the ventral horn. After 48 hours of reperfusion, 8-oxoG expression was decreased (Table).

Protein levels of DNA repair enzymes in spinal cord ischemia and reperfusion. Figs 3 to 6 show the Western blot analysis for hMYH (Fig 3), hOGG1 (Fig 4) and hMSH2 (Fig 5) in sham-control spinal cord and ischemic spinal cord during different reperfusion time points. The levels of DNA repair enzymes, such as hMYH (molecular weight, 62 kDa), hOGG1 (molecular weight, 39 kDa) and hMSH2 (molecular weight, 100 kDa), were only weakly detected in sham-control spinal cords. The levels of hMYH, hOGG1, and hMSH2 were increased as early as 3 hours after reperfusion and remained elevated as late as 24 hours (Fig 6). The peak levels of those DNA repair enzymes were found at 6 hours after reperfusion ($P < .05$, versus sham controls). Furthermore, the levels of hMYH, hOGG1, and hMSH2 were markedly decreased after the 48-hour reperfusion period ($P < .05$, versus the 6-hour period). The membrane without the primary antibody revealed no band (data not shown).

Expression and localization of DNA repair enzymes in spinal cord ischemia and reperfusion. Immunohistochemical staining indicated that positive staining of

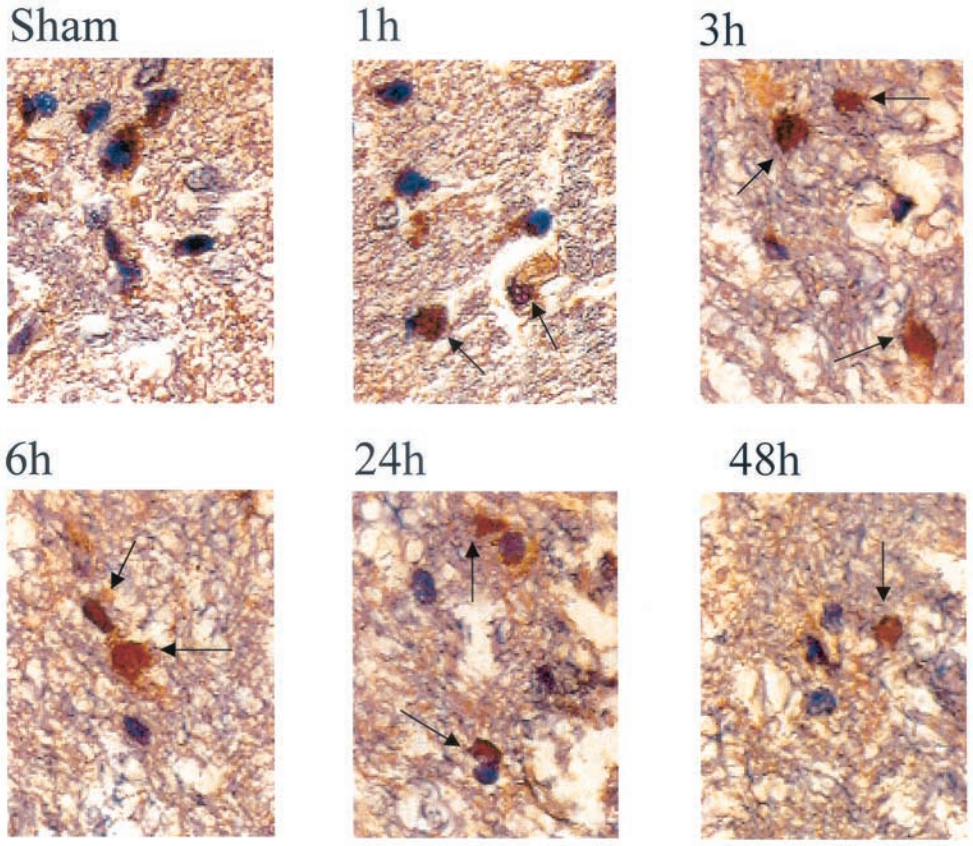

Fig 9. Representative immunostaining of hMSH2 was shown in sham-control spinal cord and at different time points of reperfusion. Protein expression of hMSH2 was detected in ischemic spinal neurons within intermediate and ventral horn of gray matter from 3 hours to 6 hours after reperfusion *(arrows)*. Peak staining level of hMSH2 was found at 6 hours after reperfusion. After 48 hours of reperfusion, level of positive neurons for hMSH2 was markedly decreased. Sections without primary antibody revealed no positive staining (data not shown). Results are representative of five individual experiments (×1000).

hMYH (Fig 7), hOGG1 (Fig 8), and hMSH2 (Fig 9) was found in spinal cord neurons of the ventral horn *(arrows)*. The proportion of positively stained neurons and the staining density of hMYH, hOGG1, and hMSH2 were both increased 3 hours to 6 hours after reperfusion (Table). At 6 hours after the reperfusion period, the peak levels of hMYH, hOGG1, and hMSH2 staining density were found in spinal cord neuron. After 48 hours of reperfusion, the staining density of hMYH, hOGG1, and hMSH2 was markedly decreased in the spinal cord. The sections without the primary antibody revealed no positive staining (data not shown).

Apoptosis and apoptosis-related gene expression in spinal cord ischemia. Representative photographs of TUNEL in spinal cords are shown in Fig 10. No positive staining neurons for TUNEL were seen in sham-control spinal cord tissue. After 3 hours of reperfusion, positive staining of TUNEL was detected in spinal cord neuron cells in the medial aspects of the ventral gray matter and the intermediate zone. The peak level of TUNEL-positive neurons in the ischemic spinal cord was found after the 48-hour reperfusion period (Table).

Fig 11 depicts levels of caspase-3 determined with Western blot analysis. Only weak expression was detected in sham controls. In contrast, the caspase-3 level was markedly increased at 3 hours and peaked at 6 hours after blood flow restoration. The band became scarcely detectable 48 hours after reperfusion. All animals awakened paralyzed.

The relationship between 8-oxoG generation and TUNEL level in the spinal cord after 24 hours of ischemia-reperfusion injury is shown in Fig 12 , *A*. Positive correlation was seen between TUNEL and 8-oxoG generation. Furthermore, Fig 12 , *B*, shows the relationship of MYH level and TUNEL staining in spinal cord. After 6 hours of ischemia-reperfusion injury, MYH level was increased and TUNEL level was decreased. In contrast, after 48 hours of ischemia-reperfusion injury, the MYH level was decreased and the TUNEL level was markedly elevated.

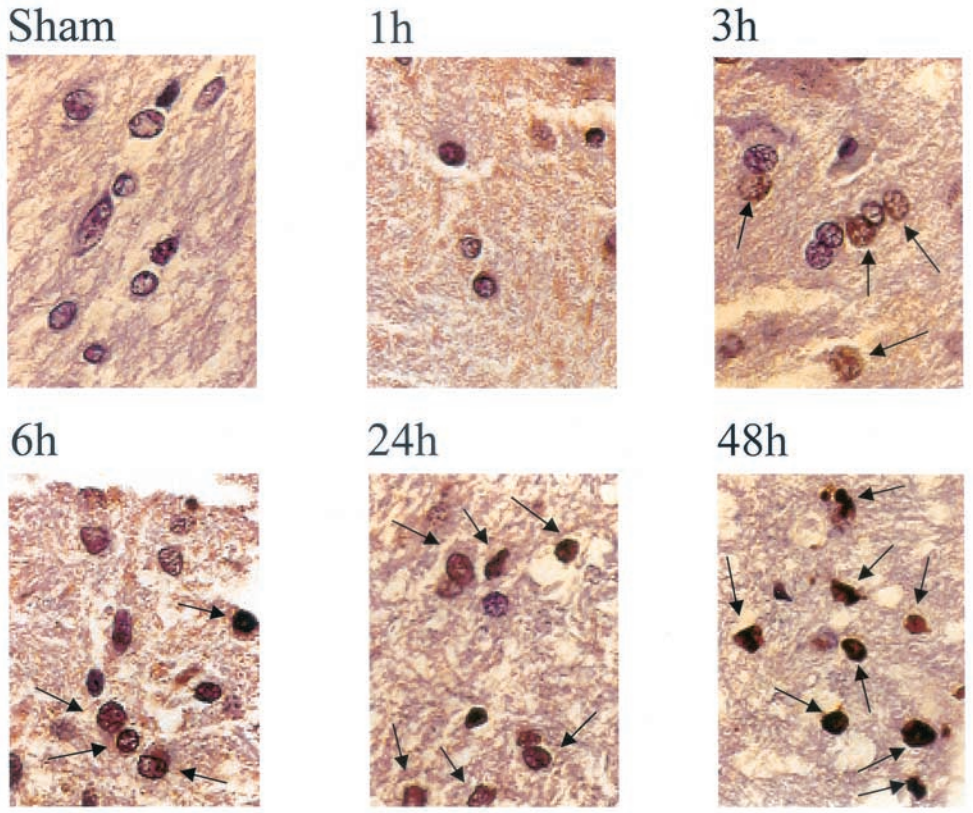

Fig 10. Representative photographs of spinal cord neurons stained with TUNEL study. No positive neurons for TUNEL staining are seen in sham-control spinal cord tissue. TUNEL-positive cells appeared in intermediate zone and medial aspects of ventral gray matter between 3 hours and 48 hours *(arrows)*. Peak level of TUNEL-positive cells in ischemic spinal cord was found at 48 hours after reperfusion (×1000).

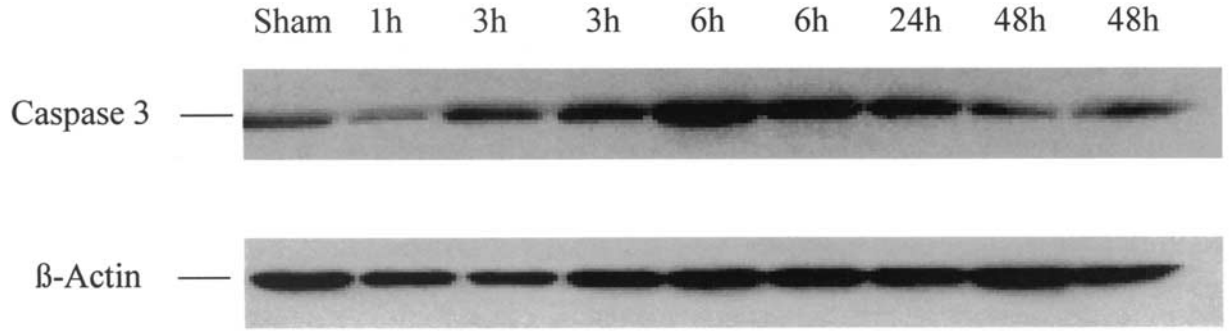

Fig 11. Western blot analysis of caspase-3 in sham-control and ischemic spinal cord. Only weak bind of caspase-3 was detectable in sham control. In contrast, protein expression of caspase-3 was increased from 3 hours to 24 hours after reperfusion. Peak protein level of caspase-3 was found at 6 hours after blood flow restoration. Band became scarcely detectable at 48 hours after reperfusion.

DISCUSSION

This study showed that DNA damage as assayed with 8-oxoG level was significantly increased in the ventral horn neurons of the gray matter after spinal cord ischemia and reperfusion. Furthermore, DNA repair enzymes, such as hMYH, hOGG1, and hMSH2, were markedly increased after DNA damage in spinal cord ischemia. These results suggest that the DNA damage and repair systems are components of spinal cord ischemia/reperfusion injury.

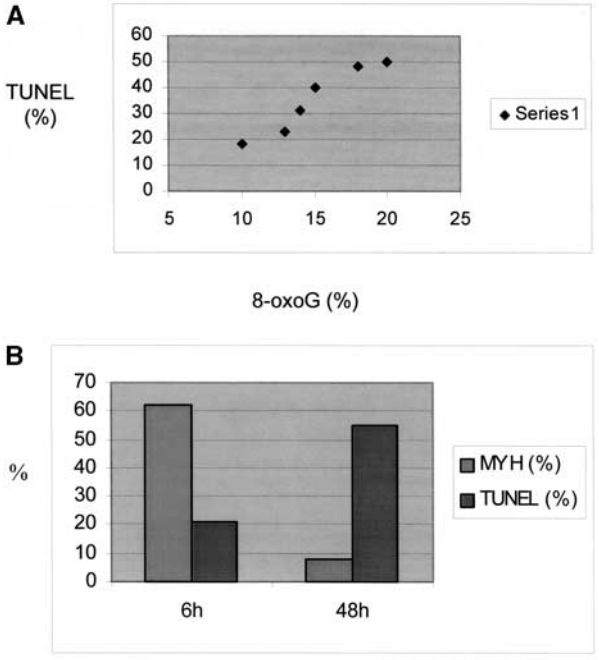

Fig 12. A, Relationship between 8-oxoG generation and TUNEL level in spinal cord after 24 hours ischemia-reperfusion injury. Positive correlation is seen between TUNEL and 8-oxoG generation. **B,** Relationship of MYH level and TUENL staining in spinal cord. After 6 hours of ischemia-reperfusion injury, MYH level was increased and TUNEL level was decreased. In contrast, after 48 hours of ischemia-reperfusion injury, MYH level was decreased and TUNEL level was markedly elevated.

Ischemia-reperfusion injury may be initiated by a host of factors.[38,39] Interactions between DNA and the hydroxyl radical produce DNA strand breaks and base modifications, which are frequently assessed with measurement of the nucleoside 8-oxoG.[40] A recent study showed that the frequency of mutation in a reporter gene increased in cortical DNA after forebrain ischemia-reperfusion.[41] Among the different oxidative-damage DNA products, 8-oxoG is the most stable and deleterious adduct.[22,23] If the 8-oxoG lesions in the DNA are not properly repaired, a high percentage of guanine/cytosine to thymine/adenine transversion will occur through the 8-oxoG/adenine replication intermediate.[24,25] To date, no information exists regarding 8-oxoG generation in spinal cord during ischemia-reperfusion injury. Furthermore, no study has investigated the relationship of DNA damage and its repair system with apoptosis during spinal cord ischemia-reperfusion injury. In this study, we showed, for the first time, that 8-oxoG level was significantly increased during spinal cord ischemia and reperfusion injury. The peak level of 8-oxoG generation was 6 hours after reperfusion. These data suggest that DNA damage such 8-oxoG generation is one of the important mechanisms during ischemia-reperfusion injury in spinal cord.

Caspase-3 has been shown to be increased in cardiac myocytes of failing hearts and is likely to be associated with apoptosis by proteolytic destruction of nuclear and contractile proteins. We found that expression of this enzyme also occurred in the spinal cord in abnormally high concentrations after the temporal sequence of DNA injury and attempted repair.[42]

The 8-oxoG repair system in *Escherichia coli*, which is composed of three enzymes, MutM, MutT, and MutY, is responsible for preventing the mutagenic effect of the 8-oxoG lesions.[43-45] In eukaryotes, the repair mechanisms analogous to *E coli* MutT, MutM, and MutY dependent pathways (MHT1, OGG1, and MYH, respectively) have been identified. After hMYH, hOGG1, and hMSH2 activation in the spinal cord, the level of 8-oxoG was significantly decreased. These data suggest that hMYH, hOGG1 and hMSH2 genes are important in the repair of DNA damage, such as 8-oxoG production. On the other hand, this level of ischemic injury outstrips repair mechanisms as all animals had paraplegia develop. Repair enzymes were only increased 6 to 24 hours after ischemia-reperfusion injury. After 24 hours, the levels of these enzymes fell and the apoptosis peaked. These data coupled with the high activity of caspase-3 show the complexity of molecular mechanisms after spinal cord ischemia-reperfusion injury. Therefore, we speculate that the future therapeutic strategies should focus on the mediators of the initial injury rather than attempts to bolster repair systems.

The specific irreversible DNA damage shown as internucleosomal DNA fragmentation, TUNEL reaction, is an active cell-death process that necessitates protein synthesis.[46] We showed with TUNEL reaction that apoptosis was elevated after 24 to 48 hours of reperfusion. These data confirmed the previous studies that indicated delayed onset of apoptosis after spinal cord ischemia and reperfusion. Importantly, we found that apoptotic levels were increased after the levels of the DNA repair enzymes (including hMYH, hOGG1, and hMSH2) decreased. These data suggest that the activation of DNA repair enzymes may not be enough to repair lesions caused by this degree of ischemia. We are unable to state that individual cells expressing 8-oxoG are those destined for nuclear fragmentation because the 8-oxoG is nearly absent at the time of greatest reactivity to TUNEL.

This study, while clearly showing DNA injury and attempted repair, may also provide potential assays predicting neurologic dysfunction. For example, as experiments are conducted to attenuate ischemic injury, it may follow that measurement of levels of 8-oxoG or caspase-3 prove indicative of success or failure.

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