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A Re-assessment of Erythropoietin as a Neuroprotective Agent Following Rat Spinal Cord Compression or Contusion Injury

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Abstract

This study was initiated due to an NIH “Facilities of Research – Spinal Cord Injury” contract to support independent replication of published studies that appear promising for eventual clinical testing. We repeated a study reporting the beneficial effects of recombinant human erythropoietin (rhEPO) treatment after spinal cord injury (SCI). Moderate thoracic SCI was produced by two methods: 1) compression due to placement of a modified aneurysm clip (20g, 10 seconds) at the T3 spinal segment (n=45) [followed by administration of rhEPO 1000 IU/kg/IP in 1 or 3 doses (treatment groups)] and 2) contusion by means of the MASCIS impactor (n=42) at spinal T9 (height 12.5 cm, weight 10 g) [followed by the administration of rhEPO 5000 IU/kg/IP/7d or single dose (treatment groups)]. The use of rhEPO following moderate compressive or contusive injury of the thoracic spinal cord did not improve the locomotor behavior (BBB rating scale). Also, secondary changes (i.e. necrotic changes followed by cavitation) were not significantly improved with rhEPO therapy. With these results, although we cannot conclude that there will be no beneficial effect in different SCI models, we caution researchers that the use of rhEPO requires further investigation before implementing clinical trials.

Keywords

spinal cord injury; erythropoietin; rat; neuroprotection; BBB analysis

Introduction

Erythropoietin (EPO) is a hematopoietic cytokine involved in the development and maturation of red blood cells (Finch, 1982). It has a molecular weight of approximately 34,000Da and is produced mainly in the kidney and in lesser amounts in the fetal liver, uterus and brain (Chikuma et al., 2000; Finch, 1982; Lacombe and Mayeux, 1999). The EPO receptor is found on a variety of non-hematopoietic cells including endothelial cells, mesangial cells, myocardial cells, smooth muscle cells, placental cells and cells of neural origin (Buemi et al., 2002; Chikuma et al., 2000; Masuda et al., 1993; Sawyer et al., 1989).

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Initial information suggested that recombinant human EPO (rhEPO) protects the spinal cord, specifically the ventral cord motoneurons in an indirect ischemic injury model in rabbits (Celik et al., 2002). A more recent study of both rat thoracic spinal cord contusion and compression injuries reported an impressive recovery in locomotor behavior and histopathological changes post-injury (Gorio et al., 2002). In addition to the anti-inflammatory properties of rhEPO, an electron microscopy study reported that acute inhibition of lipid peroxidation could be involved in the general neuroprotective effect observed with this EPO cytokine in spinal cord injury (SCI) (Kaptanoglu et al., 2004). Also, rhEPO has been suggested to work by delaying the increase in tumor necrosis factor (TNF), lowering the levels of interleukin 6 (IL-6; Agnello et al., 2002) and reducing apoptotic cell death (Cerami et al., 2002; Gorio et al., 2002; Sun et al., 2004), among others.

There could be interest in conducting clinical trials with rhEPO, given the magnitude and significance of earlier results of its administration following SCI (Gorio et al 2002). It was considered worthwhile, therefore, to replicate the study of rhEPO treatment in compressive and contusive injuries by Gorio and colleagues (2002). Attempts were made to duplicate the experimental conditions as much as possible including conversations with and scheduling a visit by Dr. Gorio to the Miami Project. The 2002 studies were performed in Izmir, Turkey (aneurysm clip model) and in Milan, Italy (University of Trieste impactor model).

Materials and methods

Compression model and treatment

Adult female Wistar rats (220–280 g; n=88; Harlan Co., Indianapolis, IN) were housed according to National Institutes of Health and United States Department of Agriculture guidelines. The Institutional Animal Care and Use Committee of the University of Miami approved all animal procedures. After the initial anesthetization with inhaled halothane, an intraperitoneal (IP) injection of pentobarbital was administered (40 mg/kg) and, following verification of an adequate level of anesthesia by assessing the corneal reflex and withdrawal reflex to painful stimuli for the hindlimbs, all animals underwent a T3 spinal laminectomy procedure as follows. The back region was shaved and aseptically prepared with betadine. The rat was placed on top of sterile gauze to elevate the surgical site, for an adequate exposure of the back, with the neck in slight flexion. During surgery, the rats were maintained on a heating pad to maintain the body temperature at $37 \pm 0.5^\circ\text{C}$.

A 2 cm longitudinal skin incision was made centered over the T3 spinous process along the midline. The rostral part of the interscapular hibernating gland (multilocular adipose tissue) was proximally dissected and lifted. There is a large median vein formed by the confluence of the veins from the hibernating gland that required proper identification to avoid injuries to the vessel and subsequent severe blood loss (Figure 1A). After identifying the vessel with the help of a surgical microscope, the dorsal fascia was incised to expose the pars thoracis of the trapezius muscle. The latter was then dissected, followed by lateral retraction of the deep thoracic vertebral muscles (splenius and spinalis muscles). Once soft tissue dissection was completed, a self-retaining retractor was placed to expose the facet joints. The spino-ligamentous complex and spinous process were removed along with the articular process and the dorsal lamina using a micro-rongeur (Figure 1B). Because of the model used in the 2002 study (60g force compression clip applied for 60 s), a 50g force clip (calibrated in and procured from Dr. M. Fehlings laboratory, Toronto, ON, Canada) was passed around the cord extradurally and, when both tips were effectively visualized (Figure 1C), the clip was released from the applicator and pressure applied for 60 s (n=40). There were four groups: (1) 1000 IU/kg rhEPO IP (Eprex, Cilag, Zug, Switzerland; n=10) immediately after surgery; (2) an initial dose of 1000 IU/kg rhEPO IP, repeated at 24 and 48 h (n=10) (3) only normal saline solution IP (n=10); and (4) injury only to be evaluated at 24 h post injury for

histopathology to reveal acute changes. Gel foam was applied on the sides of the laminectomy for hemostasis. The rats were returned to their cages with heating pads to maintain body temperature for the following 24 h and ad libitum access to water and food. Unlike the Gorio et al. study (2002) this severe injury led to a high rate of mortality that allowed only a non-statistical and brief description of observations.

Next a 20g calibrated aneurysm clip was applied for 10 s to better replicate the histological and behavioral changes reported by Gorio et al. (2002). Four groups were prepared: (1–3) as above; n=15 for each group and (4) a T3 laminectomy (no damage to the cord, n=3). Behavior was assessed in all chronic groups by two blinded examiners at 24 h and 72 h and then weekly with the locomotor open field rating scale (BBB; Basso et al., 1995).

At 4 weeks, all animals were anesthetized and intracardially perfused with cold heparinized normal saline solution and subsequently with 10% paraformaldehyde solution. Cords (n=12 per injured group) were removed and embedded in paraffin. The tissue was cut in 10 μ m sections every 90 μ m throughout the sample, and stained with hematoxylin and eosin. Data from the remaining normal cord tissue, total abnormal cord tissue and cavities were obtained for further volumetric analysis using a Zeiss Axiovert 200 M microscope and Stereo Investigator and Neurolucida Software (MicroBright Field Inc). Abnormal cord was defined as altered size and number of neurons and their normal distribution in the gray matter, small cell infiltration and cavitation (lack of tissue). Data are reported as percentages of spared tissue and cavitation areas (epicenter) and volumes (throughout the injured segment). For improved preservation and myelinated axonal morphology at the lesion epicenter, 2mm slices were removed from spinal cords (n=3 per group) and further fixed and embedded in plastic to obtain 1 μ m-thick transverse sections that were stained with Toluidine blue (Xu et al., 1995).

Contusion model and treatment

The contusion injury was induced by the weight drop device developed at New York University (Gruner, 1992) and further standardized in the MASCIS study (Young, 2002). Adult female Sprague-Dawley rats (240–260 g; n=42; Harlan Co., Indianapolis, IN), with the same preparation and care described above, underwent a T9 laminectomy under general anesthesia with halothane. Without disrupting the dura mater, the tenth thoracic (T10) spinal cord segment was exposed by removing the dorsal part of the 9th vertebra. The exposed cord was then contused by a 10 g weight dropped from a height of 12.5 mm to create a moderate contusion injury. The contusion impact velocity and compression were monitored and recorded to maintain consistency between animals. After injury, the muscles were sutured in layers and the skin was closed. Animals were categorized into four groups: rats received IP (1) a dose of 5000 IU/kg rhEPO IP (Epoetin alpha, Ortho Biotech Products, L.P. Raritan, NJ) immediately after surgery and every 24 h for the following 7 consecutive days, (2) a single dose of 5000IU/kg rhEPO IP or (3) normal saline solution following either treatment protocol. Post-operative care, locomotor behavior and paraffin sections for histological analysis were obtained and analyzed with the same methods as described for the compression model.

Statistical analysis

Data are expressed as mean percentages of spared tissue and cavitation areas and volumes +SEM. Statistical analysis of data among groups was performed using two-way ANOVA for behavioral assessment or one-way ANOVA for histology followed by Dunnett's t test where appropriate. Statistical significance was considered at $p < 0.05$.

Results

Compression model

Macroscopic observation of spinal cords from non-treated animals at 24 h post-severe compression injury at the T3 spinal level revealed an extensive area of hemorrhage in several segments in both rostral and caudal directions (Figure 2A). After sagittal sectioning of the cords, hemorrhage was predominantly visible in dorsal white matter and the extension into several segments of the cord was more evident (Figure 2B). In a cross section view of the spinal cord, a clear hemorrhagic area was visible in the white matter (Figure 2C, left). With higher magnification, there was evidence of thrombotic components in small blood vessels (Figure 2C, right). Microscopic studies of longitudinally sectioned spinal cords showed a severe lesion compromising the entire cross-sectional area at the epicenter, with evidence of severe hemorrhagic and necrotic components in both white and gray matter (Figure 2D). BBB scores of treated animals in this group appeared to be no different from the control animals (Table 1). Because mortality was high (>60%), no statistical analysis of these groups could be performed.

Following moderate compression injury, there were no significant differences in BBB scores between groups at any point in the study (Figure 3). The average value for the single 1000U EPO dose group was $11.3 (\pm 0.2)$; for the 3000 U EPO dose group, $11.1 (\pm 0.3)$ and for the control group, $10.9 (\pm 0.8)$.

Histopathological analysis of tissue following moderate compression was based on area and volume values calculated by computer extrapolation from contour analysis of single sections (Figure 4). Data from the epicenter and throughout the injured spinal cord segment are presented in Figures 5 and 6. The best tissue preservation at the epicenter was obtained in the control group (% spared tissue, 31.1 ± 2.7 ; Figure 5), although it was not significantly different from the treatment groups. The control group also had the lowest percent of cavitation ($23.3 \% \pm 5.1$; Figure 5). For the volume analysis, the largest amount of tissue preservation was in the control group (% of spared tissue throughout T3, 52.6 ± 3.3 ; Figure 6), and the lowest cavity volume also was found in the control group ($17.2 \% \pm 3.3$; Figure 6). The differences were not significant among groups.

The 1 μ m plastic sections of the epicenter of the lesion in all sampled groups revealed axonal preservation around the spinal cord perimeter (Figure 7), with some regions showing areas of axons myelinated by Schwann cells that had migrated into the epicenter. No obvious differences in the degree of white matter sparing were apparent between treatment and control spinal cord samples.

Contusion model

The locomotor open field BBB scores of the animal groups treated with multiple doses of EPO are presented in Figure 8. Data for animals treated with one dose of 5000U EPO are shown in Figure 13. There was no significant difference at any time point between the two groups. The highest average BBB score with multiple doses at any time point was $11.7 (\pm 0.3)$ for the control group and $11.4 (\pm 0.5)$ for the EPO group. Similar results were observed for the single treatment groups.

Representative samples of paraffin sections and tri-dimensional reconstructions from histological sections are presented in Figures 9 and 10, and data obtained from these sections are presented in Figures 11 and 12. The epicenter tissue was better preserved in the EPO-treated than the control group ($25.8 \% \pm 1.5$), and cavitation was greater in the control than the EPO-treated group ($37.6 \% \pm 2.5$) (Figure 11). In neither case were the differences significant. Analysis of total lesion/cavity volumes revealed that the control and EPO groups

were very similar in spared spinal cord tissue volume and cavitation following multiple dosing (Figure 12) as well as the single dose treatment (Figure 14).

Discussion

Recently, several publications have advocated the use of EPO as a neuroprotective agent for traumatic and inflammatory injuries (Agnello et al., 2002; Brines et al., 2000; Buemi et al., 2002; Celik et al., 2002; Cerami et al., 2002; Goldman and Nederguard, 2002; Gorio et al., 2002; Kaptanoglu et al., 2004; Sun et al., 2004). For SCI, it was reported that a significant improvement was obtained with the use of rhEPO in moderate compression and contusion injuries (Gorio et al., 2002). In our studies, significant chronic results were not found when similar paradigms were tested.

Whereas an attempt was made to replicate the Gorio et al., (2002) study as closely as possible, it is important to mention that there were some clear differences in the devices used to produce the injuries. For the compression injury model, we employed an aneurysm clip both calibrated in and procured from Dr. Michael Fehling's Spinal Cord Laboratory (Toronto, ON, Canada) (Fehlins and Tator, 1995; Poon et al., 2007). The closing force of the 20g clip that we used appears to have been correct. In our hands, application of this clip for 10 s led to a BBB score of ~11 at 28 days compared to 12 obtained by Poon et al., (2007) following its use for 60 s. Our data for percent volume of preserved tissue in control rats (percent spared tissue throughout T3) was $52.6 \pm 3.3\%$ and for percent volume of cavitation, $17.2 \pm 3.3\%$ (Figure 6). Poon and colleagues (2007), using similar 20g clips (for 60 rather than 10 s), observed $59.3 \pm 4.9\%$ spared tissue and 19% cavitation. The reference provided for the identity of the 60g closing force clip used by Gorio et al. (2002) was Khan et al. (1985) who mentioned a modified Kerr-Lougheed 50g clip. Whereas the source is not mentioned in the Gorio et al. (2002) or Khan et al. (1985) studies, the clip that they used had been introduced initially as a modification of this type of aneurysm clip (Rivlin and Tator, 1978).

Poon et al. (2007) found that the highest force used in their study, 35g, for 60 s led to a BBB score of 10 at 4 weeks. Gorio et al. (2002) observed 28 days following use of a 60g clip for 60 s a BBB score of 10 for the control group. We would have expected a lower score for a more severe injury. In our experiment, the 50g clip led to lower BBB scores (Table 1) and a high mortality rate that precluded statistical analysis. Clearly this model was more severe than that used by Gorio et al. (2002). Our result then led us to the application of a 20g clip to obtain behavioral results comparable to those reported by Gorio et al. (2002). With a 20g closing force applied for 10 s, our control groups appeared very similar behaviorally to those in the original report but the final results with the use of rhEPO were not significant in our case.

For the contusive injury we used the well-known NYU (MASCIS) Impactor (Gruner, 1992; Young, 2002). Given our experience with this model, obtaining the parameters to produce a moderate injury was relatively simple and accomplished without problems. We did not observe, however, significant improvements in behavior and histology. Again, it should be stressed that the MASCIS device to induce contusion differed from the impactor device developed initially at the University of Trieste. A steel rod is driven into the spinal cord with a specified velocity and displacement and the impact is assessed by a miniaturized piezoelectric dynamometer within the rod (Gorio et al., 2002). They immobilized the animals as did we although the degree of immobilization could have differed from that originally used in the Gorio et al. (2002) study. We realized that the study would not be a complete replication with a different device but reasoned that if we did not see a similar

positive effect with EPO following an injury with comparable histology and BBB scores, then the treatment did not lead to a result robust enough to consider taking it to clinical trial.

It was essential to know if the drug being applied to the animals was active. In a small group of animals (n=6; 3 control and 3 rhEPO), we tested the hematological induced changes by the injection of rhEPO into the peritoneal area. Alterations in the morphology of erythrocytes and reticulocytes as previously described (Eder et al., 1989) were clearly indicative of drug activity in the rhEPO group. In contrast, no changes were found in the non-treated control group. We assume, then, that the drug should be similar in activity to the ones originally used. The pharmaceutical companies where the drugs were produced were the same as the ones previously reported by the Gorio group (2002).

We used the same rat strains as those chosen for the Gorio et al. (2002) investigation, female Wistar rats (Harlan Co., Indianapolis, IN) for compression injury and female Sprague-Dawley rats (Harlan Co.) for contusion injury. It was important to use the same strains because it is clear that different results may be obtained depending upon the strain (e.g., Mills et al., 2001; Popovich et al., 1997). But particularly surprising is that the same MASCIS device led to twice the contusion lesion volume in female Fischer rats obtained from a California company (Harlan Co., Livermore, CA) compared to Fischer rats from Indiana (Harlan Co., Indianapolis) (unpublished observations, R. Nishi, and A. Anderson, Reeve-Irvine Research Center, University of California). Several reports have shown that the outcome of focal cerebral ischemia differs depending upon the vendor supplying the Sprague-Dawley rats (e.g., Oliff et al., 1995). Thus, the provider of animals is yet another variable in replicating studies. Unlike our animals, the rats for the Gorio et al. (2002) study were obtained from European sources.

It is evident that some variables were different in our study compared to that of Gorio et al. (2002). In a clinical scenario, a drug is expected to be effective when similar methods are used even if the pathological circumstances are not identical. We can assume at some level that the injuries produced in our laboratories were fairly similar, given the locomotor open field scores of the animals in the control groups. It would be difficult to determine if other details influenced the outcome of our results. At present, it is not completely clear if the use of rhEPO has a beneficial effect in SCI (Mathews et al., 2003). Although most reports of the use of rhEPO and some similar molecules such as asialoerythropoietin and carbamylated EPO (Erbayraktar et al., 2003; Leist et al., 2004) have shown beneficial effects, we would like to caution researchers that new studies with consistently positive findings in different laboratories around the world are recommended before implementation of any clinical trials.

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Figure 1.

Surgical approach for the T3 laminectomy and placement of the aneurysm clip. A: Visualization of the large median vein (arrow) that results from the confluence of veins in the hibernating gland. B: Spinal cord exposed after removal of soft tissues and bony structures of the posterior spine. C: The clips were calibrated to exert closing forces of 20g or 50g for either 10 sec (moderate injury) or 1 minute (severe injury), respectively. The clip was placed to compress the cord, with care not to clamp the roots. D: Spinal cord after removal of the clip.

Figure 2.

Illustrations showing the histopathological changes 24 h after severe clip compression of the cord at T3. A: External view showing hematomas in the cord parenchyma (arrow: lesion epicenter). B: The necrotic and hemorrhagic components are better observed after sagittal sectioning of the cord. Several levels rostrally and caudally exhibit secondary post-traumatic changes. C: Microvascular thrombotic events are easily visualized in transverse sections of the cord. This image is from a low thoracic level, showing that secondary injury extends far beyond the initial injury site. D: Paraffin section illustrating an almost complete disruption of the cord at the epicenter, with hemorrhagic and necrotic components on either side.

Figure 3. Locomotor open field rating scale (BBB) comparing groups in the moderate compression model. There are no significant differences at any time point.

Figure 4.

Examples of 3-D volumetric analysis of T3 sections using Neurolucida. These images are computerized reconstructions 28 days after moderate compression injury. In A, contours are defined for the analysis: green represents the contour of the cord; red, the degenerated tissue; blue, the post-traumatic cavities formed; and pink, the central canal. B and C are projections of cord samples where volumes can be easily identified and include spared gray matter (gray). D (EPO-3000 U treated), E (EPO-1000 U treated) and F (control) show samples with lesion volumes closest to the means for each group.

Figure 5. Percentages of spared normal spinal cord areas and cavity areas following moderate compression injury in the epicenter when compared to the total tissue area in the section. The best preservation of tissue and the least cavitation were found in the control group but the differences were not statistically significant.

Figure 6. Percentages of spared normal spinal cord volumes and cavity volumes following moderate compression injury throughout the T3 lesion when compared to the total tissue area in the section. The least cavitation was found in the control group but the difference was not statistically significant.

Figure 7.

Toluidine stained sections 28 days post-injury. Upper: Representative plastic section from a moderate compression injury epicenter (EPO 1000U/kg/single dose) showing peripheral axonal sparing and a large cavity in the center. Middle: Higher magnification of the upper box in the top panel (dorsolateral cord) where myelinated axons are very dense; both central and peripheral myelinated axons can be seen at higher magnification. A dorsal root is present at the top of the panel. Lower: Higher magnification of the lower box in the top panel (ventral cord) where fewer myelinated axons are seen.

Figure 8.

Locomotor open field rating scale comparing groups in the contusion model. It is evident that there was no significant difference between the EPO group and the control group. The greatest difference found was at day 21 ($p=0.08$) in favor of the control group. At 56 days, there is no difference between groups ($p=0.62$).

Figure 9.

Reconstructed samples from the moderately contused spinal cord 56 days after injury. The 3-D volumetric analysis was performed in T10 sections using Neurolucida. A–C; EPO treated group, where A shows the best tissue preservation; B, the closest to the mean; and C, the least tissue preservation. D–F: control groups showing samples corresponding to A–C.

Figure 10.

Hematoxylin and eosin stained paraffin sections from contused animals. The uppermost images correspond to the most distal areas of T10 with minimum pathological changes. The lower figures are from the same spinal cord samples at the T10 epicenter. These photomicrographs are most representative of the mean volumetric values found in the analysis of each group.

Figure 11.

Data acquired at the epicenter of the contusive lesion show that more cavitation is found in the control group, and there is better tissue preservation in the EPO-treated group although the differences were not significant.

Figure 12.
Data from throughout the contusive lesion sample shows very small non-significant differences among groups.

Figure 13. Locomotor open field rating scale (BBB) comparing control and EPO (one dose 5000U) treated groups. No significant differences between the groups was detected.

Figure 14. Percentages of spared normal spinal cord and cavity volumes in control (saline) and EPO (one dose 5000U). Differences between these groups were not statistically significant.

Table 1

BBB scores from individual survival animals with a compressive severe injury from 1 day to 21 days after the surgical procedure.

Groups	Time Post-Injury				
	1d	3d	7d	14d	21d
Control	0	0	1	0	5
Control	0	1	0	2	2
EPO1	0	0	0	0	5
EPO1	0	0	0	0	1
EPO3	0	0	0	4	1
EPO3	0	0	0	0	1

The group with the highest score corresponds to the control group. EPO1 corresponds to 1000U single dose, and EPO3 to 1000U every 24h for 3 days.