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Thalidomide fails to be therapeutic following contusive spinal cord injury in rats

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Mechanical damage to the spinal cord (SC) generates self-destructive processes that contribute to post-traumatic neurodegeneration. Because thalidomide apparently counteracts these effects its use clinically has been proposed enthusiastically. Nonetheless, we tested its action as a neuroprotectant in a clinically relevant model of SC injury in rats. We administered thalidomide intraperitoneally to rats subjected to thoracic SC contusion as single or repeated doses within the first 24 h after injury. Edema, neutrophil infiltration, and cord tissue preservation/destruction were assessed in the SC 24 h after injury and motor function for 7 weeks. Rats treated with thalidomide showed significant increase in SC water compared with naïve rats, but not vehicle-treated rats; their neutrophil infiltration and amount of spared/destroyed cord tissue was not different from vehicle-treated rats; and in no case was motor performance improved after thalidomide. In conclusion, thalidomide failed here to be therapeutic, discouraging its use clinically for SC trauma.

Key words: acute inflammatory reaction, vasogenic edema, neuroprotective drugs, secondary injury, TNF, VEGF

INTRODUCTION

It is widely accepted that spinal cord injury (SCI) causes immediate mechanical damage to spinal cord (SC) parenchyma, and is followed by a cascade of self-destructive events that worsens the primary lesion. Reducing these secondary mechanisms of injury would be expected to diminish the risk of progressive post-traumatic SC degeneration (Baptiste and Fehlings 2007). At present, there are no pharmacologic treatments with clinically established efficacy that promote neuroprotection after an acute SCI. Methylprednisolone, once regarded as the standard treatment for acute SCI, is falling into disuse due to its doubtful efficacy and associated increased risk of serious complications (Hurlbert and Hamilton 2008, Suberviola et al. 2008). The development of novel, secure, and effective therapeutic strategies to block neurological secondary injury for improved morphologic and functional outcome are of paramount importance.

Key factors in the self-destructive processes that follow cord trauma include: (1) overproduction of proinflammatory cytokines, particularly of tumor necrosis factor-alpha (TNF-α) whose detrimental role aside from its proinflammatory effect (Klusman and Schwab 1997) has also been attributed to its active role in the promotion of neuronal and oligodendrocyte apoptosis (Yune et al. 2003) and to its capacity to promote excitotoxic damage (Hermann et al. 2001); (2) edema (Wagner and Stewart 1981), attributed to increased vascular permeability at sites of traumatic injury, associated with overexpression of vascular endothelial growth factor (VEGF) (Skold et al. 2000, Akiyama et al. 2004), and (3) an inefficient immunological restorative response (Hausmann 2003).

Thalidomide, a derivative of glutamic acid, was withdrawn from the market because of its serious teratogen effects. However, due to its unique pharmacological properties including anti-inflammatory, immunomodulatory, and anti-angiogenic effects, thalidomide and

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thalidomide analogues have been reintroduced as treatment for diverse chronic immunological/inflammatory diseases and several types of cancer (Tseng et al. 1996), and suggested as a promising treatment for neurodegenerative diseases (Greig et al. 2004).

The anti-inflammatory properties of thalidomide include its capacity to inhibit migration and phagocytic activity of neutrophils and macrophages, by importantly reducing the production of TNF-α and interferon-gamma (Tseng et al. 1996, Paravar and Lee 2008). Thalidomide's immunomodulatory effects are based on its capacity to modify T helper cell phenotype from a proinflammatory Th1 to an anti-inflammatory Th2 pattern, on the basis of the type of cytokines produced (Oliver 2000). Thalidomide's anti-angiogenic and anti-edema properties correlate with its ability to significantly inhibit the VEGF secretion and the cellular response to it (Melchert and List 2007).

Because of its pharmacological properties, here we tested the potential therapeutic benefits of thalidomide in a clinically relevant model of SCI using several dose schemes of the drug to block putative key mechanisms of secondary damage. Our findings, however, do not bear out thalidomide's potential as a neuroprotectant for this condition. Very recently two studies were reported on the influence of thalidomide on experimental traumatic SCI (Genovese et al. 2008, Koopmans et al. 2009). Below, in the discussion section, we will compare their contrasting results with the observations we describe here.

METHODS

Experimental design

We evaluated the influence of short-term (24 h) and long-term (7 weeks) thalidomide on several parameters in adult female Long-Evans rats, weighing 240–260 g, subjected to SC contusion. Injured rats receiving vehicle (saline solution) and naïve rats were control animals. Thalidomide, kindly donated by Laboratorios Serral (Mexico City, Mexico), was injected intraperitoneally at 100 mg/kg per dose in 3 ml of a saline solution. Post-injury dosing schemes and corresponding studies are described in Table I.

Animal experiments were carried out in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Anesthesia, injury, and care

Before surgery, animals were anesthetized with ketamine (80 mg/kg) and xylazine (8 mg/kg) given i.m. Through a laminectomy at T9 level the dorsal aspect of the SC was exposed, maintaining meninges intact. The SCI was produced by contusion of moderate intensity using the New York University weight-drop impactor device (MASCIS impactor), as previously described (Guizar-Sahagun et al. 2005). Briefly, after laminectomy the spine was stabilized with clamps fastened to T8 and T10 spinous processes, held by a stereotaxic frame; the impactor rod was dropped onto the exposed dura, from a height of 25 mm. Post-surgical care included manual expression of bladders twice a day until bladder function returned. Animals were housed individually in a temperature-regulated environment and kept on a 12 h light/dark cycle. Food and water were provided *ad libitum*. As prophylactic for infections, 8 mg/kg of ciprofloxacin lactate (Bayer, Mexico) were given subcutaneously every 12 h, starting at the end of surgery and for 7 consecutive days. To prevent self-mutilation, acetaminophen (Cilag, Mexico) was given in the drinking water at an approximate dose of 64 mg/kg/d for 3 weeks.

Assessment of SC water content

To evaluate the effect of thalidomide on SC edema, 24 h after injury each rat was sacrificed by bolus injection of sodium pentobarbital. Immediately afterwards a 2 cm-long segment of SC was removed, with the injured area in the center. The specimen was weighed (wet weight), dried at 56°C for 6 h and reweighed (dry weight). Data are presented as percentage of water in SC specimens, which was calculated as follows: % water content = [(wet weight − dry weight)/wet weight] × 100.

Tissue preparation for morphological assessment

For evaluations (general histology, neutrophil count, and measurements of damaged/spared cord tissue), injured animals were euthanized 24 h after injury. Rats were deeply anesthetized with pentobarbital and perfused by intracardiac puncture with 200 ml saline solution, followed by 500 ml 10% buff-

ered formaldehyde. A 2 cm-long segment of cord centered at the injury zone was removed, post-fixed for 1 week in the same fixative, and then processed for paraffin embedding. Every 2 mm, coronal 6 µm-thick sections of the SC were stained with hematoxylin and eosin for further morphological and morphometric analysis.

Morphologic study of the SC

General histopathological characterization as well as morphometric analysis of damaged and spared cord tissue was performed in a blinded fashion. The lesion at the epicenter and 6 mm rostral and caudal to it, studied every 2 mm, were digitally photographed through an ×4 objective using a bright field microscope. The area occupied by each of 3 sections of different tissue quality (complete tissue destruction, spared tissue with microcysts, and healthy appearing spared tissue), as well as the entire SC cross-sectional area, were measured using the Image Pro Plus software (Media Cybernetics, Silver Spring, MD). Data are presented separately for each level and treatment condition studied.

Assessment of acute inflammatory response

To determine the rostro-caudal extent of the acute inflammatory response in the injured SC, we counted the number of neutrophils in cord sections 24 h following the lesion, when neutrophil influx into the contused rat SC is known to peak (Carlson et al. 1998).

Histologically neutrophils were identified from their morphologic characteristics, namely as round cells of approximately 10 µm diameter with multilobular nuclei and neutrophilic granular cytoplasm. Using the ×100 objective, neutrophils were counted in areas of complete tissue destruction at the epicenter (in 10 consecutive fields), as well as 4 mm rostral and caudal from it (usually between 4 and 6 fields covering the total infarct area). Data are reported as the average of neutrophils per 0.02 mm^2.

Table I

Experimental design				
Post-injury dosing schemes		Studies after injury		
		at 24 hours		at 7 weeks
Thalidomide 100 mg kg^{-1} per dose		Edema (*n*=6)	Morphology (*n*=4)	Motor function (*n*=8)
Single dose at	10 min	X	X	X
	4 h	X	X	X
	24 h			X
Repeated doses starting at	10 min[1]			X
	4 h[2]			X
	24 h[3]			X
[4] Vehicle		X	X	X
[5] Naïve		X	X	

[1, 2, 3] Subsequent doses every 24 h for 5 days; [4] single 3 ml saline solution injection 4 h post-injury; [5] non-treated intact rats

Assessment of motor function

For analysis of locomotor performance, contused rats were assessed individually for 4 min in an open field using the Basso, Beattie, and Bresnahan (BBB) rating scale (Basso et al. 1996). Rats were assessed on day one after surgery, and then once a week for 7 weeks by two blinded examiners.

Statistical analysis

Results of water content and morphometry were analyzed using the one-way ANOVA test followed by Tukey's test. Neutrophil count was analyzed using the Kruskal-Wallis test followed by Dunn's multiple comparison test. Mean and standard error of the mean scores on the BBB rating scale were plotted as a function of time post-injury, and assessed using the repeated measures ANOVA test followed by Tukey's multiple comparison test. Differences were considered significant when $P<0.05$.

RESULTS

Edema

The increase in cord water content of injured rats treated with vehicle, compared with naïve rats (average 6.24%) was not significant. Injured rats treated with thalidomide showed a significant increment of SC water compared with naïve rats (average 9.21% and 8.48% for rats given thalidomide 10 min and 4 h after injury, respectively, $P<0.05$). No significant differences were found when comparing vehicle-treated rats with thalidomide-treated groups (Fig. 1).

Acute inflammation

Neutrophils were absent in SC tissues of naïve rats. For each of the 3 cord segments in injured rats (rostral, epicenter, and caudal), rats treated with thalidomide 4 h after injury showed the lowest number of neutrophils. This trend was significant only at the epicenter when comparing 10 min vs. 4 h thalidomide-treated groups (Fig. 2).

Motor function outcome

Hind limb performance showed complete bilateral paralysis at day 1 following SCI for all rats, and gradually improved thereafter until the end of the study at week 7. Analysis of BBB performance of rats treated with a single dose of thalidomide (Fig. 3A) showed that rats given the drug 10 min after injury had the poorest functional scores compared with groups that received thalidomide 4 h and 24 h after injury, and vehicle ($P<0.05$, $P<0.001$, and $P<0.01$, respectively). For rats treated with repeated doses of thalidomide (Fig. 3B), the group that was started on the drug 10 min after injury showed a significantly better functional outcome than rats started on thalidomide 24 after injury ($P<0.01$). In no case did groups treated with thalidomide show a better motor function recovery than controls.

Histopathology and morphometry

Histopathology of cord sections from rats euthanized 24 h after injury showed variable alterations, depending on their proximity to the injury site and their location within the SC cross-sectional area. Three levels of cord tissue damage/preservation were identified: (1) complete tissue destruction showing extensive bleeding, infarcts (areas of tissue pallor with complete loss of histological architecture), and acute inflammatory infiltrate; (2) spared tissue with microcysts, and (3) healthy-looking spared tissue (Fig. 4). The healthy-looking spared tissue at the epicenter may contain scarce microcysts.

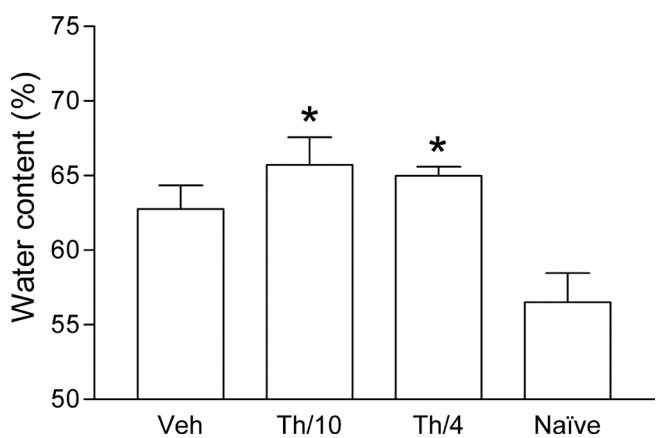

Fig. 1. Percentage of water content 24 h after SCI. Rats treated with a single dose of thalidomide 10 min (Th/10) or 4 h (Th/4) after injury showed a significant increase in cord water compared with naïve rats (*$P<0.05$). Differences among injured rats treated with vehicle (Veh), and rats treated with thalidomide were not significant. Data are expressed as mean ± SEM ($n=6$). Statistical analysis – One-way ANOVA.

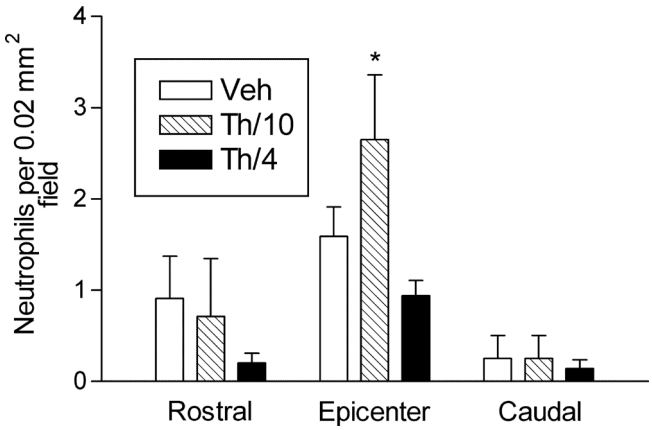

Fig. 2. Neutrophil count (per 0.02 mm^2 field) at the epicenter, as well as 4 mm rostral and caudal to it. (*) difference between 10 min (Th/10) and 4 h (Th/4) thalidomide-treated groups, $P<0.05$. Data are expressed as the mean ± SEM (n=4). Statistical analysis – Kruskal-Wallis test.

The highest amount of complete tissue destruction was seen at the lesion epicenter (Fig. 4A) as well as 2 mm rostral and caudal to it involving grey matter and adjacent white matter (Figs 4B and C). At sites 4 and 6 mm from the epicenter (Fig. 4D–G), destruction was found in the dorsal funiculus. Spared tissue with microcysts was identified only at the epicenter and 2 mm rostral and caudal to it, in the white matter surrounding necrotic tissue (Fig. 4A–C).

Morphometric analysis of damaged and spared tissue for all experimental groups showed no statistically-significant differences at any of the cord levels studied (Fig. 5A–C). The entire cross-sectional area at the epicenter was significantly higher in rats treated with thalidomide 4 h after injury compared with rats treated with vehicle and naïve rats ($P<0.05$). Six mm rostral to the epicenter, this measurement was also significantly higher in rats treated with vehicle or with thalidomide 4 h after injury compared with naïve rats ($P<0.05$). For all other segments, the increased trend observed in injured rats over naïve rats was not significant (Fig. 5D).

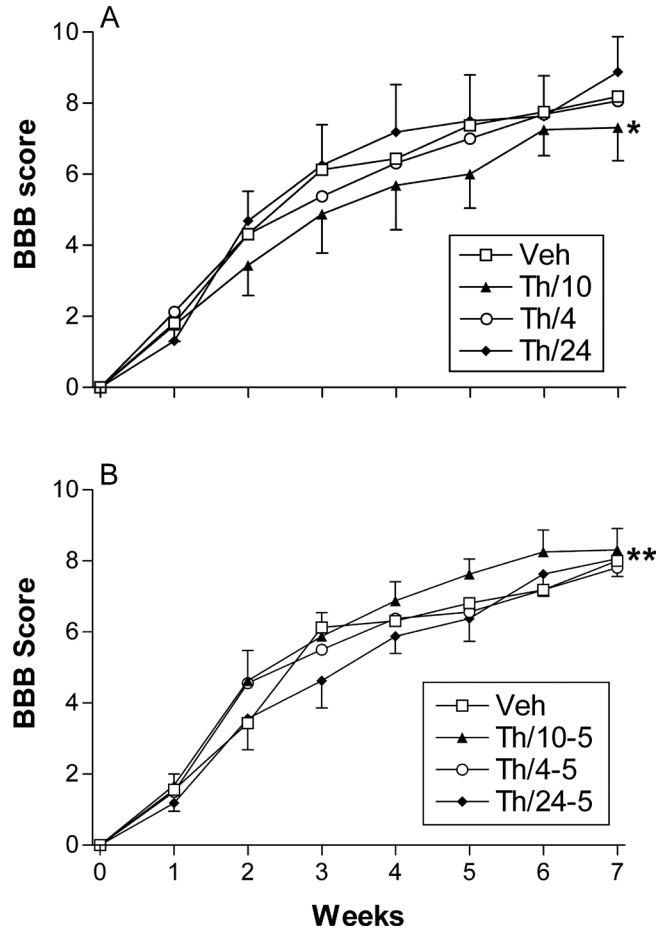

Fig. 3. Motor function outcome. For single dose (A) and repeated doses (B) thalidomide treated rat BBB scores were significantly different for: (*) Th/10 vs. Veh, $P<0.01$; Th/10 vs. Th/4, $P<0.05$; Th/10 vs. Th/24, $P<0.001$, and (**) Th/24-5 vs. Th/10-5, $P<0.01$. Single dose treatments: Th/10, Th/4, and Th/24, thalidomide given 10 min, 4 h, and 24 h after injury, respectively. Repeated dose treatments: Th/10-5, Th/4-5, and Th/24-5, first thalidomide dose at 10 min, 4 h, and 24 h after injury respectively, and then daily for 5 days; (Veh) injured rats treated with vehicle only. Plots represent mean ± SEM (n=8). Statistical analysis – repeated measures ANOVA.

DISCUSSION

Scientific evidence suggests a process of self-destruction after mechanical damage to the SC. Because thalidomide is known to possess pharmacological properties that counteract key mechanisms of cord self-destruction, we assumed that thalidomide administered during the acute stage of SCI could diminish edema and acute inflammation at the site of injury, and thereby possibly improve the long-term morpho-functional outcome. The present study was designed to determine a therapeutic window for the drug. Unexpectedly, however, our experimental evaluations did not validate our hypothesis.

In agreement with our observations, recently Koopmans and coauthors (2009) showed in adult rats subjected to SC contusion injury that thalidomide given in a single intraperitoneal 100 mg/kg dose (as in our study) immediately after contusion is not capable of improving long-term motor function, although in

contrast with our results they found that the amount of spared white substance at the epicenter was higher compared to vehicle-treated rats. Such differences could be attributed to the fact that they performed a less severe injury than we did (impact intensity of 12.5 gcm vs. 25 gcm).

On the other hand, in contrast with our results, Genovese and colleagues (2008) found that a single oral dose of 300 mg/kg given to mice 1 h after compression injury significantly reduced several markers of acute cord inflammation, apoptosis, and histological tissue damage 24 h after injury, and that the same oral dose given daily for 10 days, significantly improved motor function outcome compared to injured vehicle-treated animals. These results are remarkable in view of the gastrointestinal dysfunction associated with spinal shock (García-López et al. 1997, Guízar-Sahagún et al. 1998). Discrepancies between the results of Genovese and others and us might be attributed to differences in the pathophysiology of our experimental models: Kraus (1996) reported that hemorrhagic necrosis is the main damage after contusion (our model) and vasogenic edema (a known target for thalidomide, Melchert and List 2007) is the predominant alteration after cord compression (the model of Genovese et al. 2008). In short, from the three studies performed to determine the influence of thalidomide on functional outcome after experimental SCI it can be concluded that at doses of 100 mg/kg i.p. given to rats subjected to SC contusion (Koopmans' and our studies) thalidomide shows no motor functional benefit, contrary to the observations of Genovese and coworkers (2008) who reported functional improvement using oral doses of 300 mg/kg in mice after SC compression injury.

Rationale for thalidomide delivery and preparation was as follows: as a consequence of spinal shock, gastrointestinal tract motility is drastically reduced (Guizar-Sahagun et al. 1998), thereby altering the bioavailability of oral drugs (Garcia-Lopez et al. 1997). Therefore, in the present study, instead of administering the drug orally as usual, thalidomide was given intraperitoneally, a route used successfully in other studies (Ding et al. 2002, Kaicker et al. 2003, Daruwalla et al. 2005). Moreover, in a study of tumor growth inhibition, intraperitoneal, but not oral, thalidomide was effective (Kotoh et al. 1999). After administration, the drug is widely distributed across tissues and easily crosses the blood-brain barrier (Huang et al. 2005). Our dose scheme was arbitrarily determined based on previously published experiments using intraperitoneal thalidomide. For most rodent studies doses range from 50 to 200 mg/kg (Kotoh et al. 1999, Kaicker et al. 2003). In spite of its low water solubility, previous studies have shown a pharmacological effect after intraperitoneal thalidomide in a saline solution (Lin et al. 2005). Therefore we chose to give thalidomide as a "timed release" suspension in a saline solution instead of the widely used drug solvent, dimethyl sulfoxide, which has been described as a neuroprotectant for SCI (Turan et al. 2008), or as a thalidomide mimetic (Eter and Spitznas 2002), either of which could confound our results for thalidomide.

Using the BBB scale to monitor motor function outcome, thalidomide did not provide neuroprotection at any time after SCI. Moreover, an early single dose significantly worsened rats' motor function, possibly by interfering with early endogenous protective/reparative mechanisms. This effect disappeared with repeated doses of the drug, an observation for which we have no explanation.

Increase in cord water content (edema) after acute SCI has been positively correlated with SCI severity (Wagner and Stewart 1981). Vasogenic edema due to enhanced vascular permeability has been associated to an increased expression of VEGF after neurotrauma (Skold et al. 2000, Akiyama et al. 2004) and ischemia/reperfusion brain injury (van Bruggen et al. 1999). There is evidence that VEGF and its receptors are induced at the site of lesion within 1 day after traumatic SCI to rats (Skold et al. 2000). Considering that thalidomide has been widely reported to block VEGF and VEGF receptor expression (Melchert and List 2007), we assumed that administration of thalidomide would lower posttraumatic edema. This, however, did not occur. Actually, by comparison with the naïve group, the rise in water content in the SC of injured rats treated with thalidomide was significant, but not for the vehicle-treated group. These observations suggest that posttraumatic edema associated with SC contusion is not solely vasogenic or exclusively associated with VEGF overexpression. In fact, Herrera and coauthors (2009) have very recently shown a general reduction of VEGF isoforms following contusive SCI, for at least 1 month post injury. A hydrostatic edema attributed to altered cerebrospinal fluid pulse pressure could explain the edema of our model as suggested previously by Nemecek and colleagues (1977), and recently by Josephson and others (2001) after spinal thecal sac constriction.

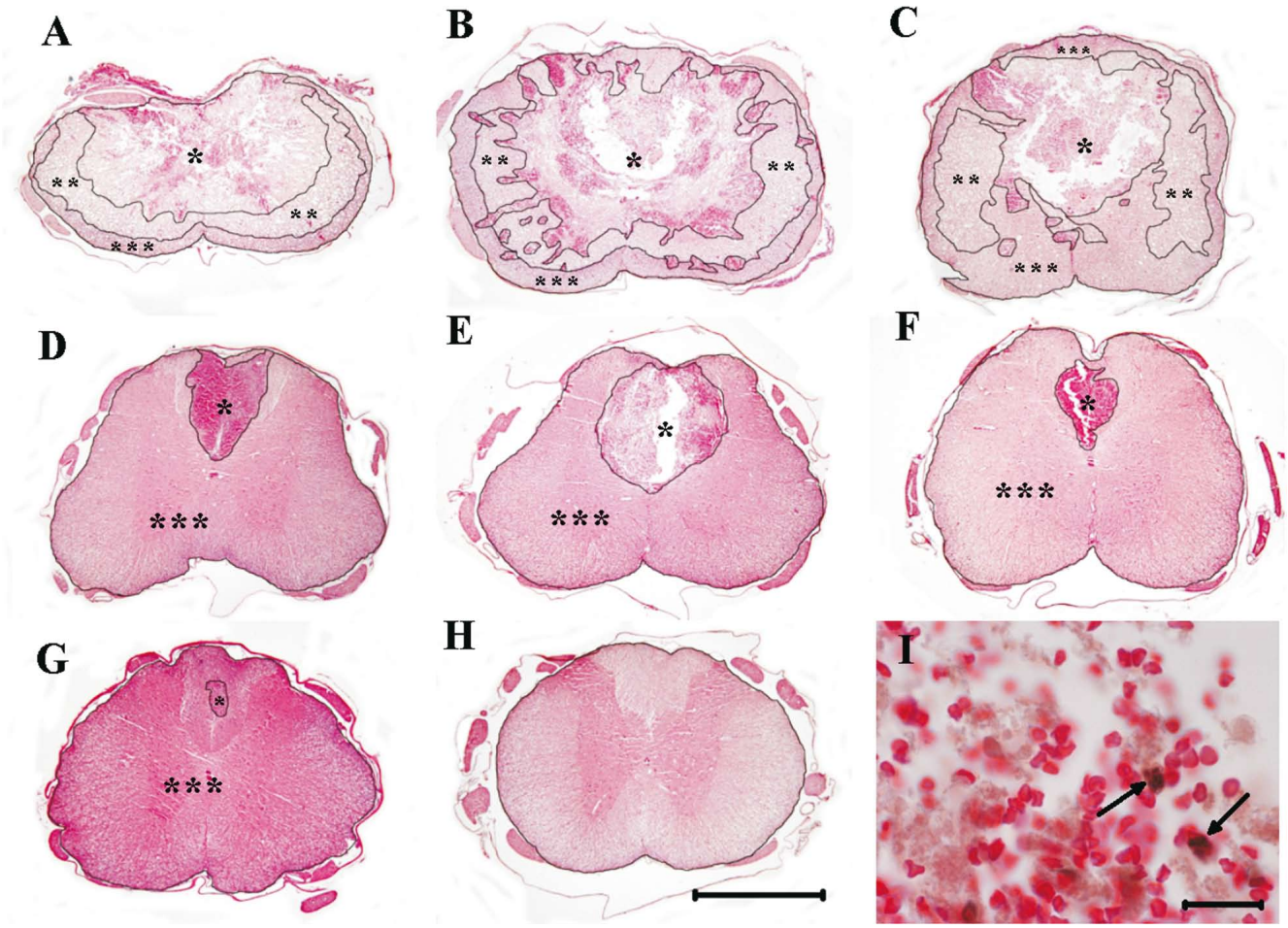

Fig. 4. Histology of injured spinal cord. Representative images of spinal cord tissue at epicenter (A), and at 2 mm (B, C), 4 mm (D, E), and 6 mm (F, G) rostral and caudal to it, respectively. (H), cord section from a naïve rat at T-9. Levels of cord tissue damage/preservation are outlined: complete tissue destruction (*); spared tissue with microcysts (**); and healthy-looking spared tissue (***). Bar in H for A–H is 1 mm. (I), Neutrophils (arrows) in a field of necrotic-hemorrhagic cord tissue at the epicenter. Bar is 20 μm. H&E stain.

The acute inflammatory response triggered by SCI (induction and release of proinflammatory cytokines, activation of microglia, and early infiltration of neutrophils) has usually been regarded as a destructive process (Carlson et al. 1998, Pan et al. 2002), although evidence is beginning to emerge suggesting that it may in fact underlie essential tissue repair mechanisms (Donnelly and Popovich 2008). Thalidomide has been reported to be an immunomodulator capable of inhibiting TNF-α synthesis, attenuating neutrophil activation and inhibiting myelo-proliferative responses (Tseng et al. 1996, Oliver 2000, Paravar and Lee 2008). Consequently, we speculated that thalidomide treatment after SCI might block secondary damage, and result in improved motor function. The absence of a demonstrable anti-inflammatory effect of thalidomide under our experimental conditions could be attributed to the low availability of the drug at the site of injury and penumbra zone due to poor tissue blood perfusion by thromboses and vasoconstriction (Tei et al. 2005). It is important to point out that early studies demonstrating the anti-inflammatory properties of thalidomide were performed *in vitro* and *in vivo* using animal models with intact blood circulation (Tseng et al. 1996, Paravar and Lee 2008). Because with the current experimental design we observed a tendency for thalidomide given 4 h after injury to reduce acute cord inflammation, we believe it is possible that by increasing the number of animals per experimental group we may then be able to detect a significant anti-inflammatory effect of the drug.

In our study, morphometric assessment of graded tissue damage, including areas of complete tissue destruction,

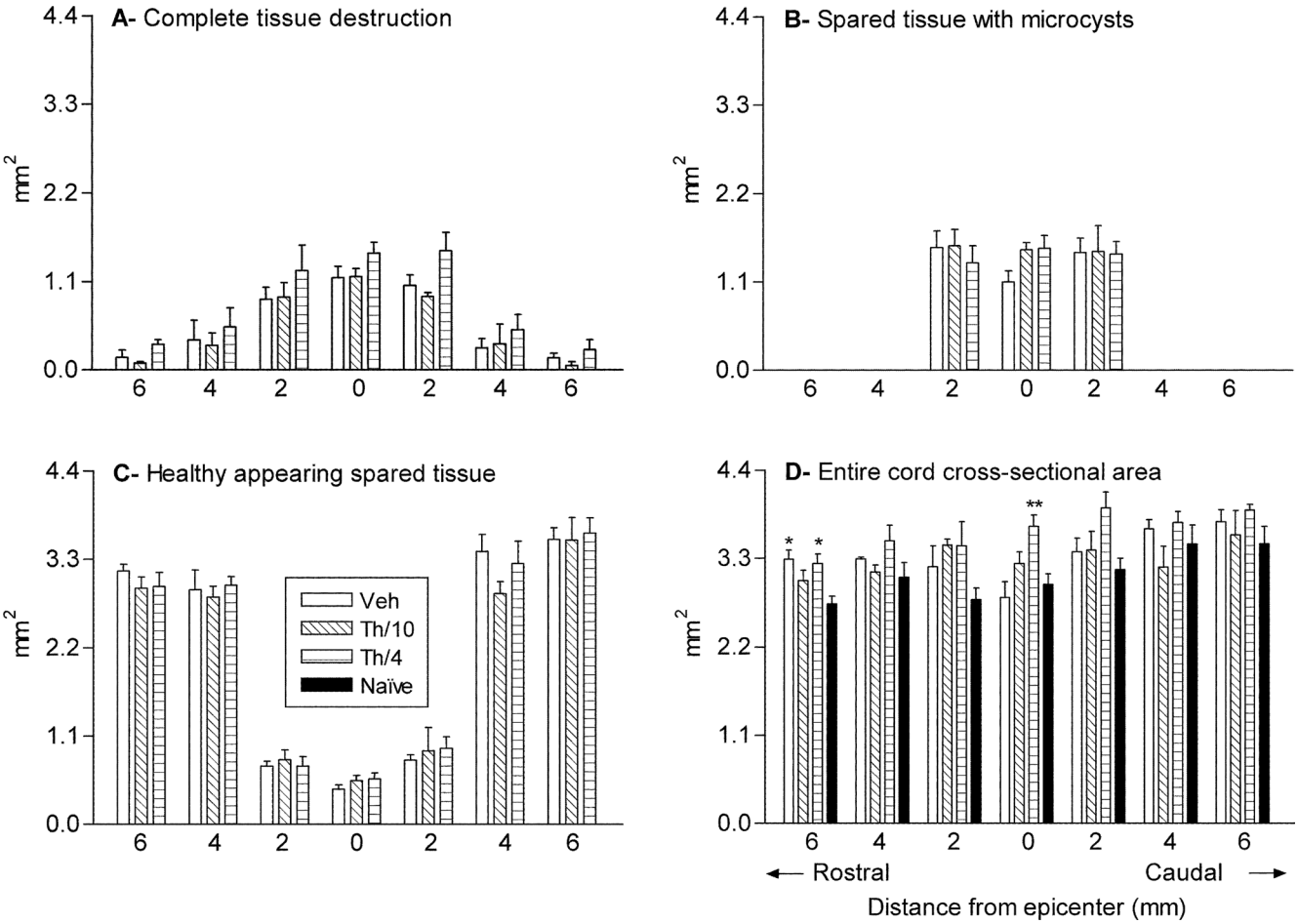

Fig. 5. Morphometric analysis. Plots show area of damaged and spared tissue in relation to its location at and from the epicenter (A–C), and total cross-sectional area (D). Thalidomide treatment 10 min (Th/10), and 4 h (Th/4) after injury; (Veh) vehicle. (*) Veh and Th/4 vs. naïve, $P<0.05$; (**), Th/4 vs. Veh and naïve, $P<0.05$. Data are expressed as the mean ± SEM, (n=4). Statistical analysis – ANOVA test.

spared tissue with microcysts, and healthy appearing spared tissue, did not show any significant differences among groups of thalidomide treated and vehicle-treated injured rats, ruling out any beneficial effect of thalidomide in cord tissue preservation. The tendency towards a lower amount of tissue seen in segments of naïve rats after examination of total cross-sectional areas compared to injured rats could be related to cord swelling after injury, although values were significantly different for some segments only when compared to the single dose 4 hour-treated thalidomide group and the vehicle-treated group.

Even though relevant properties of thalidomide have been extensively studied and exploited for therapeutic purposes, its precise mechanism of action is complex and not yet fully understood. There is evidence suggesting that metabolism of the drug may play a role. Thalidomide undergoes biotransformation by non-enzymatic hydrolysis as well as enzyme-mediated hydroxylation resulting in a multitude of metabolites (Lepper et al. 2006) which may account for its beneficial effects. If traumatic SCI were to interfere with its metabolism, as previously shown to occur with other drugs (Garcia-Lopez et al. 2007), thalidomide might be ineffective as a neuroprotectant.

CONCLUSION

In our hands, thalidomide administered intraperitoneally after acute SCI failed to improve any of the parameters we assessed. Our observations together with recent publications involving the use of thalidomide for SCI treatment are generating controversy, which must be resolved before attempting its use as a neuroprotectant in clinical trials.

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