Neuroprotective effects of sildenafil in experimental spinal cord injury in rabbits

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Abstract

Neuroprotective agents such as methylprednisolone and sildenafil may limit damage after spinal cord injury. We evaluated the effects of methylprednisolone and sildenafil on biochemical and histologic changes after spinal cord injury in a rabbit model. Female New Zealand rabbits (32 rabbits) were allocated to 4 equal groups: laminectomy only (sham control) or laminectomy and spinal trauma with no other treatment (trauma control) or treatment with either methylprednisolone or sildenafil. Gelsolin and caspase-3 levels in cerebrospinal fluid and plasma were determined, and spinal cord histology was evaluated at 24 hours after trauma. There were no differences in mean cerebrospinal fluid or plasma levels of caspase-3 between the groups or within the groups from 0 to 24 hours after injury. From 0 to 24 hours after trauma, mean cerebrospinal fluid gelsolin levels significantly increased in the sildenafil group and decreased in the sham control and the trauma control groups. Mean plasma gelsolin level was significantly higher at 8 and 24 hours after trauma in the sildenafil than other groups. Histologic examination indicated that general structural integrity was better in the methylprednisolone in comparison with the trauma control group. General structural integrity, leptomeninges, white and grey matter hematomas, and necrosis were significantly improved in the sildenafil compared with the trauma control group. Caspase-3 levels in the cerebrospinal fluid and blood were not increased but gelsolin levels were decreased after spinal cord injury in trauma control rabbits. Sildenafil caused an increase in gelsolin levels and may be more effective than methylprednisolone at decreasing secondary damage to the spinal cord.

INTRODUCTION

Traumatic spinal cord injury (SCI) negatively affects the quality of life of patients and their families and mostly affects younger adults. These injuries severely disturb motor, sensory, and autonomic functions. In most cases, complete neurologic recovery cannot be achieved in patients who experience SCI, even though advanced surgical techniques are available.

The biochemical changes and pathophysiology after SCI have been investigated in order to develop treatments that may minimize function loss. The biphasic injury process in SCI includes a primary phase with localized acute bleeding and ischemia and a secondary phase with edema, electrolyte alterations, occurrence of oxygen free radicals, inflammation, lipid peroxidation, and apoptosis [1-4].

As in other central nervous system diseases, apoptosis is the most common type of cell death, mediated by caspase, and highly related to the pathologic loss of neurons, astrocytes, and oligodendrocytes [5]. Caspase levels are increased in SCI because cell death in the spinal cord increases posttranslational activation of caspases [6]. Caspase-3 levels are increased in tissue samples of rats that have experimental SCI [7].

Gelsolin, an actin-binding plasma protein, is an important mediator of apoptosis. Gelsolin might cause changes in cell morphology and motility. When actin is released from injured cells, circulating gelsolin begins to clear the actin. Normal plasma gelsolin levels are 200 to 300 mg/L. During apoptosis, gelsolin is cleaved by caspase-3, and gelsolin levels decrease. The N-terminal gelsolin fragment (39 kDa) increases apoptosis [8]. Plasma gelsolin levels may decrease below normal

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because of tissue damage from lung injury, acute liver injury, pancreatitis, trauma, burns, and sepsis [9-12].

Neuroprotective drugs have been evaluated to minimize injury during the second phase of SCI, but none have been effective in clinical studies [13,14]. Methylprednisolone is used commonly in SCI but has serious adverse events. The phosphodiesterase type 5 (PDE5) inhibitor sildenafil may have beneficial effects on stroke, subarachnoid bleeding, dementia, learning disorders, and neurodegenerative disorders [15,16].

The purpose of the present study was to evaluate caspase-3 and gelsolin levels in cerebrospinal fluid (CSF) and plasma associated with secondary damage after SCI, and to evaluate the effects of methylprednisolone and sildenafil on caspase-3 and gelsolin levels as markers of neuroprotection after SCI. We hypothesized that sildenafil may have neuroprotective effects on the spinal cord after SCI and these effects might be monitored by following CSF or plasma caspase-3 or gelsolin levels.

MATERIALS AND METHODS

Study design

The study protocol was approved by the Ethics Committee of the Necmettin Erbakan University Experimental Medicine Research and Application Center. National Institutes of Health Guide for Care and Use of Laboratory Animals was followed in all the experiments. We included 32 female adult New Zealand rabbits (weight, 2.5 to 3 kg) that were split into 4 equal groups (sham control, trauma control, methylprednisolone, and sildenafil; 8 rabbits each). Sham control rabbits received laminectomy only, and all other rabbits had laminectomy and spinal trauma. After laminectomy and spinal trauma, trauma control rabbits had no other treatment, the methylprednisolone group received methylprednisolone, and the sildenafil group received sildenafil.

Surgery and spinal trauma

Anesthesia was induced with xylazine hydrochloride (2.5 mg/kg intramuscular) and ketamine (5 mg/kg intramuscular). The rabbits were positioned prone on the operating table and the area at T8-10 was cleaned. A midline incision was made and dermal and subcutaneous layers were developed. The fascia was opened and paravertebral muscles were removed subperiosteally. A T10 laminectomy was performed. Experimental spinal cord trauma (trauma control, methylprednisolone, and sildenafil groups) was created with the Allen heavy object falling method with an iron ball (10 g) falling through a long plastic tube (length, 30 cm; diameter, 1 cm). The rabbits in the methylprednisolone group were given methylprednisolone (30 mg/kg intravenous) and the

rabbits in the sildenafil group were given sildenafil (5 mg/kg via nasogastric tube).

Biochemistry

Venous blood (2 mL) was collected at 0, 8, and 24 hours after trauma, and CSF (2 mL) was collected at 0 and 24 hours after trauma and stored in tubes with ethylenediaminetetraacetic acid (EDTA). Samples were centrifuged (3000 rpm for 5 min). The supernatant was transferred to tubes and stored at -80°C until the assays were performed. Samples were thawed and vortexed before the assay. Gelsolin and caspase-3 levels were measured using a commercial kit (Eastbiopharm, Hangzhou, China) and microplate reader (RT-2100C Microplate Reader, Rayto, Nanshan, Shenzhen, China) with enzyme-linked immunosorbent assay in the Department of Biochemistry laboratories.

Histology

At 24 hours after spinal injury was induced, the same anesthetic agents were given and the animals were decapitated. The spinal cords were removed by transection 0.5 cm distal and 0.5 cm proximal to the observed contusion. *Medulla spinalis* samples were fixed in 10% formaldehyde and processed for histology. Paraffin blocks were sectioned (thickness, 4 to 6 μm) including the area of lesions with a microtome (Leica RM 2125RT, Leica Microsystems, Wetzlar, Germany). Sections were stained with hematoxylin-eosin and toluidine blue. All samples were examined with a trinocular microscope (Olympus BX51, Olympus, Tokyo, Japan). Sections in which lesions were clearly seen were photographed with a digital camera (Olympus DP71 camera, Olympus). The general structural integrity, meninges, structural integrity and hematoma of the white and grey matter, *dura mater* bleeding, inflammatory cells, increased vascularization, necrotic areas, and apoptosis were evaluated by 2 independent blinded histologists and graded (0, normal; 1, minimal lesion; 2, moderate lesion; 3, severe lesion; 4, very severe lesion).

Statistical analyses

Model-fitting procedures were performed to account for variation between subjects (different treatment groups) and within subjects (repeated measures of plasma at 0, 8, and 24 hours and CSF at 0 and 24 hours). Repeated measure analysis was performed with statistical software (R, version 3.0.2, R Foundation for Statistical Computing, Vienna, Austria) [17]. Ordinal logistic regression was employed to evaluate the effects of treatment on physical condition variables (obtained by scoring), removing the sham control group from the data set and setting the trauma control group as the reference group. We estimated odds ratios and 95% confidence

intervals for each variable. Goodness of fit was tested using the Pearson χ^2 test (chi-square test). Logistic regression was performed with software (Minitab, version 14, Minitab Inc. 2003) [18]. Statistical significance was defined by $p \leq 0.05$.

RESULTS

In the 32 rabbits, 4 rabbits died for unknown reasons (sham control, 2 rabbits; methylprednisolone, 1 rabbit; sildenafil, 1 rabbit) and were not included in the analysis (Table 1).

Caspase-3 level in cerebrospinal fluid

There were no differences in mean CSF caspase-3 level between 0 and 24 hours in all groups, and mean CSF caspase-3 levels were similar at 0 and 24 hours between all groups (Table 1, Figure 1).

Gelsolin level in cerebrospinal fluid

There were no differences in mean CSF gelsolin level between 0 and 24 hours in all groups. However, there was a significant time-group interaction (p =0.02); from 0 to 24 hours, mean CSF gelsolin increased in the sildenafil group and decreased in the sham control and trauma control groups (Table 1, Figure 1). This was confirmed statistically by including the sham control, trauma control, and methylprednisolone groups and repeating the interaction model; in these analyses, there was no interaction. Therefore, we concluded that the increase in mean CSF gelsolin level with time in the sildenafil group was statistically significant (Table 1, Figure 1).

Caspase-3 level in plasma

There were no differences in mean plasma caspase-3 levels at 0, 8, and 24 hours in each group, and there were no significant changes in plasma caspase-3 with time (Table 1, Figure 1).

Gelsolin level in plasma

The sildenafil group had higher mean plasma gelsolin levels at 8 and 24 hours than other groups (time-group interaction) ($p \leq 0.001$) (Table 1, Figure 1). This was confirmed statistically

TABLE 1. Effects of methyprednisolone and sildenafil on plasma and cerebrospinal fluid levels of gelsolin and caspase-3 after spinal cord injury in rabbits.*

Study	Sham control	Trauma control	Methylprednisolone	Sildenafil
No. rabbits	6	8	7	7
CSF gelsolin (ng/mL)				
0 h	615±173	586±255	622±323	606±169
24 h	464±202	491±225	724±196	818±111
Plasma gelsolin (ng/mL)				
0 h	260±73	315±63	201±101	181±84
8 h	379±158	314±85	201±101	467±44
24 h	210±115	211±118	253±113	478±108
CSF caspase-3 (ng/mL)				
0 h	6±2	8±2	8±1	8±2
24 h	6±2	9±2	8±2	8±1
Plasma caspase-3 (ng/mL)				
0 h	3±1	4±2	4.0±0.9	3.9±0.6
8 h	3.5±0.6	3.6±0.9	4.0±0.9	4±1
24 h	2±1	4±1	4±2	3.5±0.4

*Data reported as mean±SD. Abbreviations: CSF, cerebrospinal fluid..
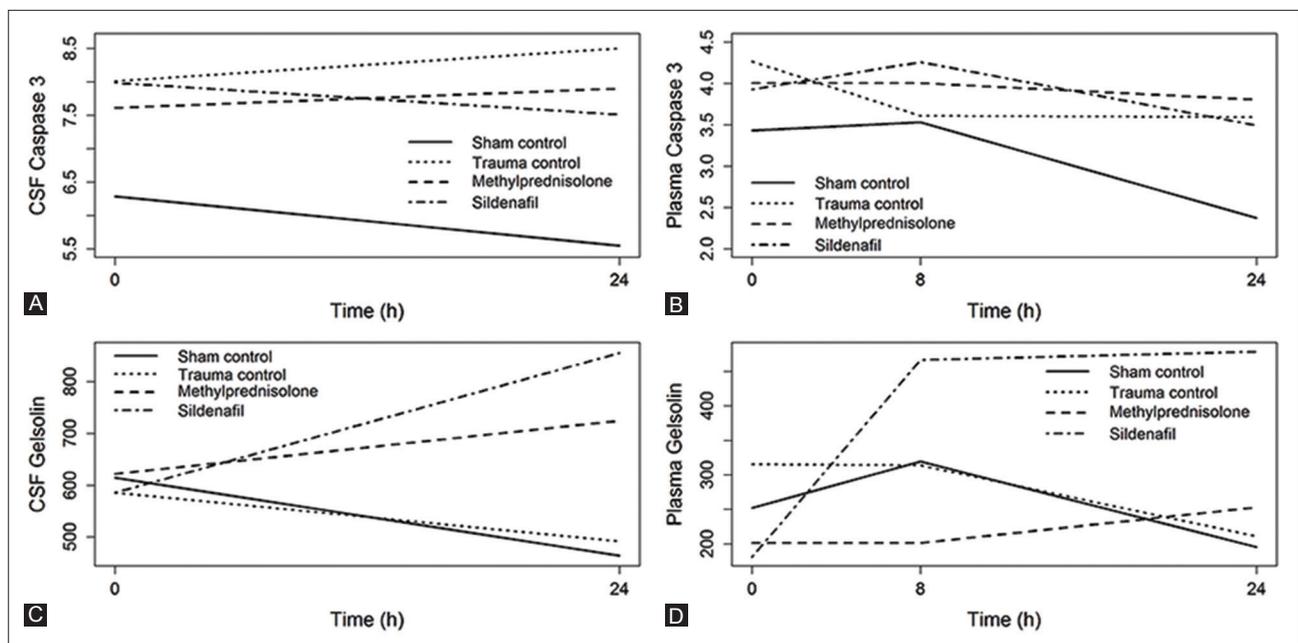
FIGURE 1. Cerebrospinal Fluid (CSF) and Plasma Levels of Caspase-3 and Gelsolin After Spinal Cord Injury in Rabbits. (A) CSF caspase-3. (B) Plasma caspase-3. (C) CSF gelsolin. (D) Plasma gelsolin.

by excluding the sildenafil group and repeating the analysis; in this analysis, there were no significant differences between the 3 groups (sham control, trauma control, and methyprednisolone), there was no difference in the temporal change of plasma gelsolin level, and there was no time-group interaction.

Histopathology

In the sham control group, neuronal morphology was normal; the general structure and structural integrity were preserved, but some samples had mild structural disturbance and mild hematoma that were attributed to damage during laminectomy.

The most marked degenerative changes were observed in the trauma control group, including marked deterioration of general structure and wide areas of hematoma in white and grey matter. The number of neurons was decreased in the grey matter. Axonal edema and myelin loss were observed in the white matter. Meningeal structure was disrupted and bleeding in the *dura mater* was frequently observed (Figure 2).

In the treatment groups, structural deterioration was partial. Signs of trauma were decreased in both methyprednisolone and sildenafil groups compared with the trauma control group (Table 2, Figure 2). In the methylprednisolone group, only general structural integrity was improved compared with that in the trauma control group. In the sildenafil group, general structural integrity, meninges, white and grey matter hematomas, and necrosis were significantly improved compared with that in the trauma control group (Table 2). General structural deterioration and white and grey matter hematomas were greater in the trauma control group (Figure 2A), and these signs were observed less in the methylprednisolone and sildenafil groups (Figure 2B and 2C).

DISCUSSION

The present results revealed that CSF and plasma caspase-3 levels were unchanged after SCI in all rabbit groups, in contrast with previous studies that showed increases in tissue caspase-3 levels in rats with SCI [7]. In the trauma control group, gelsolin levels in CSF and plasma decreased after trauma, similar to previous findings (Table 1, Figure 1). Therefore, CSF and plasma gelsolin levels, but not caspase-3 levels, may be useful to monitor SCI in rabbits. Furthermore, sildenafil caused increase in CSF and plasma gelsolin levels and decrease in histologic signs of trauma after SCI, consistent with the hypothesis that sildenafil may have neuroprotective effects after SCI.

The brain and spinal cord are very sensitive to free radical injury because of the dense lipid content. Free radicals might
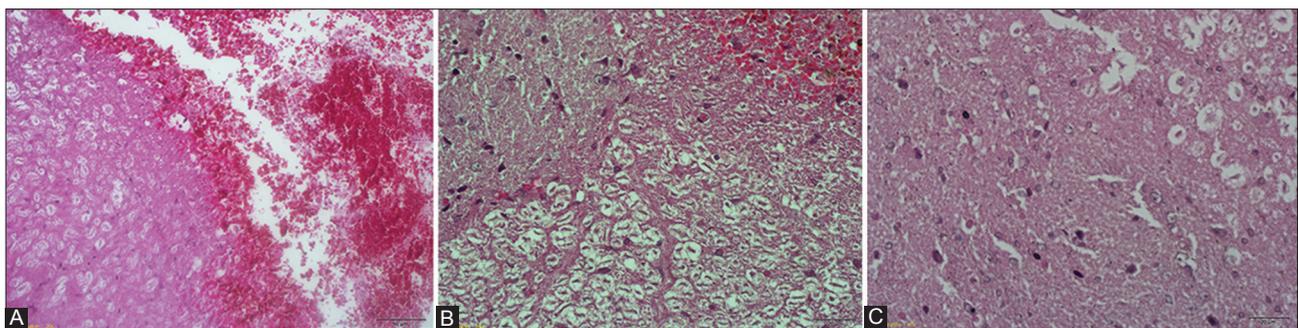
FIGURE 2. Histopathologic characteristics of the spinal cord. (A) Trauma control group; hematoma and damage to grey and white matter was noted (hematoxylin-eosin, original magnification ×20). (B) Methylprednisolone group; grey and white matter hematoma, pyknotic cells, myelin loss was noted (hematoxylin-eosin, original magnification ×40). (C) Sildenafil group; pyknotic cells, myelin loss was noted (hematoxylin-eosin, original magnification ×40).

TABLE 2. Effects of methyprednisolone and sildenafil on histopathologic features of spinal cord injury in rabbits.

Histopathologic feature	Trauma Control	Methylprednisolone	Sildenafil
Structural integrity			
General structural	3.5±0.8	2.7±0.5[a]	2.3±0.5[ab]
Meninges	3.1±0.6	2.4±0.8	2.0±0.6[ab]
White matter	2.6±0.5	2.0±0.8	2.0±0.6
Grey matter	3±1	2±1	2.1±0.9
Dura mater bleeding	2.0±0.8	1.1±0.7	1±1
Hematoma of white and grey matter	2.8±0.7	2.4±0.8	2±1a
Infiltration of inflammatory cells	1.0±0.0	1.4±0.5	1.1±0.7
Increased vascularization	1.5±0.5	2.4±0.8[a]	1.0±0.6[b]
Necrotic areas	2.1±0.8	2.0±0.8	1.0±0.6[ab]
Apoptosis and pyknosis	2.1±0.8	2.4±0.8	1.4±0.5[b]

[a] $p \leq 0.05$ compared with trauma control group, [b] $p \leq 0.05$ compared with methylprednisolone group.

damage proteins, lipids, and DNA. Microvascular damage and tissue hypoperfusion may cause secondary ischemic damage from lipid peroxidation after SCI [3]. In SCI, the primary phase is fast and irreversible, and the main treatment strategy is to stop or limit tissue damage during the secondary phase [19]. Clinical studies indicate that pharmacologic treatments may be effective in decreasing negative outcomes of SCI, and substances that have antioxidant and anti-inflammatory effects may be beneficial in treating clinical conditions associated with neuronal damage [3]. However, despite promising experimental studies, there is no treatment that improves secondary damage after SCI [20].

Steroids are first-line treatment for SCI, and methylprednisolone is an effective antioxidant and anti-inflammatory agent that might limit secondary damage and improve neurologic outcomes after SCI [13,21-24]. Steroids have antioxidant, anti-inflammatory, and membrane stabilizing effects; they decrease edema and vessel permeability; and the effects of steroids are time- and dose-dependent [25]. In high doses, methylprednisolone decreases posttraumatic lipid peroxidation and free radical production. Methylprednisolone also protects cell membrane proteins such as adenosine triphosphatase and neurofilaments that are responsible for the integrity of the cellular cytoskeleton. Therefore, methylprednisolone is an effective treatment for SCI when it is used within 8 hours after injury [20]. However, high-dose steroids have serious adverse events that might limit their use after SCI.

The neuroprotective strategy after SCI includes protection from the effects of secondary damage and preventing loss of tissue and functional capacity. Various neuroprotective agents are being evaluated including riluzole, minocycline, polyethylene glycol, anti-Fas antibodies, autologous activated macrophages (ProCord, Proneuron Biotechnologies, New York, NY), and PDE5 inhibitors [26,27]. The PDE5 inhibitors may improve reperfusion injury and increase the tolerance of the spinal cord against ischemia. In the present study, we used the short-acting PDE5 inhibitor sildenafil as a neuroprotective agent. Sildenafil may protect against damage from temporary spinal cord ischemia because PDE5 is present in the spinal cord. Sildenafil has a half-life of 4 hours, reaches maximum plasma concentration in 30 to 120 minutes, and helps maintain high levels of cyclic guanosine monophosphate which increases the effects of nitric oxide in causing vasodilation [28,29]. Therefore, sildenafil might cause facial flushing, hyperemic nasal congestion, nosebleed, conjunctivitis, and cardiovascular vasodilation [30,31]. Experimental studies showed that sildenafil improves the prognosis after ischemic stroke, increases angiogenesis and neurogenesis, and increases neurologic function [15].

In the present study, we compared the effects of sildenafil and methylprednisolone on secondary injury as indicated by

caspase-3 and gelsolin levels in plasma and CSF. Caspase-1 and caspase-3 are increased in neurons after SCI, and inhibition of caspase reduces the posttraumatic lesion size and improves motor performance [7,32]. However, we did not detect any significant differences in plasma or CSF caspase-3 levels between groups or at different times after SCI in the present rabbit model (Table 1, Figure 1). The differences between the previous and present findings about caspase-3 levels after SCI are unknown, but possible factors may include interspecies differences between rats and rabbits and differences in caspase-3 levels in tissue, CSF, or plasma after SCI [7]. Previous reports suggested that caspase inhibitors may be beneficial [7,33], but sildenafil was not effective in changing caspase-3 levels in our study.

Gelsolin binds to inflammatory substances and modulates the excessive host response to sepsis, burns, and trauma. The primary function of plasma gelsolin is to protect the body against the autoinflammatory response in people who have severe diseases [34]. Low plasma gelsolin levels worsen the prognosis of systemic inflammation [35,36]. Therefore, gelsolin is associated with the severity and outcomes of critical disease and is used as a prognostic marker in acute diseases. In the present study, we used plasma and CSF gelsolin levels to compare the effects of sildenafil and methylprednisolone treatments. Plasma and CSF gelsolin levels were not affected by methylprednisolone but were increased significantly in the sildenafil group (Table 1, Figure 1). Therefore, sildenafil might be neuroprotective and might increase plasma and CSF gelsolin levels after SCI.

A previous study of SCI showed that glial and neuronal apoptosis may occur in grey and white matter in the injured area by 24 hours after SCI [37]. In the present study, we detected apoptosis in the trauma control group during microscopic examination. There was no improvement in apoptosis in the methylprednisolone group, but apoptosis level was significantly low in the sildenafil group, possibly because of the anti-inflammatory and antiapoptotic effects of sildenafil (Table 2).

Limitations of the present study include the short period of time (24 hours) studied after SCI. Therefore, long-term plasma and CSF levels and histopathologic findings were not evaluated; in future studies, it would be useful to evaluate these parameters for longer periods of time after trauma. The number of rabbits was small, especially because 4 rabbits died, and further study is justified with a larger sample. We did not evaluate tissue levels of caspase-3 and gelsolin. In addition, the histopathologic examinations were limited because of limited facilities; further evaluation of tissue damage might include use of electron microscopy and immunohistochemical techniques. Although the histology was evaluated by 2 independent blinded histologists, interobserver variation was not

tested and could be assessed in future studies. In addition, it is unknown whether the dose of sildenafil was optimal, and future study could evaluate effects of different doses.

CONCLUSION

In summary, the present study revealed that CSF and plasma caspase-3 levels after SCI did not increase in this rabbit model, but gelsolin levels were decreased after SCI in trauma control rabbits. Sildenafil demonstrated better neuroprotective effect than methylprednisolone because sildenafil increased gelsolin levels and was associated with less histopathologic findings of tissue damage than methylprednisolone.

DECLARATION OF INTERESTS

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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