

Neuroprotection from inflammation: Experimental allergic encephalomyelitis facilitates traumatic spinal cord injury recovery.

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ABSTRACT: Passive immunization with T cells activated against central nervous system (CNS) - associated myelin antigens has been found to provide neuroprotection following CNS trauma, leading to the concept of protective autoimmunity. However, limited research exists about whether actively induced CNS autoimmunity may offer any similar benefit. In this study, the kinetics and the effect of endogenously anti-myelin activated T cells following spinal cord injury (SCI), were investigated. Experimental allergic encephalomyelitis (EAE) was actively induced in Lewis rats following immunization with myelin basic protein (MBP). In vivo 5-Bromo-2-deoxyuridine (BrdU) incorporation from activated T cells was used as a marker of T cell- proliferation. BrdU was injected on 5th, 6th and 7th day post-induction (DPI) at all EAE-animals. On DPI 8, spinal cord compressive injury was induced by a transient extradural application of an aneurysm clip at the T8 spinal level. SCI resulted in spastic paralysis of hindlimbs, in all but sham-injured animals. Recovery from SCI was significantly better in EAE-animals. Activated mononuclear cells were selectively accumulated at the site of the injury. Axonal loss was less in the EAE group following SCI. Our findings indicate that actively induced autoimmunity against CNS myelin antigens may protect spinal cord pathways from mechanical injury.

Key Words: Spinal cord injury, Experimental allergic encephalomyelitis, Neuroprotection, Autoimmunity.

INTRODUCTION

Spinal cord injury results in significant neuronal loss with dramatic, irreversible functional deficit. “Secondary degeneration” is a term that describes the expansion of neuronal degeneration to cell populations that exist in the vicinity of the initial mechanical trauma, thus escaping the lesion¹⁻³. This phenomenon is attributed to the release from the injured cells of high levels of toxic molecules which act on the cell bodies, dendrites and axons of the adjacent, non-injured, neurons⁴⁻⁶. Glutamic acid, free radicals and other neurotoxic factors have been implicated for the development of secondary degeneration^{3,6}. This finding yields

the necessity for treatment modalities directed to the rescue of neurons that initially escape the mechanical injury but potentially die due to the deleterious effects of secondary degeneration.

It is well established that surgical alignment, in the presence of fracture, and methylprednisolone administration are both important modalities in the management of acute spinal cord injuries. Methylprednisolone diminishes the cascade of secondary degeneration and this is the reason for which previous studies concluded that the accumulation of inflammatory cells in the area of trauma is a key factor for the inhibition of neural tissue regeneration^{7,8}. Other stud-

Table 1. Experimental design.

Group A (acute EAE)	Group B (acute EAE + SCI)	Group C (SCI)
Day 0	Day 0	5 th , 6 th , 7 th day
EAE induction (MBP)	EAE induction (MBP)	BrdU 10mg/rat/day
5 th , 6 th , 7 th day	5 th , 6 th , 7 th day	8 th day
BrdU 10mg/rat/day	BrdU 10mg/rat/day	SCI (T8-T9 level)
15 th or 20 th day	8 th day	15 th or 20 th day
Sacrifice	SCI (T8-T9 level)	Sacrifice
Spinal cord fixation		Spinal cord fixation
Histological study	15 th or 20 th day	Histological study
Immunocytochemistry	Sacrifice	Immunocytochemistry
	Spinal cord fixation	
	Histological study	
	Immunocytochemistry	

ies in the field of neurodegeneration have questioned this assumption claiming that presence of inflammatory cells after the injury may benefit the regeneration process of the neural tissue, the final outcome being actually the result of a competition between a “protective” and a “destructive” role of the inflammatory cells⁹⁻¹⁵. More recently, it has been shown that T cells directed against myelin antigens (autoreactive T cells) express a similar double role: they are destructive in case of demyelinating diseases, but protective in case of spinal cord injury¹⁶⁻¹⁸.

Most of the aforementioned studies dealt with exogenously administrated auto-reactive T lymphocytes. In our study, we investigate the role of the endogenously activated anti-myelin T cells due to active immunization, following experimental spinal cord injury. Experimental autoimmune encephalomyelitis was used in order to induce active immunization. According to this experimental model, CD4 T lymphocytes are activated against CNS myelin antigens, such as MBP. After their activation they cross the blood-brain barrier (BBB) and produce diffuse inflammatory reaction in the white matter of the brain and spinal cord. The disease is monophasic that is after a while it remisses without any subsequent deficit. Depending to the experimental model (antigen nature, animal specie), demyelination follows the inflammatory reaction.

Our results revealed that the presence of activated

T cells promotes the clinical improvement and restores the neurologic deficit of the injured animals.

MATERIALS AND METHODS

Animals

Female adult Lewis rats (6-8 weeks old, 180-200gr) were supplied by Charles River GmbH, Sulzfeld, Germany, and maintained in our pathogen-free animal facility. The rats were housed in a light- and temperature-controlled room and matched for age in each experiment.

Thirty Lewis rats were divided into three groups (A, B, and C) of ten (Table 1).

Group A consisted of 10 rats without SCI. In this group EAE was induced and was used as a control group in order to evaluate the grade of muscle weakness produced by the inflammation itself. Group B consisted of 10 rats with both EAE and SCI. Group C consisted of 10 rats with SCI only that is without previous EAE induction.

Fifteen of these rats (5 of each group) were sacrificed on DPI 15, whereas the other 15 were sacrificed on DPI 20 following the induction of EAE.

Induction of EAE.

EAE induction was performed by subcutaneous injection of 50mg MBP 72-85 (guinea pig myelin basic protein, Sigma, St. Louis, MO) in the plantar surface of one hindlimb¹⁹. MBP was dissolved in 200µl of

Table 2. Clinical scale for the evaluation of EAE.

Clinical score	Clinical feature
0	No overt disease
1	Limp tail
2	Paresis of 1 or 2 limbs
3	Unilateral hindlimb paralysis
4	Bilateral hindlimb paralysis
5	Bilateral hindlimb paralysis and incontinence
6	Death

incomplete Freund's adjuvant containing mycobacterium tuberculosis H37Ra (6mg/ml) (Difco Laboratories, Detroit, MI) and PBS (Phosphate Buffered Saline, pH 7.4).

Spinal cord injury at the midthoracic level

Experimental SCI was performed under general anesthesia (Xylazine, Ketamine) using a stereotactic microscope (Zeiss). After skin incision and spine exposure at the midthoracic level, a laminectomy of T8 and T9 was performed. Following that, the spinal cord was compressed by applying an extradural temporary aneurysmatic clip with closure force of 35g for 1min²⁰⁻²². According to the relative literature this protocol results in a medium-to-severe injury.

Post-operatively all of the animals received antibiotic treatment (intraperitoneal injection of Gentamycin).

Clinical assessment.

Clinical signs of EAE were graded daily on an arbitrary scale of 1 to 6¹⁹. (Table 2). Functional recovery was determined by locomotor hindlimb performance. This was scored with the open-field locomotor rating scale of Basso, Beattie, and Bresnahan (BBB) on a scale of 0 (complete paralysis) to 21 (normal mobility)²³. Twice a week, the locomotor activity of the trunk, tail, and hindlimbs was evaluated, in an open field by placing the animal for 4 min in the centre of a circular enclosure (90 cm in diameter, 7 cm wall height). Each hind limb was scored by two investigators blinded to the treatment protocol. Individual hind limb scores were averaged to provide a single score for each animal per session.

Animal care

In injured rats the bladder expression was assisted manually twice a day (three times per day during the first 48 hours after injury) until the end of the second week, by which time automatic voidance had been recovered. Rats were monitored carefully for evidence of urinary tract infection or any other sign of systemic disease. Daily inspections included examination of the laminectomy site for evidence of infection and assessment of the hindlimbs for signs of autophagia or pressure. Such animals were excluded from the study. Animals were followed up for 15 or 20 days following the induction of EAE.

In vivo BrdU incorporation of activated T lymphocytes

In order to identify T cell proliferation, 5-Bromo-2'-Deoxyuridine (BrdU, 10 mg/ml PBS) was intravenously administered in animals in vivo, during the last 3 days prior to the SCI. Those days were actually the 5th, 6th and 7th day following EAE induction in the corresponding subgroups. BrdU study of the animals with EAE induction only, provided evidence of the presence or absence of SCI-induced T cell activation/proliferation.

Histopathological study

Rats were sacrificed at DPI 15 and 20. All animals were anaesthetized with ether and were perfused with ice-cold PBS followed by ice-cold 4% paraformaldehyde in PBS, through transcatheterial perfusion. Spinal cords were removed by surgical excision of vertebra lamella and kept in 4% paraformaldehyde in PBS for a 4-hour post-fixation. Tissues were then prepared for

cryostat sections according to standard procedures. Sections of 6µm were cut and used for histological and immunohistological evaluation.

Quantification of the inflammatory process in both acute and chronic groups was performed in Hematoxylin-Eosin sections at arbitrary predefined levels of hemispheres, brain stem and spinal cord. At least 5 sections were evaluated for each one of above CNS areas. Sections were evaluated under 10x optical fields. An observer blinded to treatment and clinical severity, evaluated the total number of perivascular mononuclear infiltrates for each section. Data were then used to evaluate a) the number of infiltrating cells per perivascular infiltrations, per group, b) number of perivascular infiltrations per group, c) number of infiltrating cells per group.

Quantification of the inflammatory process and axonal pathology in both acute and chronic groups was performed in sections stained with a modified Bielschowsky silver stain method counterstained with Hematoxylin, performed in our laboratory and based on the modification suggested by Litchfield and Nagy²⁴. According to this modification incubations were performed in 4°C for 35 minutes and incubation and developing solutions were carefully prepared to reach a specific pH for each solution. All sections were then counterstained with Hematoxylin. This modification allowed simultaneous evaluation of the axonal pathology along with the inflammatory process in each section. Comparisons of the results obtained from our Bielschowsky modification with those of the classic Hematoxylin-Eosin revealed that EAE infiltrations were estimated reliably with either of the two methods and both methods revealed the same number of infiltrations and infiltrated cells in adjacent sections. Thus, our modification of Bielschowsky silver staining with Hematoxylin counterstaining was preferred for the simultaneous evaluation of inflammation and axonal pathology, on the same section, in both groups.

The evaluation of inflammation was performed by counting the total number of perivascular mononuclear infiltrates for each section and data were used to evaluate a) the number of infiltrating cells per perivascular infiltrations, per group, b) number of perivascular infiltrations per group, c) number of infiltrating cells per group. For axonal injury we used an ordinal graded scale from 0 to 4, where we applied 0 for normal axonal

morphology and 4 for severe axonal injury. Moreover, for axonal loss we also used a similar ordinal graded scale from 0 to 4, where 0 was for normal axonal density and 4 for total absence of axons.

BrdU immunohistochemistry revealed the proliferating T-cells. Briefly, 6 µm spinal cord sections were incubated in 0.3% H₂O₂ in methanol for 15 min. After TBS washes, the sections were incubated in 0.1 mg/ml proteinase K for 10min. The sections were treated with 2N HCl for 30 min and washed to neutralize the pH. Sections were incubated with anti-BrdU (clone Bu20a, Dako, 1:400 dilution) overnight at 4°C. A goat anti-mouse IgG secondary antibody (Serotec) was added for 1 hour at room temperature. Counterstaining was done with Hematoxylin.

Statistics

Statistics were performed using the SPSS 11.5 software. For clinical and histopathological scale data we initially tested their normality using Shapiro-Wilk and Kolmogorov-Smirnov normality tests in order to assess the criterion of normality for t-student test application. Wherever data were found to violate the normality assumption and a logarithmic transformation could not apply normality we used the non-parametric equivalent Mann-Whitney U test for comparison of the two groups. Wherever the criteria for t-test were met we performed the t-test for comparison of the two groups taken care to use the right p depending on the equality or not of the variances. For nominal or ordinal data we used a chi-square test for comparison of the two groups. Results were considered as “statistical significant” at the level of $p < 0,05$ and values are expressed as mean \pm SD.

RESULTS

Clinical evaluation

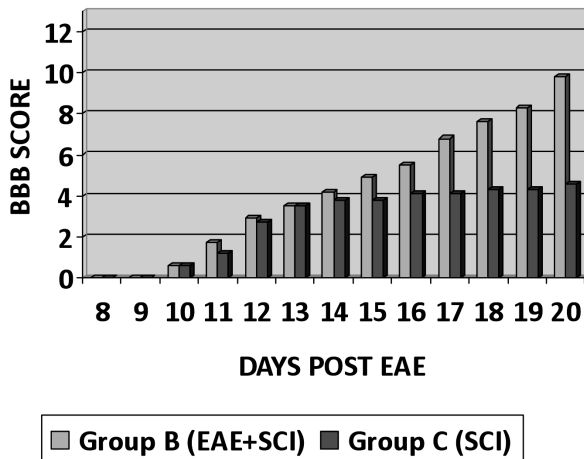
In the group with pure SCI without previous active EAE induction the mean BBB varied from 0 (on DPI 1) to 3,8 (on DPI 15) and 4,6 (on DPI 20). In the other group, in which SCI was preceded by active EAE induction, the mean BBB varied from 0 (on DPI 1) to 4,8 (on DPI 15) and 9,8 (on DPI 20).

Mean values of BBB for both groups are shown in Table 3 and are schematically represented in Figure 1.

On DPI 15 there was no significant difference in lo-

Table 3. Mean values of BBB score of Group B and C.

DAYS POST INDUCTION OF EAE	DPI 1	DPI 8	DPI 10	DPI 12	DPI 15	DPI 18	DPI 20
GROUP B	0	0	0,6	2,9	4,9	7,6	9,8
GROUP C	0	0	0,6	2,7	3,8	4,2	4,6

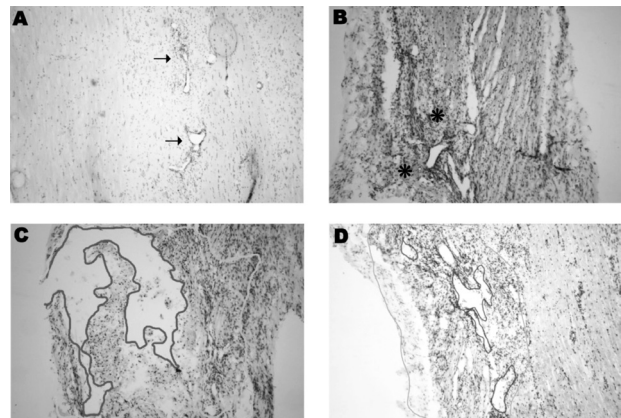
**Figure 1.** Clinical assessment (BBB score) of group B (EAE + SCI) animals which exhibited significantly better clinical scores when compared to group C (SCI) animals.

comotor activity between the animals of group B (SCI + EAE) and C (SCI only). Thereafter, the animals of group B presented a remarkable improvement of their locomotor skills, whereas the animals of group C continued deteriorating. This difference was more prompt between DPI 18 and 20 ($p < 0.05$).

The animals of group A (EAE only) presented a score of no more than 1 in the relative scale for EAE evaluation which is equal to a flaccid paralysis of the limb. This finding suggests that EAE did not influence the clinical condition of the animals.

Histological study

The animals with pure EAE presented typical diffuse perivascular infiltrations in the white matter of spinal cord (Figure 1A). In the animals with EAE and SCI

**Figure 2.** Distribution of inflammatory cells, A: in EAE animals inflammatory infiltrates were distributed mainly perivascularly (arrows) whereas, B: in EAE+SCI animals inflammatory cells were distributed diffusely (asterisks). The size of the traumatic cavity was bigger in SCI animals (C), compared to those where EAE was also induced (D). Red-dotted line highlights the injury related cavity, whereas the Green-dotted-one indicates the outer limits of the trauma.

such infiltrations were shown to accumulate in the area of trauma enriching the trauma-induced inflammation with extra inflammatory elements (Figure 1B).

The cavity due to the trauma was larger in animals without EAE (Figure 1C), but the extension of infiltrations in this group was similar or even smaller compared to the animals with SCI plus EAE (Figure 1D). These findings suggest that the size of the trauma cavity and inflammatory infiltration are not necessarily linearly correlated (Figure 1C and D, 3A and C).

BrdU is a useful index for identifying cells that are actively divided. This way we were able to locate reactive T lymphocytes in the examined spinal cord tissue (Figure 2A). In periphery, EAE-induced T lymphocytes initially activated against MBP and then

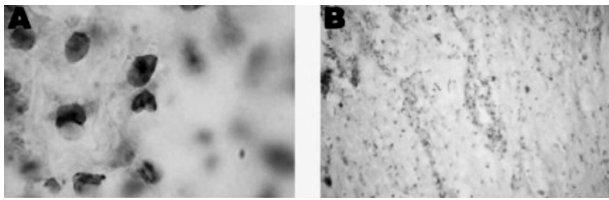


Figure 3. Proliferating inflammatory cells marked with BrdU immunohistochemistry: BrdU is incorporated in the nucleus of the divided cell (A). BrdU – positive cells may be identified as perivascular infiltrates (B).

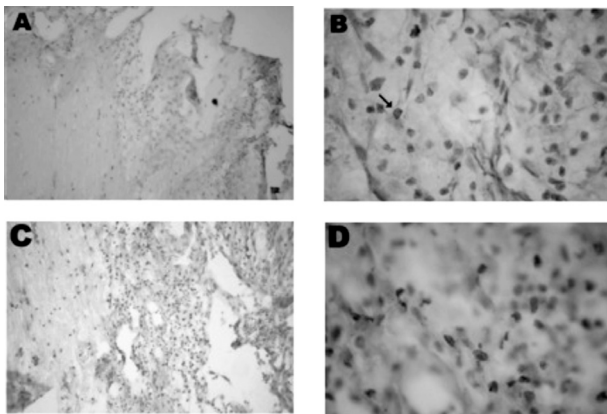


Figure 4. Inflammatory reaction in SCI (A; B, higher magnification) and SCI plus EAE-induction (C; D, higher magnification) as indicated by BrdU incorporation. In animals with SCI-only, monocytes positively stained for BrdU could hardly be detected (arrow in B) whereas the co-existence of SCI with EAE was characterized by massive accumulation of BrdU – positive inflammatory cells at the area of trauma, indicating their clear relation to EAE, though not the SCI. Cells that were negative for BrdU were counterstained with Hematoxylin.

start to invade the CNS. These lymphocytes, due to their high proliferation rate, incorporate BrdU and can be located by immunocytochemistry under light microscope (Figure 2B).

Our findings suggest that BrdU⁺ cells were almost exclusively related to EAE and not to SCI (Figure 3A,B). The SCI itself failed to provide massive inflammatory infiltrations. On the other hand, the EAE-induced inflammatory cells accumulated in the area of trauma (Figure 3C,D) and not diffusely in the spinal cord as normally expected (Figure 2B). These findings are consistent with the fact that the EAE-induced inflammatory cells exacerbate the inflammatory infiltration process in the area of trauma.

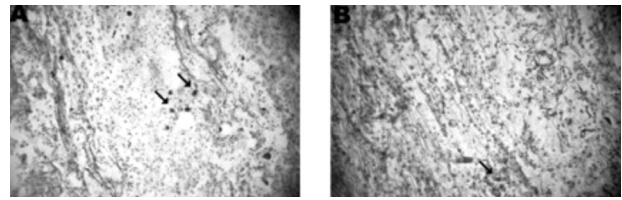


Figure 5. Axonal injury and loss as indicated with Bielschowsky's silver impregnation staining, in SCI (A) and during the co-existence of SCI with EAE (B). Evidently, higher axonal preservation as well as reduced number of axons undergoing degeneration (arrows) (B) is attributed to EAE-related inflammatory cells (Hematoxylin counterstaining).

On DPI 20 using Bielschowsky staining we found that animals of group C (SCI without EAE) presented extensive neuronal injury and loss (Figure 4A). On the other hand, animals of group B (SCI plus EAE) had a significant number of preserved neurons (Figure 4B).

DISCUSSION

Our results imply that autoimmunity can be potentially neuroprotective after a sustained spinal cord injury. Endogenously produced inflammatory cells activated against myelin antigens are selectively accumulated in the area of trauma thus boosting the initial trauma-induced inflammatory process. Under these circumstances the animals present better clinical outcome with less neurologic deficit. This clinical finding was further supported by the identification of significantly more preserved axons in the animals with SCI plus EAE.

The research about the role of inflammation in the neurologic recovery following CNS trauma was triggered by the finding that regeneration of neural tissue is much more prominent in peripheral nervous system (PNS) than in CNS.

Previous studies have shown that significant differences exist in the inflammatory process taking place in PNS and CNS after trauma. Macrophage invasion in trauma area is slower and less extensive following PNS trauma²⁵. Additionally, macrophages invading the CNS are less capable for phagocytosis of the injured myelin²⁶. Macrophages of the CNS environment, that is microglia, even though activated following mechanical trauma have minor compared to the macrophages in periphery and lasts less long²⁷.

Finally, transplantation of activated macrophages in the area of trauma has been proved to be beneficial for the regeneration process¹³.

Summarizing, it seems that we have to reconsider the role of inflammation in cases of CNS trauma. Traditionally this role was believed to be destructive but there are upcoming evidences for a different, protective action. Inflammatory cells may promote the recovery and regeneration of traumatized neural tissue recruiting mechanisms that are still under investigation.

During the last decade there's been a growing debate about the protective role of autoimmunity in CNS lesions.¹⁸ It has been shown that lymphocytes activated against CNS antigens release neurotrophic factors such as Brain Derived Growth Factor (BDNF) and promote the survival of neural cells in vitro^{28,29}.

Our results support the assumption that there is a neuroprotective role of inflammation after CNS trauma.

We confirmed the presence of significant percentage of preserved axons in the case of EAE and SCI coexistence. This finding was correlated with a better clinical outcome of the animals.

There are various experimental questions that need to be addressed. Which elements of inflammation must be promoted? Which of them must be eliminated? It's obvious that we have to find the appropriate "therapeutic window" for a given intervention and also the equilibrium between neuroprotective autoimmunity and induction or exacerbation of an autoimmune disorder. A rational approach would be a well controlled, time-limited boosting of selective autoimmune reactions.

Abbreviations: BrdU = 5-Bromo-2-deoxyuridine; CNS = central nervous system; DPI = day post-induction; EAE = experimental allergic encephalomyelitis; MBP = myelin basic protein; SCI = spinal cord injury.

Νευροπροστασία από τη φλεγμονή. Η Πειραματική Αλλεργική Εγκεφαλομυελίτιδα διευκολύνει την αποκατάσταση μετά από τραυματική κάκωση του νωτιαίου μυελού.

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ΠΕΡΙΛΗΨΗ: Η φλεγμονή που αναπτύσσεται μετά από τραυματική κάκωση του κεντρικού νευρικού συστήματος (ΚΝΣ) συμβάλλει σημαντικά στην καταστροφή που επέρχεται στον τραυματισμένο ιστό. Βρέθηκε ωστόσο ότι εξωγενώς χορηγούμενα T-λεμφοκύτταρα τα οποία προηγουμένως έχουν εργοποιηθεί έναντι αντιγόνων της μυελίνης, προσφέρουν νευροπροστασία μετά από κάκωση του ΚΝΣ. Οι παρατηρήσεις αυτές οδήγησαν στην άποψη περί του προστατευτικού ρόλου της φλεγμονής. Υπάρχει όμως περιορισμένη έρευνα σχετικά με το αν η ενδογενώς προκαλούμενη φλεγμονή, δηλαδή η ενεργητική, αυτοανοσοποίηση, μπορεί να προσφέρει τα ίδια αποτελέσματα. Στη μελέτη αυτή ερευνάται η κινητικότητα και η επίδραση των ενδογενώς ενεργοποιημένων T-λεμφοκυττάρων έναντι της μυελίνης στην αποκατάσταση του νωτιαίου μυελού (NM) μετά από κάκωση. Προκλήθηκε πειραματική αυτοάνοση εγκεφαλομυελίτιδα (ΠΑΕ) σε επίμυες φυλής Lewis μετά από ανοσοποίηση έναντι της βασικής πρωτεΐνης της μυελίνης, (MBP). Υπό τις συνθήκες αυτές, τα ενεργοποιημένα T-λεμφοκύτταρα σημάνθηκαν με Βρόμο-δεοξυουριδίνη (BrdU) in vivo. Η BrdU χορηγήθηκε στα πειραματόζωα κατά τις ημέρες 5, 6 και 7. Την 8^η μέρα, προκλήθηκε ημιδιατομή του NM με τοποθέτηση ανευρυσματικού κλιπ στο Θ8 επίπεδο. Τα πειραματόζωα εξετάστηκαν κλινικά καθημερινά μετά την ημέρα της κάκωσης. Η κάκωση του NM είχε ως αποτέλεσμα τη σπαστική παράλυση του ομόπλευρου οπισθίου άκρου. Η κλινική αποκατάσταση ήταν σημαντικά καλύτερη στα πειραματόζωα με ΠΑΕ. Ενεργοποιημένα μονοπύρρηνα κύτταρα ήταν συγκεντρωμένα εκλεκτικά στην περιοχή της κάκωσης. Η απώλεια των νευραξόνων λόγω της κάκωσης ήταν μικρότερη στην ομάδα των επίμυων με ΠΑΕ. Τα αποτελέσματα αυτά είναι ενδεικτικά του ότι η ενεργητική αυτοανοσία κατά του ΚΝΣ μπορεί να ασκεί νευροπροστασία μετά από μηχανικό τραυματισμό.

Λέξεις Κλειδιά: Τραυματική κάκωση νωτιαίου μυελού, Πειραματική αλλεργική εγκεφαλομυελίτιδα, Νευροπροστασία, Αποκατάσταση.

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