

Comparative neuroprotective effects of methylprednisolone and rosiglitazone, a peroxisome proliferator-activated receptor- γ following spinal cord injury

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ABSTRACT

الأهداف: عمل مقارنة بين عقاري ميثيل بريدنيزولون (methylprednisolone) وروزيجليتازون (rosiglitazone) وتأثيرهما الوقائي بعد إصابة العمود الفقري.

الطريقة: أُجريت هذه الدراسة في مركز تقويم العظام وهو المستشفى الأول التابع لجامعة سوكو، زوشو، الصين وذلك خلال الفترة من يونيو 2009م إلى مارس 2010م، وشملت هذه الدراسة 120 جرذيا من النوع سبراغ دولي. لقد تم تقسيم الجرذان بعد إصابة العمود الفقري إلى 4 مجموعات (في كل مجموعة 30 جرذيا) وهي كالتالي: مجموعة الجرذان المتضررة بعد حادث مركبة والتي لم تُعطى شيئا، ومجموعة الجرذان التي عولجت بعقار ميثيل بريدنيزولون، ومجموعة الجرذان التي عولجت بعقار روزيجليتازون، ومجموعة الجرذان التي عولجت بالحلل الملحي فقط. وبعد ذلك تم تحليل النتائج ارتكازا على العناصر الخمسة التالية: التهاب العمود الفقري وتضرر الأنسجة، وارتشاح الخلايا العدلة، والموت الخلوي، وسيتوكينات الأثر الصافي لكلا من معامل ألفا لتنخر الأورام، وإنترلوكين-بيتا، بالإضافة إلى بروتينات باكس، وBcl-2، وبروتينات الصدمة الحرارية (HSP70).

النتائج: أشارت النتائج إلى أن عقار روزيجليتازون مشابه لميثيل بريدنيزولون في التأثير الوقائي للجهاز العصبي، كما أنه يقلل بنسبة كبيرة من تضرر العمود الفقري، والموت الخلوي، والسيتوكينات. ولم يكن هناك اختلافا واضحا بين نتائج المجموعة المعالجة بعقار ميثيل بريدنيزولون أو تلك المعالجة بعقار روزيجليتازون.

خاتمة: أثبتت الدراسة أن العلاج بعقار روزيجليتازون يقوم بتقليل تطور الالتهابات وتضرر الأنسجة المرتبطة بإصابات العمود الفقري.

Objectives: To compare the neuroprotective effects of methylprednisolone (MPSS) and rosiglitazone (ROSG) following spinal cord injury (SCI).

Methods: This study was carried out at the Institute of Orthopedics, the First Affiliated Hospital of Soochow University, Suzhou, China between June 2009 and March 2010. One hundred and twenty Sprague-

Dawley rats after SCI were divided into 4 different groups (30/group): i) SCI-vehicle group, ii) MPSS group, iii) ROSG group, and iv) sham saline group. The following 5 aspects were evaluated: 1) spinal cord inflammation and tissue injury; 2) neutrophil infiltration; 3) cell apoptosis; 4) the expression of proinflammatory cytokines tumor necrosis factor- α and interleukin- β ; and 5) the expression of tissue Bax, Bcl-2, and HSP70 proteins in situ.

Results: Notably, ROSG showed similar neuroprotective effects to MPSS, and significantly decreased spinal cord damage, apoptosis, and cytokine expression. There were no significant differences between the MPSS or ROSG-treated groups.

Conclusion: Administration of ROSG after SCI reduces the development of inflammation and tissue injury associated with spinal cord trauma.

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Spinal cord injury (SCI) is considered to be the factor primarily responsible for inducing disability, affecting millions of people in the general population all over the world. Several pathological events, including inflammation, apoptosis, oxidative stress, and excitotoxicity, are known to precipitate the neuronal

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death and neurological dysfunction^{1,2} during the acute stage after an injury. Inflammation that starts within minutes and continues for days after the injury is known to significantly result in secondary neuronal damage, which is a major cause of motor dysfunction after SCI in rodents.³ Hence, therapeutic compounds that target multiple pathophysiological mechanisms might be extremely useful in preventing post-SCI neuronal death, and represent one of the most challenging areas in regeneration medicine. The peroxisome proliferator-activated receptor- γ (PPAR γ) is a ligand-activated transcription factor of the nuclear hormone receptor superfamily.⁴ The 15-d-Prostaglandin J2 is the natural ligand of PPAR γ ,⁵ while several thiazolidinediones (TZDs) are potent synthetic agonists.⁶ It is well known that 2 TZDs, rosiglitazone (ROSG) and pioglitazone are approved for type-2 diabetes treatment by the United States Food and Drug Administration (FDA).^{7,8} Since PPAR γ is vital in glucose and lipid metabolism,⁹ PPAR γ agonists have been applied in various models of CNS injury and disease because of their anti-inflammatory and anti-oxidant properties. For example, in amyotrophic lateral sclerosis,¹⁰ Parkinson's disease,¹¹ cerebral ischemia or hemorrhage,¹² and traumatic brain injury.¹³

In this study, we explored the neuroprotective effect of PPAR γ agonist ROSG in rat models after SCI. Meanwhile, we compared the effect of ROSG and methylprednisolone (MPSS) as therapeutic roles in a SCI model. We determined the following end points 1) motor recovery, 2) infiltration of neutrophils, 3) proinflammatory cytokines, 4) apoptosis, and 5) the expressions of Bax, Bcl-2, and heat-shock protein.

Methods. One hundred and twenty adult male Sprague-Dawley rats, 10-12 weeks old, weighing 200–250g were used in this study. This study was carried out at the Institute of Orthopedics, the First Affiliated Hospital of Soochow University, Suzhou, China between June 2009 and March 2010. All study procedures were conducted in agreement with the guidelines for the use of experimental animals of the US National Institutes of Health. All efforts were made to minimize the quantity of animals used in the experiment, and to alleviate their suffering. Moreover, all surgical procedures were performed in an aseptic manner and were approved by the Research Animal Resources and Care Committee of Soochow University.

Spinal cord injury. Rats were anesthetized using chloral hydrate (100 mg/kg body weight) and the SCI models were established as previous described.¹⁴ Rats were randomly divided into the following groups: i) SCI-vehicle group. Rats were subjected to SCI plus administration of vehicle (N=30); ii) MPSS group. The same as the SCI-group, but MPSS was administered intraperitoneally (i.p.) (one hour 30 mg/kg, 15 mg/kg

at 24 and 48 hours) after SCI (N=30); iii) ROSG group. The same as the SCI-saline group, but ROSG (2mg/kg, Avandia™, GlaxoSmithKline, Raleigh, SC, USA) was administered i.p. one hour after SCI (N=30). iv) Sham saline group. Rats were subjected to the same surgical procedures as the previous groups, except that the aneurysm clip was not applied (N=30). Drug solutions were made fresh for each administration; ROSG or vehicle injections (i.p.) were given one hour post-injury, every 12 hours for 7 days. The dose regimen used (2mg/kg ROSG; 30 mg/kg MPSS) was chosen on the basis of a previous dose-dependent study in our laboratory.

Motor function assessment. Motor function recovery after SCI was studied with the Basso-Beattie-Bresnahan (BBB) scoring system that uses a 21-point open-field locomotor scale as described previously.¹⁴ This scale is based on the precise observation of hind limb movements, stepping, and coordination in an open field. Uninjured animals with normal locomotion exhibit a locomotor score of 21, whereas animals that exhibit complete hind limb paralysis are scored as 0. Spinal cord injured animals were tested on post-operative day 3 and then weekly for 4 weeks. The movements were scored for 4 minutes by 2 evaluators blinded to the study groups. Scores are represented as the average score of all animals (n = 5) in a group at a certain time point.

Histopathology. The paraffin-embedded spinal cord samples were cut (4 μ m thick) transversally, and then stained with hematoxylin and eosin (H&E). Damaged neurons were counted, and the histopathological changes of the gray matter were scored on a 4-point scale.¹⁵

Myeloperoxidase (MPO) activity assay of spinal cord tissues. Myeloperoxidase (MPO) activity, an indicator of polymorphonuclear leukocyte (PMN) accumulation, was measured in the spinal cord tissues. The frozen samples were weighed, and a 20% homogenate was extracted from each sample. The MPO activity was measured in each sample according to the manufacturer's instructions (Nanjing Jiancheng Biological Institute, Nanjing/Jiangsu, China) and was recorded in U/g wet tissue.

Immunohistochemistry. After deparaffinization, endogenous peroxidase was quenched with 0.3% (v/v) hydrogen peroxide in 60% (v/v) methanol for 30 minutes. The sections were permeabilized with 0.1% (w/v) Triton X-100 in phosphate buffer solution (PBS) for 20 minutes. Non-specific adsorption was minimized by incubating the section in 2% (v/v) normal goat serum in PBS for 20 minutes. The sections were incubated overnight with primary antibodies. The antibodies used were: tumor necrosis factor (TNF)- α and interleukin (IL)-antibody (1:100, rat monoclonal; Santa Cruz Biotechnologies, Santa Cruz, CA, USA). The sections were incubated with the primary antibody for one

hour at room temperature, or overnight at 4°C. The streptavidin-peroxidase (SP) method was used. Control experiments, in which isotype antibodies (1:100, rat monoclonal; Santa Cruz Biotechnologies, Santa Cruz, CA, USA) were substituted for the primary antibody, were performed to ascertain the specificity of antibody staining. Independent scoring was also performed by a blinded investigator.

Terminal deoxynucleotidyl transferase-mediated UTP end labeling (TUNEL) assay. Terminal deoxynucleotidyl transferase-mediated UTP end labeling (TUNEL) assay was conducted to measure neuronal apoptosis by using the TUNEL detection kit according to the manufacturer's instruction (Roche, Basel, Switzerland). Neurons with brown-stained nuclei, or those containing apoptotic bodies, were considered as apoptosis.¹⁶ Independent scoring was performed by a blinded investigator and data presented as mean \pm SD.

Total protein extraction and western blot analysis for Bax, Bcl-2, and heat-shock proteins. Tissue samples from SCI-injured animals were collected and homogenized on ice in 10 mM Tris-HCl buffer (PH 7.4), 10 mM EDTA, 30% Triton-1000, 10% SDS, and NaCl using a homogenizer. Homogenates were centrifuged at 12,000g for 30 minutes at 4°C. The supernatant was collected and stored at -80°C. The protein concentration was assayed with BCA reagent (Sigma, St. Louis, MO, USA). Samples then were loaded on a 12% SDS polyacrylamide gel (SDS-PAGE) and followed by electrophoresis for 1.5 hours at 120 V. Proteins were transferred from gel to nitrocellulose membrane for 6.5 hours at 16 V. For immunoblotting, membranes were blocked with 10% non-fat dried milk in saline buffer for one hour and then, incubated with primary antibodies against Bax, Bcl-2, HSP70, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), and stored at 4°C overnight in 1 \times PBS, 5% w/v non-fat dried milk, 0.1% Tween-20. The membranes were washed 3 times for 10 minutes in Tris buffered saline with 0.1% Tween-20 and incubated with peroxidase-conjugated bovine anti-rabbit IgG secondary antibody (1:2000). All antibodies were from Santa Cruz Biotechnologies, Santa Cruz, CA, USA. The intensity of each band was quantitatively determined using Gel-Pro Analyzer Software (Media Cybernetics, Silver Spring, MD, USA) and the density ratio represents the relative intensity of each band against those of controls in each experiment.

Statistical evaluation. Results are expressed as means \pm standard errors of the mean and the differences between groups were considered significant at $p < 0.05$. The results were analyzed by one-way ANOVA followed

by a Bonferroni post hoc test for multiple comparisons. The histology scale data and the locomotor tests data were analyzed by the Mann-Whitney test. Analyses were conducted with GraphPad Prism 4.0c software for Windows XP (Graph Pad).

Results. The ROSG and MPSS improved the locomotor function following SCI. To detect if histological damage to the spinal cord was associated with loss of motor function, the modified BBB hind limb locomotor rating scale score was evaluated. The BBB score starts from 21 points. Spinal cord contusion resulted in a score of 0 with bilateral hind limb paralysis in all of the rats in day one. Frankly, lines representative of MPSS and ROSG-treated groups were steadily ascending from day one to day 28. Although there was no noticeable difference in MPSS and ROSG-treated groups when administered as a single treatment, a significant difference was shown between 2 treatment groups (ROSG, MPSS) and trauma groups ($p = 0.035$ and $p = 0.021$, Figure 1). After 3 days, the hind limbs of rats could slowly move. Rats were treated by ROSG and MPSS and reached a plateau after day 14. However, rats treated by ROSG or MPSS nearly improved further until day 28. Rats subjected to SCI had significant deficits in hind limb movement though their motor functions were just slightly impaired in sham rats. Control rats progressively improved from day 3 to day 28.

The ROSG and MPSS treatment both decreased the tissue damage following SCI. The severity of the trauma at the level of the perilesional area, assessed as the presence of edema as well as alteration of the white matter, was evaluated at 24 hours after injury. Spinal cords were collected and stained by H&E. Damaged neurons were counted, and the histopathological

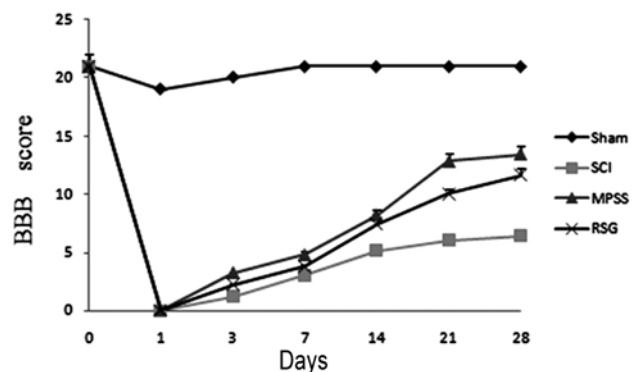


Figure 1 - Locomotor activity was evaluated during the 4 weeks following SCI using the 21-point Basso Beattie Bresnahan (BBB) locomotor scale in rats. Treatment with rosiglitazone (ROSG) or methylprednisolone (MPSS) significantly ameliorated the hind limb motor disturbances compared with the other groups. Means of 5 animals for each group. The values are the mean \pm SD. SCI - spinal cord injury.

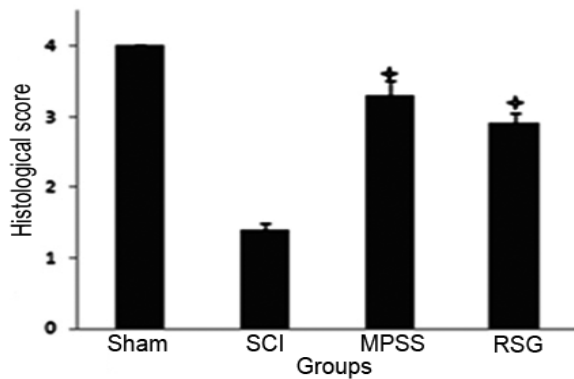


Figure 2 - Effect of rosiglitazone (RSG) on histological alterations of the spinal cord tissue at 24 hours after injury. Significant damage to the spinal cord in nontreated spinal cord injury (SCI)-operated rats was assessed 24 hours after SCI. Rosiglitazone or methylprednisolone (MPSS) reduced the spinal cord damage. Significant differences from vehicle values are indicated by $+p < 0.05$.

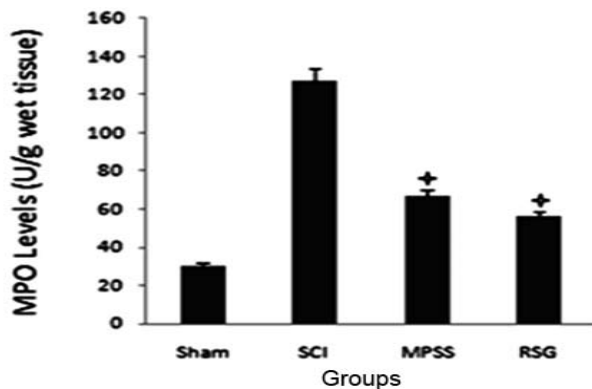


Figure 3 - Effect of rosiglitazone (RSG) on myeloperoxidase (MPO) activity. The MPO activity in the spinal cord of nontreated spinal cord injury (SCI)-operated rat was significantly increased at 24 hours after the damage in comparison to the sham rats. Treatment with RSG or methylprednisolone (MPSS) significantly reduced the SCI-induced increase in MPO activity. Data are expressed as means \pm SEM. $+p < 0.05$.

changes of the gray matter were scored on a 4-point scale. Significant damage to the spinal cord was observed in the spinal cord tissue of control rats subjected to SCI when compared with sham-operated rats (Figure 2). Treatment with ROSG resulted in significantly less damage compared to the vehicle control ($p = 0.022$, Figure 2). Similarly, treatment with MPSS resulted in significantly less damage compared to vehicle control ($p = 0.019$, Figure 2). Taken together, these observations showed that ROSG attenuated the spreading of the necrotic lesion by suppressing the overall spinal cord tissue damage and loss of myelin. However, there was no difference in the MPSS and ROSG treated groups.

The ROSG and MPSS both decreased inflammation after SCI. Neutrophils play an important role in

inflammation by expressing surface markers and cytokines. The infiltration of neutrophils was assayed by MPO for a quantitative indication of the presence of inflammation in the ipsilateral frontal lobes from 4 groups. Compared to the sham surgery group, there was a statistically higher MPO in the vehicle-treated group. In the ROSG or MPSS treated groups, there was a significant decrease in MPO compared with the vehicle-treated groups, demonstrating anti-inflammatory properties ($p = 0.011$ and $p = 0.013$, Figure 3). However, there was no noticeable difference in MPSS and ROSG-treated groups. Immunohistochemical analysis of well-known inflammatory markers TNF- α and IL-1 β indicated that these markers were increased in the neurons in the affected spinal tissue in the vehicle-treated rats. However, in animals treated with ROSG or MPSS, there appeared to be decreased levels of both inflammatory markers in the affected spinal tissue, further illustrating that treatment with ROSG or MPSS decreased inflammation ($p = 0.044$ and $p = 0.038$, Figure 4). However, there was no noticeable difference in the MPSS or ROSG-treated groups (Figure 4).

ROSG and MPSS curtailed post-SCI apoptosis by changing the expressions of Bax, Bcl-2, and HSP70. Cell death after SCI maybe due to one of 2 mechanisms: necrosis or apoptosis.

The TUNEL-positive neurons are reported to be restricted primarily to the lesion epicenter 4–24 hours after injury, followed by a second wave of oligodendroglial apoptosis one week after SCI.¹⁷ Apoptotic neurons and oligodendrocytes in SCI change the axon-myelin structural unit, and therefore deteriorate impulse conduction, inducing functional loss finally. Corresponding to previous research, TUNEL-

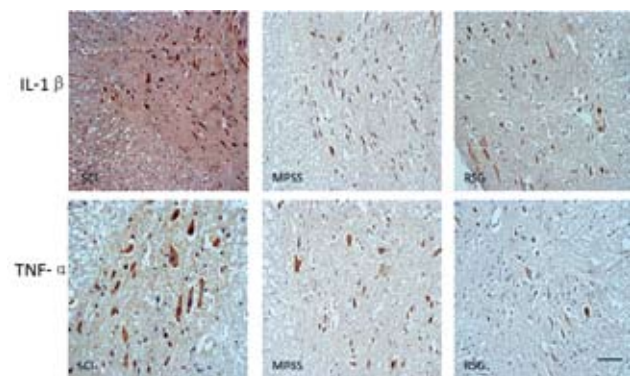


Figure 4 - Effects of rosiglitazone (RSG) on spinal cord levels of tumor necrosis factor (TNF)- α and interleukin (IL)-1 β . A significant increase of the TNF- α and IL-1 β levels was observed in the spinal cord tissues 24 hours after spinal cord injury (SCI). In the spinal cord tissues of RSG-treated SCI rats, the TNF- α and IL-1 β levels were significantly reduced in comparison to those of SCI in the same conditions. A similar pattern of staining was observed in methylprednisolone (MPSS)-treated rats. Scale bar = 100 μ m, positive cells stained in brown.

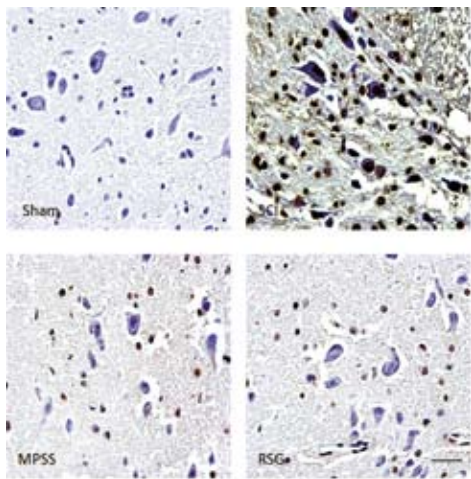


Figure 5 - The PPAR γ agonist rosiglitazone (ROSG) treatment decreases TUNEL+ cells in the injured spinal cord at 24 hours after spinal cord injury (SCI). The figure is representative of at least 3 experiments performed on different experimental days. A similar pattern of staining was observed in methylprednisolone (MPSS)- treated rats. Scale bar=100um. Positive cells stained in brown.

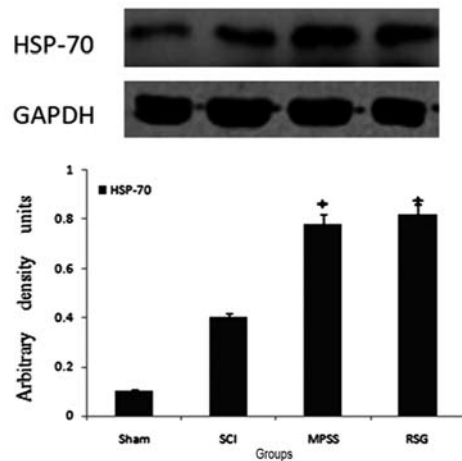


Figure 7 - Representative Western blots of HSP70 levels. Western blot analysis was realized in spinal cord tissue collected at 24 hours after injury. The GAPDH was used as a loading control. Significant difference between vehicle- and rosiglitazone (ROSG) or methylprednisolone (MPSS)- treated rats was indicated by $+p<0.05$, SCI - spinal cord injury

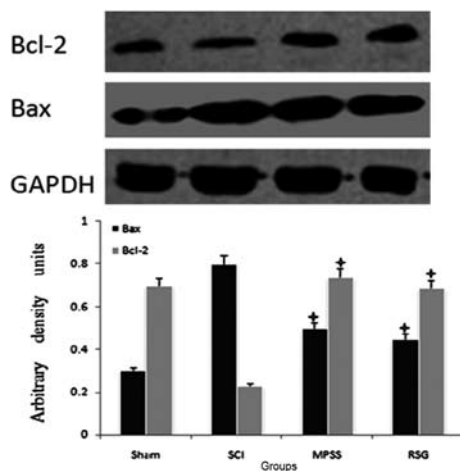


Figure 6 - Representative Western blots of Bax and bcl-2 levels. Western blot analysis was realized in spinal cord tissue collected at 24 hours after injury. The GAPDH was used as a loading control. A significant difference between vehicle- and rosiglitazone (ROSG) or methylprednisolone (MPSS)-treated rats was indicated by $+p<0.05$. SCI - spinal cord injury

positive cells were appeared 24 hours post-SCI along the lesion margins in the dorsal column of spinal cord sections from the lesion epicenter in vehicle-treated SCI (Figure 5). The ROSG-treatment significantly reduced the number of TUNEL+ cells compared to the vehicle ($p=0.031$, Figure 5). The number of TUNEL+ cells in the rats treated by MPSS was also reduced. However, there was no significant difference between the MPSS or ROSG-treated groups (Figure 5).

The Bcl-2 family members are crucial regulators of cell apoptosis. The expressions of Bax and Bcl-2 of cells can determine the outcome of cells. At 24 hours after SCI, the appearance of Bax in spinal cord homogenates was investigated by Western blot. The Bax levels were appreciably increased in the spinal cord from rats subjected to SCI. However, the ROSG or MPSS reduced the SCI-induced Bax expression (Figure 6). The Bcl-2 expression was also analyzed in whole extracts from the spinal cord of each rat by Western blot analysis. A basal level of Bcl-2 was detected in the spinal cord from sham-operated rat. Twenty-four hours after SCI, Bcl-2 expression was significantly reduced in whole extracts obtained from SCI rats. Treatment of rats with ROSG or MPSS significantly blunted the SCI-induced inhibition of Bcl-2 expression ($p=0.026$ and $p=0.014$, Figure 6). In all, the expression ratio of Bax/Bcl-2 was increased in the groups treated with ROSG or MPSS.

Heat shock proteins (HSPs) and anti-oxidant enzymes are known to promote neuroprotection. We presently observed that SCI significantly increased the expression of HSP70 one day after injury in the vehicle treated rats compared with sham controls. Treating rats subjected to SCI with ROSG or MPSS led to a further significant enhancement of the expression of HSP70 in the injured tissue ($p=0.008$ and $p=0.006$, Figure 7). There was no significant difference between MPSS or ROSG-treated groups (Figure 7).

Discussion. In brief, the present study strongly demonstrates the feasibility of this treatment, and treatment with PPAR γ agonists ROSG and MPSS

decreased secondary neuronal damage and myelin loss, and also improved motor function recovery after SCI.¹⁸ Glucocorticoid drugs, such as MPSS, are particularly potent immune-suppressive and anti-inflammatory agents that are therapeutically used in several inflammatory pathologies. Glucocorticoid drugs have effects on different cell types involved in the inflammatory process. For example, proliferation, differentiation, and the function of macrophages and fibroblasts could be inhibited by glucocorticosteroids. In addition, their effects also include inhibiting production and releasing of cytokines, such as interleukin-1, interleukin-6, and TNF- α .¹⁹ Furthermore, reduction of lipid peroxidation has been postulated to be a major factor in the improvement of outcome with MPSS.²⁰ Previous animal studies have provided key evidence that antagonizing TNF- α is a viable therapeutic strategy.^{19,21,22} High doses of MPSS have a clinically proven beneficial effect on functional recovery after SCI in humans,²³ and improved neurological recovery after experimental trauma to the spinal cord.²⁴

The PPAR γ is a transcription factor of the nuclear hormone receptor superfamily.²⁵ The PPAR protein is a complex formed with several domains such as a DNA-binding domain (DBD) and a ligand binding domain (LBD), it also has a D-domain, which links the DBD and LBD.^{26,27} In the absence of a ligand, the PPAR will be associated with a corepressor complex to trans-repress the target genes. Whereas, when a specific ligand binds to the LBD, the PPAR complex dimerizes with retinoic acid-X-receptor (RXR) leading to release of the corepressor.²⁸ The PPAR/RXR heterodimer translocates to the nucleus and binds to the promoters of several genes that contain a cis-acting element known as peroxisome proliferator response element (PPRE) to either initiate or suppress the transcription of the target genes.²⁵

The ROSG have been identified by the United States Food and Drug Administration to control blood glucose levels in millions of type 2 diabetics. The TZDs were also reported to be beneficial in several neuro inflammatory conditions. In patients with early Alzheimer's disease, ROSG promotes cognitive preservation.²⁹ The neuroprotective effects of TZDs may be a general characteristic of PPAR γ activation, as this phenomenon has been noted in other models of CNS gray matter injury. Both Pioglitazone and ROSG were shown to decrease the infarct volume in rodent focal ischemia models.³⁰⁻³² The PPAR γ natural agonist 15-d-PGJ2 was shown to decrease neurological deficits after experimental intracerebral hemorrhage,³³ moreover, the generation of inflammation and tissue injury could be largely palliative associated with spinal cord trauma.³⁴ These studies indicated that TZDs have significant therapeutic potential in treating neuro inflammatory disorders, likewise, diabetes.

Spinal cord injury initiates a sequence of events that lead to secondary neuronal cell damage. Basically, an inflammatory response develops within hours after injury. It could be characterized by the infiltration of neutrophils and the activation of microglia.³⁵ After that, it is followed with a second response to localize and to dampen the inflammatory reaction within the spinal cord. Moreover, it has been well proven that the expression of proinflammatory cytokines at the site of injury including TNF- α and IL- β could regulate the precise cellular events after SCI. Previous studies⁶ proved that low microglial activation and lower expression of inflammatory cytokines and chemokines might be the major mechanism of TZD-induced neuroprotection. It has also been monitored by local and peripheral inflammatory cells during the first week post-injury.³⁶ Our results showed that ROSG reduced the degree of spinal cord damage, infiltration of neutrophils, and cytokine expressions (TNF- α and IL-1 β). On the contrary, it increased the amount of apoptosis (TUNEL staining). However, there was no significant difference between the MPSS and ROSG-treated groups. Nevertheless, HSPs prevent aggregation and denaturation of many proteins. Song³⁷ and Tachibana³⁸ showed that SCI leads to significant up-regulation of several neuroprotective HSPs. It is well known that HSP70 could attenuate increased glutamate and release to limit excitotoxic neuronal damage after CNS injury.³⁹ Our results showed improved outcome after SCI in rats treated with a peroxisome proliferator-activated receptor agonist. We thought that the ROSG neuroprotective action was partially mediated by the upregulation of HSPs.^{9,40}

In conclusion, we have detected that SCI led to an increase in the expression of PPAR γ . The treatment with PPAR γ agonist, ROSG, induces significant neuroprotection, as does MPSS, by reducing the development of inflammation and tissue injury associated with spinal cord trauma. The ROSG may be considered a novel target of therapeutic applications in the treatment of SCI.¹⁸ It could be a more promising remedy in the absence of combined treatment modalities based on the complexity and redundancy of the inflammatory response associated with SCI.

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