ORIGINAL ARTICLE

Lack of neuroprotection with pharmacological pretreatment in a paradigm for anticipated spinal cord lesions

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Background: In humans elective spine surgery can cause iatrogenic spinal cord injury (SCI). Efforts for neuroprotection have been directed to avoid mechanical injury by using intraoperative monitoring and improving surgical techniques. There is, however, uncertainty regarding the efficacy of neuroprotective drugs.

Study design: Experimental study on the effectiveness of pharmacological neuroprotection in an animal model of spine surgery simulating anticipated mechanically induced neurological damage.

Objective: To compare the efficacy of four drugs to protect against the neurological effects of iatrogenic SCI.

Setting: Research Unit for Neurological Diseases, IMSS-Proyecto Camina, Mexico City, Mexico.

Methods: Erythropoietin, melatonin, cyclosporine-A and methylprednisolone were administered to rats before, during and after controlled spinal cord contusion of mild intensity. Dosage was in accordance with their pharmacokinetic properties and experience gained with experimental SCI. Drug efficacy was assessed by motor function recovery over a period of 6 weeks and by spinal cord morphometry.

Results: Compared with animals treated with saline, the drug-treated groups showed no differences in their locomotor performance, nor in the amount of spared cord tissue. Notably, spontaneous activity was significantly reduced in rats treated with cyclosporine-A.

Conclusion: The neuroprotectant drugs used here perioperatively did not reduce the extent of neurological damage in a model simulating iatrogenic SCI. Therefore, for now, the only protection in elective spine surgery is avoidance of primary injury altogether.

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Introduction

Elective surgical procedures involving extensive manipulation of the spinal cord (SC) such as resection of intramedullary tumors and scoliosis surgery can result in iatrogenic transient or permanent neurological deficits.^{1,2}

Efforts have been made to reduce the risk of mechanically induced damage to SC during elective spine surgery by intraoperative electrophysiological monitoring and the development of less traumatic and more hemostatic surgical techniques.^{2,3} However, few attempts have been made to pharmacologically prevent or reduce this complication.

It is widely accepted that a mechanic and/or ischemic insult to the SC (primary injury) is followed by a complex array of cellular and molecular autodestructive events (secondary mechanisms of injury), which have been linked to the progressive degeneration of the injured SC.^{4,5} Many pharmacological agents targeting known pathophysiological events of the secondary injury cascade have proven to be neuroprotectant in animal models of traumatic and ischemic spinal cord injury (SCI).⁵

Looking for pharmacological tools to protect SC tissue from secondary insults simulating iatrogenic SCI, here we tested four drugs that comply with the following criteria: (a) proven capacity to block at least one of the pathophysiological mechanisms involved in secondary damage; (b) experimental evidence of therapeutic benefit when used in models of traumatic and/or ischemic SCI; (c) approval for use in humans

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in the treatment of neurological or non-neurological pathologies and (d) a safety profile record. Drugs that fulfilled these criteria included methylprednisolone sodium succinate (MPSS), erythropoietin, cyclosporine-A and melatonin.

Since the report of the Second National Acute Spinal Cord Injury Study in 1990 on the moderately beneficial effects of megadoses of MPSS for the treatment of acute SCI,⁶ the use of this drug has been considered the standard of care for the treatment of this emergency. However, recent clinical trials have not shown the expected benefits and the risk for serious complications from the use of the steroid have become evident.⁷

Recent basic and preclinical investigations have suggested that erythropoietin can behave as a tissue-protective cytokine in addition to its key role in hematopoiesis. In models of traumatic and ischemic SCI, erythropoietin has improved functional neurological status. In fact, a single intraperitoneal dose of erythropoietin (1000 or 5000 U kg⁻¹) given 1 h after SCI to rats improved their neurological recovery as effectively as daily doses.⁸

In addition to its potent immunosuppressive activity, cyclosporine-A has been described as a neuroprotectant drug in several models of neural pathology, such as ischemic brain damage, traumatic brain injury and traumatic SCI.⁹ A single dose of cyclosporine-A given intravenously (10 mg kg⁻¹) produced neuroprotection against diffuse axonal injury after traumatic brain injury in the rat.¹⁰ Besides, cyclosporine-A appears to promote axonal growth.¹¹

Given to models of traumatic and ischemic SCI, melatonin has been effective in preventing oxygen- and nitrogen-based oxidative damage, neutrophil infiltration and apoptosis thereby facilitating functional recovery and tissue preservation to levels comparable with those achieved with MPSS.¹²

Our working hypothesis was that any of these drugs given before, during and after, in a single treatment scheme, to rats with controlled mild traumatic SCI (used here as a model of elective spine surgery of high risk for neurological damage) could behave as an effective neuroprotector by blocking autodestructive mechanisms.

Materials and methods

Experimental design

We evaluated the neuroprotective effects of MPSS, erythropoietin, melatonin and cyclosporine-A compared with a saline solution (control) in adult female Long-Evans rats subjected to SC contusion of mild intensity ($n = 7$ per group). Drugs were administered intraperitoneally to rats pre-, intra-

and postoperatively using the treatment schemes described in Table 1.

Anesthesia, injury and care

For SCI, animals were anesthetized with a mixture of ketamine (80 mg kg⁻¹) and xylazine (8 mg kg⁻¹) given intramuscularly. A laminectomy was performed aseptically at T9 level to expose the dorsal aspect of the SC, maintaining integrity of the meninges. The spinal column was then stabilized with angled clamps, held by a stereotaxic frame, fastened to T8 and T10 spinous processes. The SC contusion was inflicted by dropping onto the exposed dura, a stainless steel rod weighing 10 g with a flat tip of 2 mm in diameter, from a height of 12.5 mm using the New York University weight-drop impactor device. Post-surgical care included manual expression of bladders twice a day until bladder function returned. Animals were housed individually in a temperature-regulated environment and kept on a 12-h light/dark cycle. Food and water were provided *ad libitum*.

Drug administration

Dosage regimen and route of drug administration for each drug (Table 1) were determined according to their pharmacokinetic properties and previous experience gained in animal models of SCI.

Drugs tested were (a) recombinant human erythropoietin (Recormon, Roche, Mexico), (b) melatonin (Sigma Chem. Co., St Louis, MO, USA), (c) cyclosporine-A (Sandimmune, Novartis, Mexico) and (d) MPSS (Solu-Medrol, Pfizer, Mexico). Melatonin was dissolved in ethanol and further diluted in saline solution; the final ethanol concentration was 5%. All other drugs were dissolved only in saline solution. The final volume of each dose was 1 ml.

As prophylactic for infections, 8 mg kg⁻¹ of ciprofloxacin lactate (Bayer, Mexico City, Mexico) were given subcutaneously every 12 h, starting at the end of surgery and for 7 consecutive days. To prevent autophagia, acetaminophen (Cilag, Mexico City, Mexico) was given in the drinking water at an approximate dose of 64 mg per kg per day for 3 weeks.

Assessment of motor function

For analysis of locomotor performance, contused rats were assessed individually for 4 min in an open field using the Basso, Beattie and Bresnahan (BBB) rating scale and the BBB-derived subscoring scale.¹⁶ Motor function was assessed on the BBB rating scale on day 1 after surgery, and then once a week for 6 weeks.

Table 1 Treatment schemes

Drugs (given intraperitoneally)	Preoperative (dose, hours before injury)	Intraoperative (dose, during surgery)	Postoperative (dose, hours after injury)
Recombinant human erythropoietin ^{8,13}	2000 U kg ⁻¹ , 12 h	2000 U kg ⁻¹	2000 U kg ⁻¹ , 24 h
Melatonin ^{12,14}	10 mg kg ⁻¹ , 20 min	10 mg kg ⁻¹	10 mg kg ⁻¹ , 1 h
Cyclosporine-A ^{10,15}	5 mg kg ⁻¹ , 12 h	2.5 mg kg ⁻¹	2.5 mg kg ⁻¹ , 12 h
Methylprednisolone ⁶	30 mg kg ⁻¹ , 2 h	30 mg kg ⁻¹	30 mg kg ⁻¹ , 2 h
Saline solution	1 ml, 12 h	1 ml	1 ml, 24 h

Spontaneous activity, in particular rearing events, was assessed as a measure of trunk muscle activity. Measures were collected as described by Mills *et al.*¹⁷ using direct observations instead of computerized equipment. Briefly, each animal was placed in an open cage (80 × 80 × 20 cm). Each time the animals reared with or without forelimb support from the walls of the animal's cage, the event was recorded. The total number of rearing events performed during 4 min was counted for further analysis. This test, like the BBB subscoring scale, was carried out only on weeks 5 and 6 after injury, when most animals were able to perform them.

Histological study

After the last functional tests (6 weeks after injury), the rats were deeply anesthetized, and then given 1000 U of heparin intraperitoneally and perfused intracardially using a peristaltic pump at 30 ml min⁻¹ with 100 ml of saline solution followed by 500 ml of 10% formaldehyde. A 1-cm-long SC specimen, with the injured area in the center, was dissected and placed in the same fixative for 1 week; the tissue was then processed for histology and embedded in paraffin. In the coronal plane, 8-μm-thick sections were obtained sequentially from the central portion of the specimen; they were then mounted on gelatinized slides, and dyed with Masson stain for further morphological and morphometric analysis.

Morphometric analysis of spared SC parenchyma was carried out in the most central portion of the SC judging from the presence of ependyma in the examined tissue. A picture was obtained using the × 2 objective, centered in the portion of tissue showing greater destruction. Area measurements were performed using the Image-Pro Plus image analysis software from Media Cybernetics. All slides were assessed blindly with respect to treatment.

Statistical analysis

Mean and standard error of the mean scores on the BBB rating scale were plotted as a function of time postinjury and assessed using the repeated measures analysis of variance (ANOVA) test followed by Tukey's multiple comparison test. The scores on the BBB subscoring scale and rear events, as well as morphometric results, were analyzed using the one-way ANOVA test followed by Dunnett's test. Differences were considered significant when $P < 0.05$.

Ethics

We certify that all applicable institutional and governmental regulations concerning the ethical use of animals were followed during the course of this research.

Results

Motor function outcome

Hind limb performance assessed with the BBB scale showed serious deterioration of motor function at day 1 following SC contusion for all groups. Scores improved importantly during the first 2 weeks, and then were maintained with

Figure 1 Hind limb performance during free locomotion. (a) Scores on the BBB scale for each experimental group ($n = 7$) over a period of 6 weeks, starting the day after injury. No significant differences were found among experimental groups using the repeated measures ANOVA test. (b) Scores on the BBB derived subscoring scale 5 (□) and 6 (■) weeks after SCI. No significant differences were found among experimental groups using the one-way ANOVA test. Plots represent the mean $\pm$ s.e. MPSS, methylprednisolone sodium succinate; MEL, melatonin; EPO, recombinant human erythropoietin; CsA, cyclosporine-A; SS, saline solution; BBB, Basso, Beattie and Bresnahan; ANOVA, analysis of variance.

minor changes until the end of the study at week 6 (Figure 1a). Although without statistical significance among the groups, rats treated only with saline solution showed the greatest degree of recovery, whereas rats treated with cyclosporine-A had the poorest functional scores.

Results using the BBB subscoring scale at weeks 5 and 6 after injury are shown in Figure 1b. Scores were not significantly different among groups, although here again, the lowest score was obtained for rats treated with cyclosporine-A, and the highest was shared by rats treated with saline solution and MPSS.

The number of rearing events during 4 min in the open field at weeks 5 and 6 after injury is plotted in Figure 2. Activity of rats treated with cyclosporine-A was significantly lower compared with control (saline solution group).

Morphologic outcome

Specimens from all groups showed cysts of variable size and shape in the central portion of the cord, often trabeculated

and containing foamy macrophages. Although showing microcysts, white and gray substances surrounding the larger cysts were considered spared tissue (Figures 3a–e). The amount of spared SC tissue was similar for all groups;

Figure 2 Number of rearing events during 4 min in the open field 5 (□) and 6 (■) weeks after injury. Data represent the mean $\pm$ s.e., $n=7$ per group. (*), CsA versus SS, $P<0.05$, one-way ANOVA. Abbreviations as in the legend of Figure 1.

differences were not statistically significant. Results are illustrated in Figure 3f.

Discussion

Even though refinement of surgical procedures and intraoperative electrophysiological monitoring have helped to prevent damage to the SC during elective spine surgery,^{2,3} excessive surgical manipulation of the SC is bound to result in SCI.^{1,2} In search for pharmacological tools to reduce the risk of SCI under these circumstances, here we evaluated the capacity of several agents known to be neuroprotectant to modify functional and histological outcome in rats subjected to mild SC damage, a model simulating SCI that may occur during elective spine surgery. Notably none of the drugs tested produced any detectable benefit.

The damage that can be inflicted to the SC during elective surgical procedures is in essence the outcome of traumatic and ischemic injuries.^{1,2} Because of the lack of specific experimental models resembling neurological injury caused by elective spine surgery, we chose the animal model of mild

Figure 3 Examples of SC specimens from methylprednisolone sodium succinate (MPSS) (a), melatonin (MEL) (b), erythropoietin (EPO) (c), cyclosporine-A (CsA) (d) and saline solution (SS) (e) treated groups; bar = 1 mm. Trabeculated cysts (*) in the central portion of the cord containing foamy macrophages; spared nervous tissue with microcysts (+). (f) Differences in the amount of spared SC tissue for all groups were not statistically significant. SC, spinal cord.

contusion SCI for this study for two reasons: it reproduces the majority of iatrogenic injuries that could occur after elective spine surgery, and as an incomplete injury allows for the detection of beneficial and detrimental changes, as well as no changes at all in response to treatments. Furthermore, by starting pharmacological treatment before injury, we converted this model of traumatic SCI into a paradigm for anticipated SC lesions.

In contrast to accidental traumatic SCI where the short therapeutic time window for most neuroprotectants limits their clinical usefulness,⁵ it is conceivable that patients undergoing elective spine surgery receiving neuroprotectants perioperatively could be protected from iatrogenic lesions by blocking the sequence of autodestructive events (secondary injury cascade) that might occur during spine surgery.

We therefore attempted to prevent autodestructive secondary injuries associated with mild SCI by pharmacological prophylaxis. Contrary to our expectations, however, pharmacological preconditioning of rats with the four drugs tested here was not neuroprotective. With the exception of cyclosporine-A that worsened functional outcome, results were similar to those obtained in the saline-treated rats.

Our unexpected results suggest that pharmacologic manipulation of secondary events occurring after cord injury is futile, regardless of the mechanisms targeted here. Therefore, it is reasonable to speculate that the primary and not secondary injuries, better predict the final outcome. This is in accordance with those authors who believe that the extent of damage after SCI is determined by the intensity of primary injury with minimal contribution from the secondary mechanisms of injury.¹⁸

Furthermore, if some very early events following SCI represent endogenous mechanisms of protection and self-repair, rather than toxic mechanisms of autodestruction,^{18,19} and are sensitive to cyclosporine-A, this could account for the poor outcome observed in the group of rats receiving cyclosporine-A, in contrast with its multiple neuroprotective effects when given after neural injuries.^{9–11} For example, it is possible that cyclosporine-A could worsen post-traumatic ischemia by blocking the early spontaneous production of the potent vasodilator nitric oxide from an endothelial source.²⁰

In spite of the excitement generated by recent reports showing that a variety of drugs behave as neuroprotectant agents by diminishing secondary damage and improving functional outcome in animal models of traumatic SCI, their usefulness to prevent extensive tissue damage when given prior to or simultaneously with SCI is questionable. The future of pharmacological manipulation to reduce risk of iatrogenic cord lesions during elective spine surgery in humans lies in the better understanding of the cellular and molecular changes associated with SCI.

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References

- Delank KS, Delank HW, König DP, Popken F, Furdere S, Eysel P. Iatrogenic paraplegia in spinal surgery. *Arch Orthop Trauma Surg* 2005; **125**: 33–41.
- Costa P, Bruno A, Bonzanino M, Massaro F, Caruso L, Vincenzo I *et al*. Somatosensory- and motor-evoked potential monitoring during spine and spinal cord surgery. *Spinal Cord* 2007; **45**: 86–91.
- Stellar S, Polanyi TG. Lasers in neurosurgery: a historical overview. *J Clin Laser Med Surg* 1992; **10**: 399–411.
- Dumont RJ, Okonkwo DO, Verma S, Hurlbert RJ, Boulos PT, Ellegala DB *et al*. Acute spinal cord injury, part I: pathophysiologic mechanisms. *Clin Neuropharmacol* 2001; **24**: 254–264.
- Baptiste DC, Fehlings MG. Pharmacological approaches to repair the injured spinal cord. *J Neurotrauma* 2006; **23**: 318–334.
- Bracken MB, Shepard MJ, Collins WF, Holford TR, Young W, Baskin DS *et al*. A randomized, controlled trial of methylprednisolone or naloxone in the treatment of acute spinal-cord injury. Results of the Second National Acute Spinal Cord Injury Study. *N Engl J Med* 1990; **322**: 1405–1411.
- Hughenoltz H. Methylprednisolone for acute spinal cord injury: not a standard of care. *C Med Assoc J* 2003; **168**: 1145–1146.
- Gorio A, Gökmen N, Erbayraktar S, Yilmaz O, Madaschi L, Cichetti C *et al*. Recombinant human erythropoietin counteracts secondary injury and markedly enhances neurological recovery from experimental spinal cord trauma. *Proc Natl Acad Sci USA* 2002; **99**: 9450–9455.
- Díaz-Ruiz A, Ríos C, Duarte I, Correa D, Guizar-Sahagún G, Grijalva I *et al*. Cyclosporin-A inhibits lipid peroxidation after spinal cord injury in rats. *Neurosci Lett* 1999; **266**: 61–64.
- Okonkwo DO, Melon DE, Pellicane AJ, Mutlu LK, Rubin DG, Stone JR *et al*. Dose-response of cyclosporin A in attenuating traumatic axonal injury in rat. *NeuroReport* 2003; **14**: 463–466.
- Ibarra A, Hernández E, Lomeli J, Pineda D, Buenrostro M, Martinon S *et al*. Cyclosporin-A enhances non-functional axonal growing after complete spinal cord transection. *Brain Res* 2007; **1149**: 200–209.
- Cayli SR, Kocak A, Yilmaz U, Tekiner A, Erbil M, Öztürk C *et al*. Effect of combined treatment with melatonin and methylprednisolone on neurological recovery after experimental spinal cord injury. *Eur Spine J* 2004; **13**: 724–732.
- Yoon WH, Park SJ, Kim IC, Lee MG. Pharmacokinetics of recombinant human erythropoietin in rabbits and 3/4 nephrectomized rats. *Res Commun Mol Pathol Pharmacol* 1997; **96**: 227–240.
- Yelleswaram K, McLaughlin LG, Knipe JO, Schabdach D. Pharmacokinetics and oral bioavailability of exogenous melatonin in preclinical animal models and clinical implications. *J Pineal Res* 1997; **22**: 45–51.
- Ibarra A, Reyes J, Martínez S, Correa D, Guizar-Sahagún G, Grijalva I *et al*. Use of cyclosporin-A in experimental spinal cord injury: design of a dosing strategy to maintain therapeutic levels. *J Neurotrauma* 1996; **13**: 569–572.
- Basso DM. Behavioral testing after spinal cord injury: congruities, complexities, and controversies. *J Neurotrauma* 2004; **21**: 395–404.
- Mills CD, Johnson KM, Hulsebosch CE. Role of group II and group III metabotropic glutamate receptors in spinal cord injury. *Exp Neurol* 2002; **173**: 153–167.
- Streit WJ, Semple-Rowland SL, Hurley SD, Miller RC, Popovich PG, Stokes BT. Cytokine mRNA profiles in contused spinal cord and axotomized facial nucleus suggest a beneficial role for inflammation and gliosis. *Exp Neurol* 1998; **152**: 74–87.
- Bolano JP, García-Nogales P, Almeida A. Provoking neuroprotection by peroxynitrite. *Curr Pharm Des* 2004; **10**: 867–877.
- Díaz-Ruiz A, Vergara P, Pérez-Severiano F, Segovia J, Guizar-Sahagún G, Ibarra A *et al*. Cyclosporin-A inhibits constitutive nitric oxide synthase activity and neuronal and endothelial nitric oxide synthase expressions after spinal cord injury in rats. *Neurochem Res* 2005; **30**: 245–251.