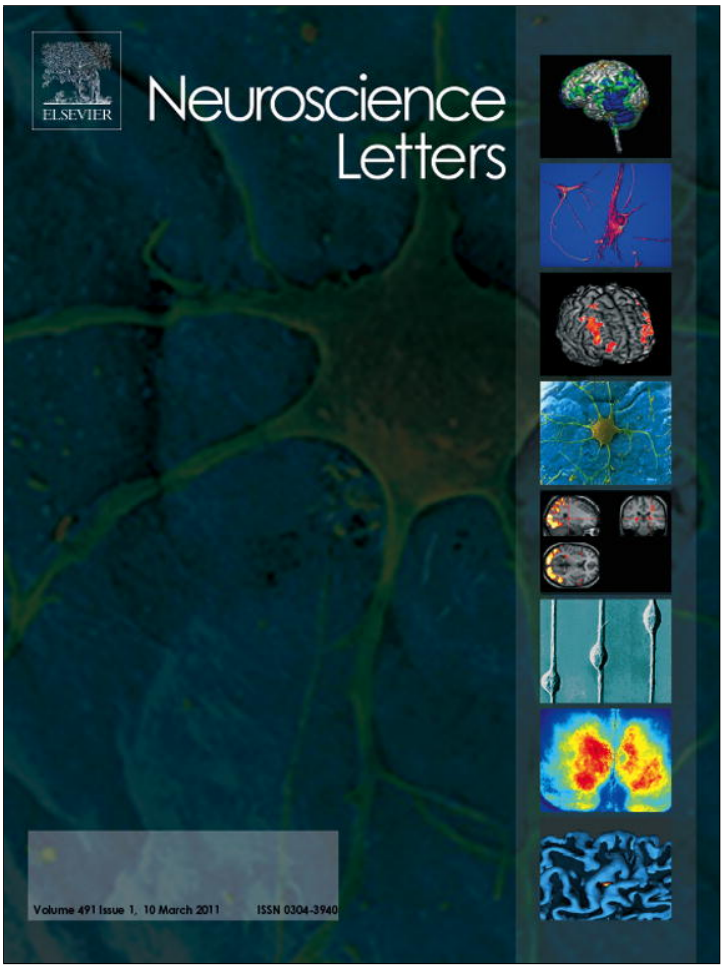

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Quadriplegia recovery after hemi-section and transplant model of spinal cord at the level of C5 and C6

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ABSTRACT

A spinal cord hemi-section with a homologous transplant of medullar tissue at the level of C5–C6 and preservation of the anterior spinal artery was used to evaluate the histological characteristics such as quantity and quality of axons, myelin index and blood vessels after quadriplegia recovery. Vascular changes after spinal injury results in severe endothelial damage, axonal edema, neuronal necrosis and demyelinization as well as cysts and infarction. Preservation of the anterior spinal artery has demonstrated clinical recuperation; therefore, in addition to the lesion we included a homologous transplant to visualize changes at a cellular level. Two groups of dogs (hemi-section and transplant) went through a traumatic spinal cord hemi-section of 50% at the level of C5–C6. The transplant group formed by animals which simultaneously had 4 mm of spinal cord removed and the equal amount substituted from a donor animal at the level of C5–C6 corresponding to the half right side; both preserving the anterior spinal artery. Histological evaluation of all groups took place at days 3 (acute) and 28 (chronic) post-operation. Changes of degeneration and axonal regeneration were found in the hemi-section and transplant groups at acute and chronic time, as well as same quadriplegia recovery at chronic time in the hemi-section and transplant groups which closely related to mechanisms which participate in regeneration and functional recuperation due to the preservation of the anterior spinal artery and presence of new blood vessels.

"Spinal Shock" is a term applied after an anatomic injury of the spinal cord [14,16]. Ditunno describes in a four-phase model the evolution mechanisms of the spinal shock. These include: Phase 1, 0–1 day, hyporeflexia (motoneurons hyperpolarized); Phase 2, 1–3 days, reflex return (denervation supersensitivity, NMDA receptor up-regulation); Phase 3, 1–4 weeks, early hyper-reflexia (synapse growth, short axons and axon supplied); Phase 4: 1–12 months, late hyper-reflexia (synapse growth, long axons and soma supplied) [7]. However, little attention has been placed concerning the relationship between SCI and vascularization–revascularization.

Vascular events following SCI are characterized by (a) breakdown of blood-spinal cord barrier, (b) edema formation, (c) ischemia and hypoxia and (d) release of vasoactive substances and alteration of spinal cord perfusion [9].

Whetstone, 2003, demonstrated the importance of the vascular response after SCI, this being the first report that defines and characterizes barrier permeability in injured and regenerating blood vessels after SCI in the mouse [17]. The use of vascular endothelial growth factor (stimulator of angiogenesis and vascular permeability), acutely administered, hastens neurobehavioral recovery by day 28 post-SCI [12], showing the importance of vascularization after SCI [12,17].

Previous work done at our laboratory has demonstrated the importance of preserving adequate vascularity after SCI. One of our micro-vascular studies demonstrated clinical recuperation after preserving vascular flow to the anterior spinal artery (C5–C6 levels) [3]. However, with this model it was not possible to visualize changes at a cellular level at acute and chronic time produced by hemi-section of the spinal cord. In addition to the lesion, a homologous transplant of the spinal cord is included to increase and describe the importance of the vascularity. Therefore, the results may help to resolve the importance of the mechanisms involved in the SCI and in this way contributing with some of the plastic response observed after spinal injury and the importance of vascularization.

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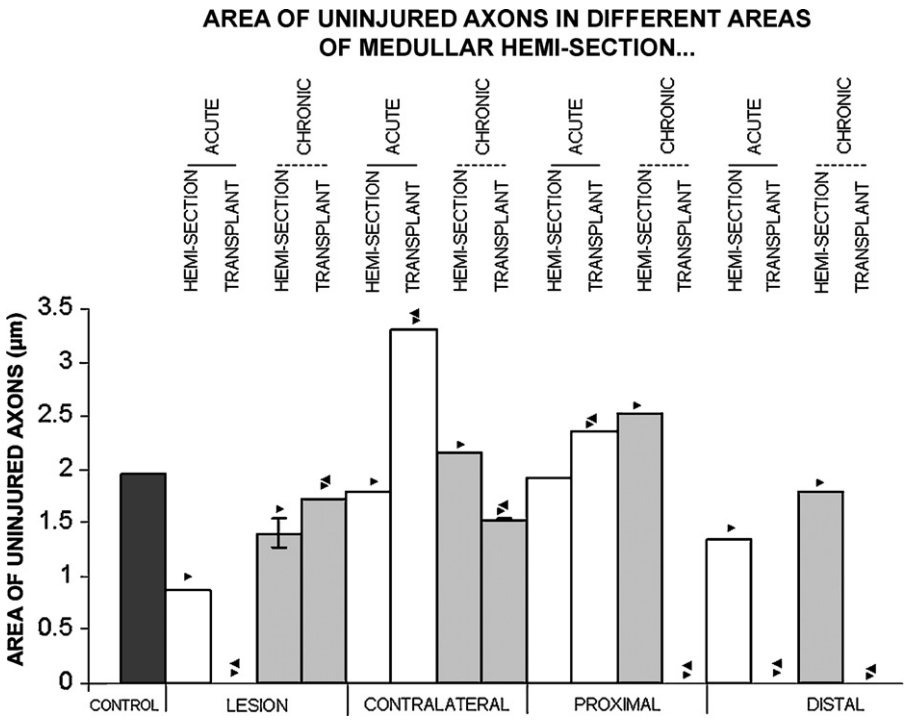


Fig. 1. Area of uninjured axons. Statistical comparisons are showed by ANOVA using a Dunnett's *t*-test between control animals with spinal cord intact (►) and sectioned or sectioned and transplanted animals. For specific paired comparisons between sectioned versus sectioned and transplanted animal groups a Student's *t*-test was employed (◄). A $p \leq 0.05$ was considered significant.

The handling of animals was in accordance with the Mexican official Norm (NOM-0062-ZOO-1999) in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals (NIH Publications No. 82-23, Revised 1978). Male mongrel dogs weighing between 25 and 30 kg were divided in two working groups besides the control group (spinal cord intact). The experimental group: transplant ($n = 4$), animals simultaneously had 4 mm of spinal cord removed and the equal amount substituted from a donor animal at the level of C5–C6 corresponding to the half right side. Comparative group: hemi-section ($n = 4$), animals which had the spinal cord sectioned, corresponding to the half right side at the C5–C6 level. After treatment the animals were kept under optimal housing conditions for a period of acute (3 days) and chronic (28 days) time.

We studied the morphology of the spinal cord with semi-fine cross sections embedded in epoxy resin (poly/bed) and stained with toluidine blue method. Areas studied: (1) transplanted area, (2) contralateral area of the transplant, (3) 0.5 cm proximal to the transplant and (4) 0.5 cm distal to the transplant.

The myelin index, which is the ratio of axon diameter to axon diameter plus its myelin sheath [10,18], was measured at 400× with the aid of an Image Analyser (Carl Zeiss 3) on ten fields.

By using the same tool, axons where quantified and their dimension was obtained (area, perimeter, diameter). Uninjured axons were oval in shape with a smooth contour and an even, non-disrupted myelin sheath surrounding them, with no vacuoles present, just as they appeared in the histological samples of the control group of animals.

Number, size and distribution of blood vessels as appear in results are the mean of ten fields explored with the Image Analyzer, which served to account for the number of vessels per field and to measure their areas.

Daily we recorded the following clinical variables: muscular contractions, upper and lower limb motor activity, tail mobility, response to painful stimuli, sphincter control function and possible sequel; using Daniels motor scale and the American Spinal Injury Association (ASIA) Impairment Scale [1,8].

In the hemi-section acute cases the histology shows a decrease in the area of uninjured axons (AUAs) in the lesion, proximal, contralateral and distal zones at acute time, $p \leq 0.05$ (Fig. 1). Furthermore, the lesion and distal zones in the transplant cases show a decrease in the AUA at acute time, $p \leq 0.05$ (Fig. 1). At chronic time in the hemi-section cases a decrease was shown in the AUA in the lesion and distal zones. Furthermore, lesion, contralateral, proximal and distal zones in the transplant chronic cases show a decrease in the AUA $p \leq 0.05$ (Fig. 1). An axonal decrease area in the transplant cases in the lesion and distal zones was higher in comparison with the section cases at acute time, $p \leq 0.05$ (Fig. 1). On the other hand, at chronic time the lesion zone didn't show difference in transplant and hemi-section cases, $p \leq 0.05$ (Fig. 1). At chronic time the transplant cases in the proximal and distal zones show a decrease in the AUA in comparison with the hemi-section cases, $p \leq 0.05$ (Fig. 1). Furthermore, at chronic time the transplant cases in the contralateral zone show a decrease in the AUA in comparison with the hemi-section cases, $p \leq 0.05$ (Fig. 1).

The analysis of myelin index in uninjured axons (MIUA) in the hemi-section cases shows a decrease in the lesion, contralateral and distal zones at acute time, $p \leq 0.05$ (Fig. 2). However, the proximal zone showed an increase in MIUA in the hemi-section at acute time, $p \leq 0.05$ (Fig. 2). At chronic time in the hemi-section cases a decrease was observed in the lesion and distal zones, $p \leq 0.05$ (Fig. 2). Nevertheless, at chronic time an increase was observed in MIUA in the hemi-section in contralateral and proximal zones, $p \leq 0.05$ (Fig. 2). Furthermore, in the transplant cases a decrease in MIUA was observed in the lesion and distal zones at acute time, $p \leq 0.05$ (Fig. 2). However, in the contralateral and proximal zones an increase in MIUA at acute time was observed, $p \leq 0.05$ (Fig. 2). On the other hand, at acute time the transplant cases in the lesion and distal groups show a decrease in the MIUA in comparison with the hemi-section cases, $p \leq 0.05$ (Fig. 2). However, at chronic time

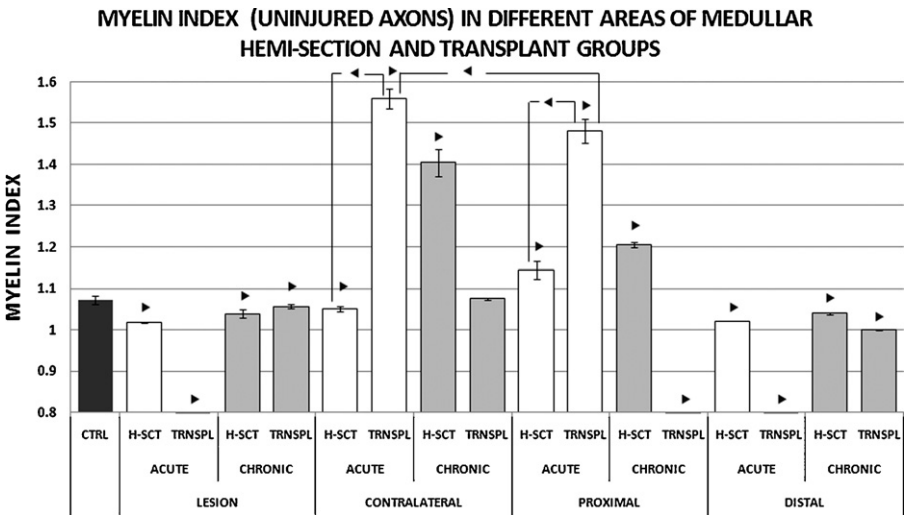


Fig. 2. Myelin index in uninjured axons. Statistical comparisons are showed by ANOVA using a Dunnett's *t*-test between control animals with spinal cord intact (►) and sectioned or sectioned and transplanted animals. For specific paired comparisons between sectioned versus sectioned and transplanted animal groups a Student's *t*-test was employed (◄). A $p \leq 0.05$ was considered significant.

the transplant cases in the contralateral and proximal groups show a decrease in the MIUA in comparison with the hemi-section cases, $p \leq 0.05$ (Student's *t*-test) (Fig. 2).

The control group presents well defined cordons exhibiting a normal cyto-architecture. On the other hand, in the lesion zone at acute time we found wide necrotic areas with small vacuolized axons accompanied by cyto-architectural alterations, in the contralateral zone the transplant exhibited a similar pattern and in the proximal zone, we observed a preservation of the cyto-architecture with multiple degenerated axons and the presence of large blood vessels. And lastly, in the distal zone axonal degeneration and cellular death was observed (Fig. 3). Furthermore, in the acute transplanted group in the lesion site we observed axonal death, degeneration and extensive necrotic areas. In the contralateral zone we observed preservation of the cyto-architecture with vacuolar axonal damage and the presence of blood vessels, in the proximal zone we observed isolated axonal degeneration and in the zone distal to the site of injury we observed massive axonal death and degeneration as well as areas of predominantly perivascular necrosis and the presence of large blood vessels (Fig. 3).

On the other hand, in the hemi-section cases at chronic time: the lesion zone shows a macro and micro-vascular axonal degeneration with large areas of necrosis. In the contralateral zone we observed regional changes in the cyto-architecture with uninjured and injured axons in different stages of degeneration and death, as well as the presence of blood vessels, in the proximal zone cyto-architecture changes were observed, with isolated and diffused axonal alterations interspersed with multiple normal axonal areas. In the distal zone we observed multiple areas of vacuolar degeneration and axonal death with partial loss of cyto-architecture, with preservation of some axonal fascicles as well as the presence of blood vessels (Fig. 3). In the chronic transplanted group, in the zone of lesion generalized vacuolar degeneration was found, but to a lesser degree to that observed in the acute group of the same segment. In the contralateral zone, we observed massive degeneration and death as well as areas of intense necrosis and multiple blood vessels. In the proximal zone to the site of the lesion, we observed multiple axons, in the stages of degeneration and death as well as diffused necrotic areas. Lastly, at the distal zone, we found the presence of well defined fascicles that nevertheless exhibited severe axonal destruction. Likewise we found large areas of necrosis as well as the presence of blood vessels (Fig. 3).

The clinical evaluation in hemi-section and transplant cases exhibit on the 1st and 3rd day spontaneous respiratory movements and muscular contractions. On the 3rd day tail movements and motor activity in upper and lower limbs was observed. After 6th and 8th day there was a recovery of the sphincter control. On the 8th and 16th maximal neurological coordination was observed. Daniels motor scale = 5 (normal), American Spinal Injury Association (ASIA) Impairment Scale = E (normal) (Table 1).

The number of blood vessels in the different segments of medullar transplant and section exhibit a higher increase with respect to the control group, $p \leq 0.05$. On the other hand, the different segments of acute section and acute transplant didn't show higher difference in the number of blood vessels. However, the segments of chronic transplant exhibit a higher increment (50%) in comparison with chronic section, $p \leq 0.05$.

This is the first study to examine the adequate vascular blood supply to the zone of hemi-section and/or transplant. In this manner, an adequate blood supply is a critical factor for the recuperation of traumatic lesions of the spinal cord. A well vascularized lesion allows the inward growth of glial and other cells which provide the framework for cell regeneration and the proceedings that induce revascularization or angiogenesis which in turn reduce the progressive chain of events that lead to tissue necrosis [11].

The first group (hemi-section) had a severe traumatic lesion as the one performed to a fine and delicate m edullar segment (C5–C6 level), and having caused a medullar section of 50% of the right side, involving motor as well as sensitive regions. In the second group (transplant) the lesion was even larger (a 4-mm resection). It was a resection of a medullar segment, in fact, which means two axial sections, a significant greater trauma. That explains why uninjured axons were decreased both in proximal and in distal zones in transplanted groups. The resected piece of tissue was then replaced with homologous spinal cord tissue (taken from another dog operated simultaneously). But we did not expect a better response in this experimental group and we are not proposing the transplant as a treatment. We are demonstrating that when we resected a segment of spinal cord and replaced it with a similar segment tissue, recuperation occurs only if an adequate blood flow exists.

The importance of preserving adequate vascularity in the lesion zone has been demonstrated in work carried out in our laboratory [3] which is strictly correlated with the importance of the vascular response after SCI shown by Whetstone et al. [17]. There-

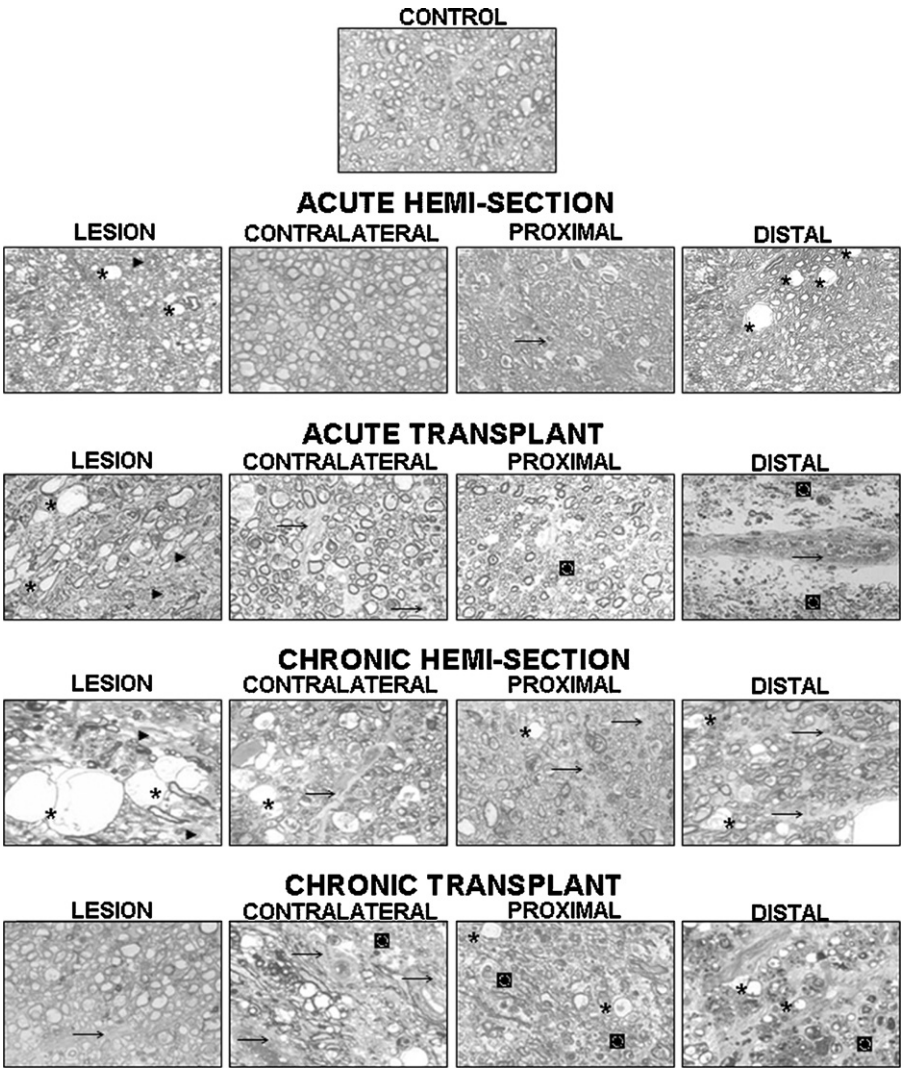


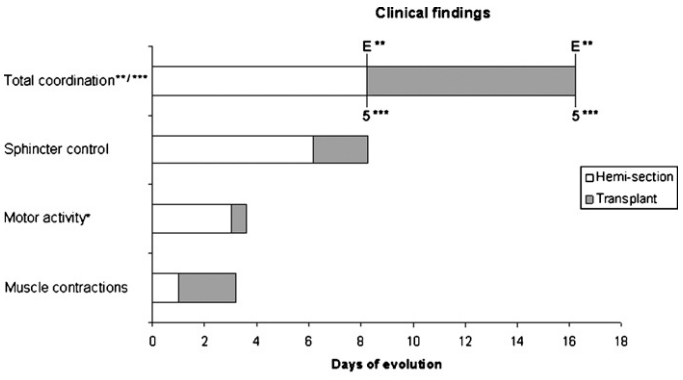
Fig. 3. Coronal sections of the spinal cord at the level of C5–C6 (400×). Samples were obtained from: control and the areas of the hemi-section and transplant cases, site transplant, the contralateral zone, the proximal zone and at the distal zone. The control group present well defined cordons exhibiting a normal cyto-architecture. The acute hemi-section, acute transplant, chronic hemi-section and chronic transplant exhibit different grades of degeneration: necrotic areas (►), degenerated axons (◙), vacuolized axons (*) and vascular recovery: blood vessels (→).

fore, the vascularization after SCI hastens neurobehavioral recovery [3,12,17].

An unusual size of axons join to abnormal shape and high metabolic activity make them more susceptible to injury, transport defects, ischemia, oxidative damage and protein-turnover defects [5]. Furthermore, the central nervous system (CNS) has the capacity to remyelinate after a demyelinating event has occurred [15]. A process that has different important functions, including lesional repair, protection of axons, and restoration of conduction velocity [4].

A change in the axonal area and degenerative process was observed in different zones of both the acute hemi-section group and the acute transplant group. In the acute transplant group an important change was observed in the lesion, contralateral, proximal and distal zones which show an increase in the injury to the spinal cord in comparison with the acute hemi-section by this medical proceeding (Figs. 1 and 3). Furthermore, in the contralateral and proximal zones corresponding to the acute transplant group, a significant increase $p \leq 0.05$ (ANOVA), in myelin index was observed. We speculate that this event could represent a compensatory mechanism until a complete degenerative process is settled in, as it was shown in these same zones during the chronic stages.

Table 1
Clinical evaluation. Maximal neurological coordination was observed on the 8th (hemi-section) and 16th (transplant) day.



*Upper and lower motor activity. Tail movement
**ASIA Impairment Scale = E (normal)
***Daniels motor scale = 5 (normal)

We do not think these changes could be edema or swelling produced by trauma, because myelin swelling due to spinal cord trauma has been described in different terms: attenuated myelin sheaths, splaying of the myelin lamellae, and a marked increase in the periaxonal spaces. At 4 h after contusion approximately one-fourth of the fibers showed breakage of the myelin sheaths and consequent denuding of axons or marked attenuation of the myelin sheaths, greatly enlarged periaxonal spaces, and degeneration of the associated axons.

The increase of myelin index in the contralateral and proximal areas corresponds to regular, smooth myelin sheaths suggesting a site of nervous conduction, myelin being an electric insulating material that forms a layer, the myelin sheath, which is usually around the axon of a neuron facilitating the transmission of nerve impulses maintaining a protective process besides recuperating electrical activity. This was observed permitting gradual clinical recuperation (as observed at 8th and 16th day) (Table 1).

Enhanced regeneration and compensatory fiber growth in the brain and spinal cord after injury have been achieved by a local improvement of the growth environment for regenerating and sprouting fibers [13]. Such an improvement of the growth environment is provided primarily by maintaining an adequate vascular flow, as we think.

As a matter of fact, this is the starting point of other studies using animal models (rats or cats) which clearly indicate that in large but incomplete spinal cord injuries, locomotion training may lead to enhanced recovery of function. Adding a treatment that facilitates regenerative and compensatory fiber growth can, therefore, is expected to further augment the effect of rehabilitative training [6] (Fig. 3). However, the acute section and acute transplant exhibit an increase in the presence of blood vessels with normal characteristics (Fig. 3) this corresponding to an increase in vascularity as a manifestation of probable regenerative activity.

On the other hand, in the chronic transplant groups we observed severe axonal area change, decrease of myelin index and degenerative process in the contralateral and proximal zones (Figs. 1–3). Likewise, distal zone shows axonal death and degeneration (Fig. 3). Nevertheless, normal appearing vessels were observed (Fig. 3).

In contrast, in the acute transplant group we observed an important change in axon size (Fig. 1), higher decrease in myelin index (Fig. 2) and degenerative process (Fig. 3), especially in the distal zone where blood vessels were seen occupying the size of the whole visual field (74,000 μm). In the chronic transplant group severe degeneration was observed also, but accompanied by a great amount of normal vessels (Fig. 3). This would seem to indicate medullar regeneration mainly in the distal zone to the site of injury.

With respect to the zones which exhibited damage and regeneration, both in the transplant as well as the hemi-section groups, the precise mode of action by which damage, regeneration and functional recuperation occurs is unknown. However, a previous work from our laboratory reflected that an excessive activation of Glu-R leads to NMDA subunit expression modification [2]. These facts might reflect an abnormal structure by assembling a different NMDA-R subunit composition, mainly NR2C and NR2A, which could influence changes in permeability properties for these receptors and promote an excessive activation of Glu-R [2]. This could be closely related to mechanisms which participate in damage, regeneration and functional recuperation [2] correlated NMDA receptor up-regulation (NR1 and NR2A subunits) as proposed by Ditunno, in phase 2 (1–3 days), on his four-phase model of the evolution mechanisms of the spinal shock [7].

Furthermore, the histological evaluation indicates simultaneous degeneration and regeneration changes which are evidenced in the zones (Figs. 1–3). Therefore, we may presume that the preservation integrity of the anterior spinal artery could play an important role in the recuperation after a SCI and the vascularization response after SCI hastens neurobehavioral recovery, this vascular neurobehavioral recovery being dependent of the aggressiveness of the trauma (Figs. 1 and 3).

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