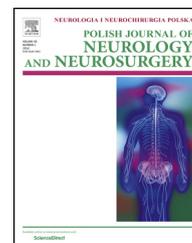


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Original research article

An in vivo model of anti-inflammatory activity of subdural dexamethasone following the spinal cord injury



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ARTICLE INFO

Article history:

Received 27 May 2015

Received in revised form

3 September 2015

Accepted 14 October 2015

Available online 27 November 2015

Keywords:

Spinal cord injury

Phagocytosis of myelin debris

Cavity of CNS injury

Subdural infusion

Inhibition of secondary inflammation

ABSTRACT

Current therapies to limit the neural tissue destruction following the spinal cord injury are not effective. Our recent studies indicate that the injury to the white matter of the spinal cord results in a severe inflammatory response where macrophages phagocytize damaged myelin and the fluid-filled cavity of injury extends in size with concurrent and irreversible destruction of the surrounding neural tissue over several months. We previously established that a high dose of 4 mg/rat of dexamethasone administered for 1 week via subdural infusion remarkably lowers the numbers of infiltrating macrophages leaving large amounts of unphagocytized myelin debris and therefore inhibits the severity of inflammation and related tissue destruction. But this dose was potentially toxic to the rats. In the present study the lower doses of dexamethasone, 0.125–2.0 mg, were administered via the subdural infusion for 2 weeks after an epidural balloon crush of the mid-thoracic spinal cord. The spinal cord cross-sections were analyzed histologically. Levels of dexamethasone used in the current study had no systemic toxic effect and limited phagocytosis of myelin debris by macrophages in the lesion cavity. The subdural infusion with 0.125–2.0 mg dexamethasone over 2 week period did not eliminate the inflammatory process indicating the need for a longer period of infusion to do so. However, this treatment has probably lead to inhibition of the tissue destruction by the severe, prolonged inflammatory process.

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<http://dx.doi.org/10.1016/j.pjnns.2015.10.006>

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1. Introduction

A compression spinal cord trauma results in a massive localized injury that in a rat model becomes a defined, fluid-filled cavity infiltrated by a large number of macrophages [1,2]. A large proportion of macrophages contain intracytoplasmic myelin debris [2] and it is considered that the severe inflammation in the injury cavity causes additional damage to myelin in the surrounding tissue thus propagating the macrophage chemotaxis and their phagocytosis of damaged myelin leading to the expansion of the cavity in a progressive fashion (Kwiecien, unpublished).

To limit the severity of destructive inflammation, we previously infused subdurally dexamethasone, a stable, potent anti-inflammatory synthetic glucocorticoid [3] at 4 mg/rat per week [2]. On histological analysis we determined that the numbers of macrophages in the cavity of injury were dramatically reduced in dexamethasone-infused rats leaving large amount of un-phagocytized myelin debris. However, this dose of dexamethasone caused remarkable toxicity to infused rats, therefore we performed an experiment with lower doses of this drug for the duration extended to two weeks to determine a dose that would inhibit macrophage phagocytosis of myelin and that would be tolerated by the recipient rats.

All doses of dexamethasone used; 0.125–2.0 mg/rat over 2 weeks were well tolerated and on histological analysis appeared to inhibit phagocytosis of myelin debris, apparently in a dose-dependent fashion.

The subdural administration of a potent anti-inflammatory compound for a sufficiently long period of time may have a direct neuroprotective effect by inhibiting a severe destructive inflammation.

2. Materials and methods

Experimental procedures were performed on 3–7 month old Long Evans rats ($n = 30$) of both sexes with the approval from the Animal Research Ethics Board, McMaster University along the guidelines from the Canadian Council for Animal Care.

2.1. Balloon compression spinal injury

The rats were induced and maintained under isoflurane anaesthesia. Balloon crush injury was adopted from Vanicky et al. [4,5]. Laminectomy was performed over the lumbar region of the spinal cord and a 3F embolectomy catheter (ZTS Hagmed, Poland) inserted epidurally over the dorsal spinal cord to place the balloon in the rostral thoracic segment of the spinal cord [2]. The sleeve of the catheter was filled with saline and the balloon was inflated with 0.2 mL saline using a 25 μ L Hamilton syringe (VWR International) for 5 s. After the compression evidenced by sudden movement of the hind legs, the balloon was deflated and the catheter carefully removed.

2.2. Subdural infusion

In the laminectomy site in the lumbar spine the dura was cut with a 25 ga needle and a rat intrathecal catheter (Alzet, Durect

Corporation, Cupertino, CA) was carefully inserted into the subdural space over the dorsal spinal cord to approximate the end of the catheter with the site of the balloon crush. After the catheter was affixed with a single suture to a spinal muscle at the site of laminectomy, the steel wire was removed from the catheter. The free end of the catheter was attached to a 2 mL osmotic pump with infusion time of 2 weeks (2ML2, Alzet) that was pre-loaded with 2 mL of a saline dilution of dexamethasone (Dexamethasone 2, 2 mg/mL, Vetoquinol N-A. Inc, Lavartrie, Quebec, Canada) resulting in administration of total doses of 0.125, 0.25, 0.5, 1.0 and 2.0 mg of dexamethasone to rats in 5 treatment groups ($n = 5$) or 2 mL of saline ($n = 5$). The attached pump was placed subcutaneously on the flank of the rat. The spinal muscles were apposed with absorbable sutures over the laminectomy and the skin closed with stainless steel staples. The surgery rats were injected subcutaneously with 5 mL saline and 0.3 mL ketoprofen (10 mg/mL, Anafen[®], Merial Canada, Inc., Baie d'Urfe, Quebec, Canada) for analgesia prior to recovery from anaesthesia. The injections of anaesthetic were repeated once daily for 2 more days and the administration of saline was performed 1–2 \times daily as indicated by the hydration status.

2.3. Clinical observations

All 30 rats with the balloon crush injury recovered well and had marked paresis or complete paralysis of the hind end throughout the 2 weeks of the survival. Large proportion of rats had markedly enlarged urinary bladder that was expressed manually twice daily. Most of the rats with urinary bladder dysfunction regained control over the urination within the second week post-op. Rats treated with dexamethasone and with saline showed no adverse clinical signs associated with steroid overdose [2].

2.4. Perfusions

At day 14 post-op, the surgery rats were overdosed with 100 mg/kg body weight sodium barbital (Ceva, France), the chest opened, and 100 I.U. sodium heparin injected intracardiac. The blood was washed out by lactated Ringer's solution (Baxter, Canada) via the left cardiac ventricle with the outflow created by cutting the right heart auricle [6]. The animals were perfusion-fixed in 10% buffered formalin, carcasses post-fixed at 4 °C for 1–3 h and the spinal cord removed carefully and post-fixed in 10% formalin. Other routine tissues were also sampled for histological analysis.

2.5. Histology, immunohistochemistry

The length of the spinal cord was sectioned transversely into 2 mm thick segments with the cranial face placed down in the tissue cassette. The tissues were treated by the raising concentrations of ethyl alcohol and xylene, embedded in paraffin, cut 4 μ m thick and mounted on glass slides. A Luxol fast blue (LFB) stain for myelin [7] was counterstained with haematoxylin and eosin.

For the anti-CD68 labelling, tissue sections were cut at 5 μ m and collected onto charged slides and dried overnight prior to starting the IHC staining procedure. The sections were

dewaxed in 2 changes of xylene and then rehydrated through a graded alcohol series from 100%, through 95%–70% alcohol. Sections were then incubated in methanol containing 0.3% H₂O₂ for 15 min to quench endogenous peroxidase activity, followed by washes in dH₂O and then rinsed in PBS-T twice for 3 min each. Antigen retrieval was performed in a pressure cooker using citrate buffer pH 6.0 for 5 min, followed by a 10 min cool down period and 5 min washing in running tap water. Sections were rinsed twice in PBS-T and placed in a slide staining tray. Non-specific antibody binding was prevented by incubating sections in 2.5% normal horse serum (Vector Laboratories, U.K.) for 20 min at room temperature. Blocking serum was poured off and the sections were then incubated with Mouse Anti-CD68 (Abcam, U.K.) diluted to 1:100 in PBS-T + 1% BSA in a humidified chamber for 1 h at room temperature.

Sections were thoroughly washed in PBS-T (3 × 3 min each) and subsequently incubated in ImmPRESS peroxidase polymer Anti-Mouse Ig reagent for CD68 (Vector Laboratories, U.K.) for 30 min at room temperature. The standard washes were performed and then incubated in a working solution of DAB (Vector Laboratories, U.K.) for approximately 3 min. Sections were washed thoroughly in running water for 5 min prior to counterstaining in Mayer's haematoxylin for 30 s. Again the sections were thoroughly washed in dH₂O prior to dehydrating through a graded series of alcohol solutions, clearing through

multiple changes of Xylene and mounted coverslips with Permount (Fisher Scientific, USA).

For the anti-GFAP labelling, the glass-mounted sections were heated at 58 °C overnight and deparaffinised in a Target Retrieval Solution, pH buffer (DAKO Corp., Denmark) at 97 °C for 20 min in a DAKO PT Link Pre-Treatment Module for Tissue Specimens PT101 apparatus. Antibodies against the glial fibrillary acidic protein (GFAP) were obtained from the DAKO Corp. and were applied at a 1:50 dilution and the positive reaction visualized with a DAKO EnVision+System-HRP (DAB +). The histological analysis was performed under a Nikon Eclipse 50i microscope and the abnormalities in the spinal cord and in other tissues photographed.

3. Results

3.1. Clinical observations

Although all experimental rats had remarkable neurological deficits including hind end paresis or paralysis and distended urinary bladder within the first week post-op, these neurological deficits were less pronounced or disappeared completely within the second week in rats in all treatment groups. Steroid toxicity due to the administration of dexamethasone such as

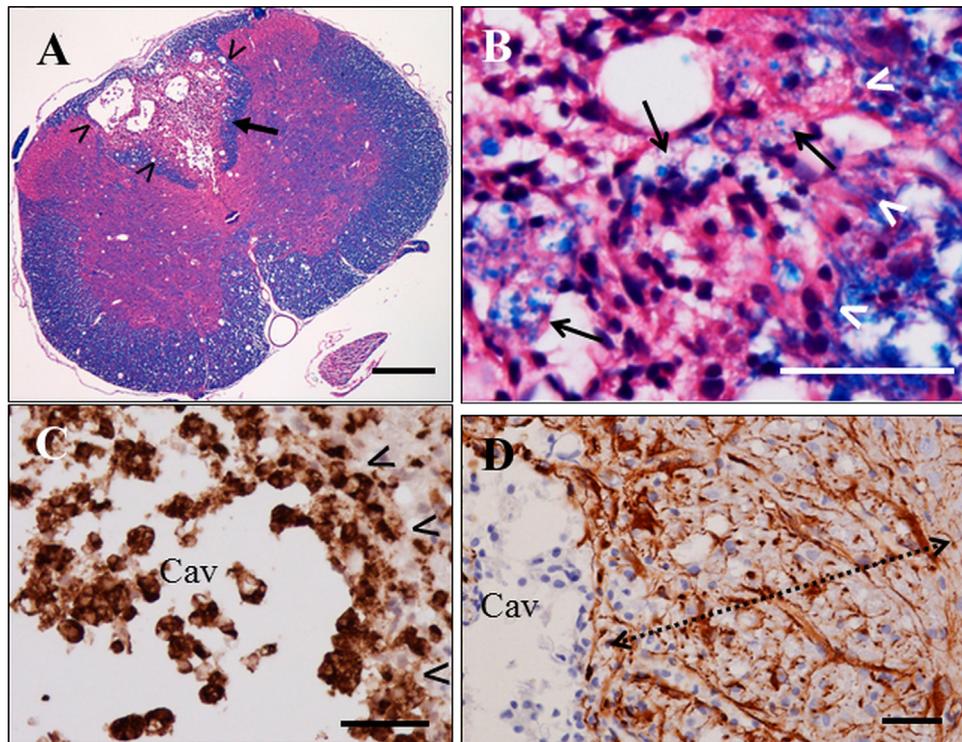


Fig. 1 – Spinal cord 2 weeks post balloon compression injury resulted in obliteration of the most of the dorsal column. In the untreated rat (A–D), severe infiltration by mononuclear phagocytic cells fill the cavity of the lesion (Cav) delineated by surrounding spinal cord tissue (arrow heads). The arrow in A indicates the area shown in B–D. The phagocytic cells are mononuclear, have large vacuolated cytoplasm and often luxol fast blue (LFB)-positive granules (arrows in B) interpreted as phagocytized myelin debris. Many such cells are positive for the anti-CD68 antibody (C) and infiltrated the surrounding spinal cord tissues that have remarkable astrogliosis (D) surrounding the cavity of the injury at the thickness (double headed interrupted arrow) of approximately 150–250 μm. A and B – LFB counterstained with haematoxylin and eosin (H&E), C – anti-CD68 antibody, a macrophage marker, D – anti-glial fibrillary acidic protein (GFAP), an astrocyte marker. Scale bars; A – 500 μm; B–D – 50 μm.

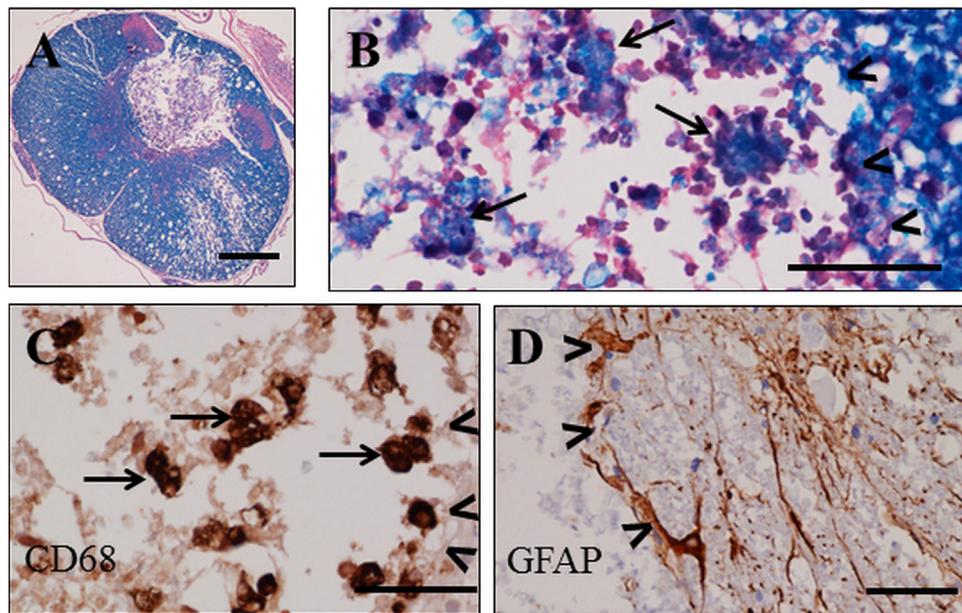


Fig. 2 – Spinal cord 2 weeks post balloon compression injury. In the rat treated with subdural infusion of 2.0 mg of dexamethasone at the rate of 5 μ L/hr, the lesion cavity contains fewer macrophages (arrows in B and C) that are surrounded by un-phagocytized LFB-positive material (B) that is interpreted as myelin debris. Astrogliosis in the surrounding tissue (arrowheads in D) appears less pronounced than in the untreated rat (Fig. 1D). A and B – LFB+H&E, C – anti-CD68 antibody, D – anti-GFAP. Scale bars; A – 500 μ m; B–D – 50 μ m.

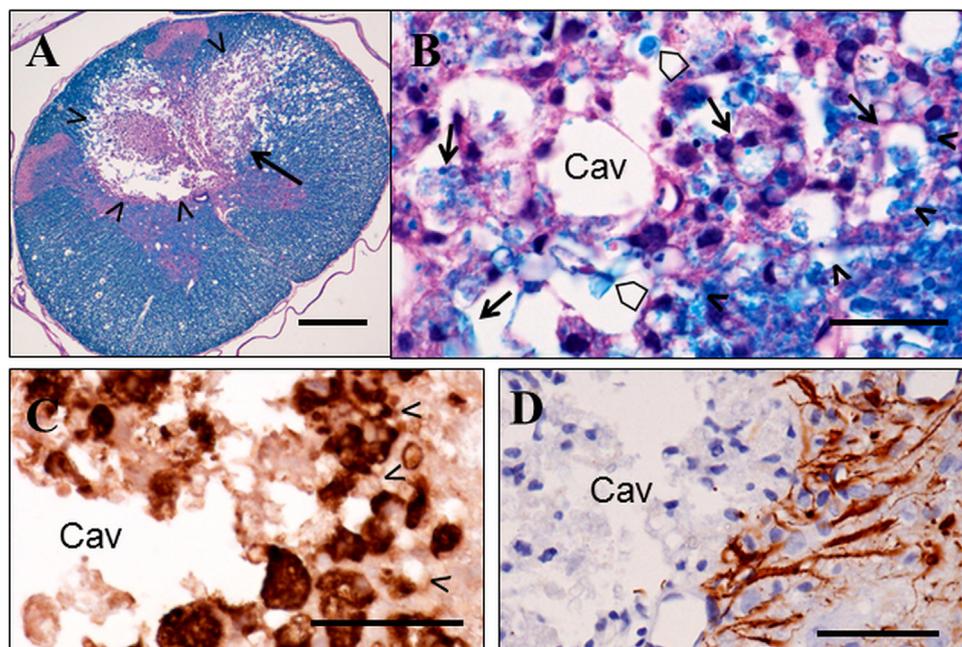


Fig. 3 – The spinal cord with the balloon crush injury infused subdurally with 0.125 mg dexamethasone over 2 weeks. Large area in the dorsal column and adjacent dorsal horn and lateral column are obliterated by a cavity (Cav) delineated by arrowheads in (A–C) and by astrogliosis in (D). The large arrow in (A) indicates the area magnified in (B–D) where in the cavity of the crush injury numerous large macrophages internalized numerous, LFB-positive granules of myelin debris (arrows in B) and most of the cells interpreted, as macrophages are positive for anti-CD68 antibody (C). Small fragments of cell-free LFB-positive myelin debris are present in the cavity of the crush injury (open arrows in B). LFB+H&E – A and B; anti-CD68 antibody – C; anti-GFAP antibody – D. Size bars; A – 500 μ m, B–D – 50 μ m.

generalized weakness, dehydration and poor doing was not observed in any rat.

3.2. Neuropathology

Two weeks post-op, the balloon compression injury resulted in the massive damage to the white matter in the dorsal column with its partial or total obliteration by a cystic cavity (Figs. 1–6) that sometimes extended to involve the adjacent dorsal horn and the lateral column (Fig. 5). The cavity was infiltrated by a pure population of numerous large cells often containing LFB-positive granules in the cytoplasm (Figs. 1B, 2B, 3B, 4B and 6A). The large proportion of the large cells in the cavity stained with an anti-CD68 antibody (Figs. 1C, 2C, 3C, 4C, 5C and 6C) and were interpreted as macrophages. While cell-free LFB-positive myelin debris were not observed in the cavity in non-treated rats (Fig. 1B), in all rats treated with the subdural infusion of 0.125–2 mg dexamethasone for 2 weeks non-phagocytized myelin debris were evident (Figs. 2B, 3B and 6A) indicating a degree of inhibition of phagocytosis of damaged myelin by subdural administration of dexamethasone as demonstrated previously [2]. However, in dexamethasone treated rats there were small pockets of the cavity at the distance from the site of the balloon crush where extracellular LFB-positive myelin debris was not observed (Fig. 4B), indicating a less than optimal diffusion of a therapeutic concentration of dexamethasone throughout the entire cavity.

In some sections of the spinal cord at or near the balloon crush injury, the large area of the crush, connecting to the meningeal space was obliterated by granulomatous tissue (Fig. 5A and B) rich in CD68-positive macrophages (Figs. 5C and 6C) and scattered macrophages with internalized LFB-positive granules of myelin debris (Fig. 6B). The granulomatous infiltration was negative for astrocytes and was delineated from the surrounding neural tissue by intense astrogliosis (Fig. 5D) and was adjacent to the cavity (Fig. 5).

The neural tissue surrounding the cavity of injury invariably had pronounced astrogliosis (Figs. 1D, 2D, 3D, 4D and 5D) that was 150–250 μm thick (Fig. 1D). Individual reactive astrocytes were the most pronounced in saline treated spinal cords (Fig. 1D) whereas the administration of dexamethasone was associated with somewhat fewer and thinner processes labelled by anti-GFAP antibody (Fig. 2D) suggesting a partial inhibitory effect of dexamethasone on astroglial hypertrophy.

4. Discussion

The balloon crush injury to the rat spinal cord resulted in a severe inflammatory response with a cavity filled by a pure population of macrophages at 1 week post-op [2] and at 2 weeks post-op in the present study indicating that this severe destructive inflammatory process runs well beyond 2 weeks post-injury. Large proportion of macrophages internalized

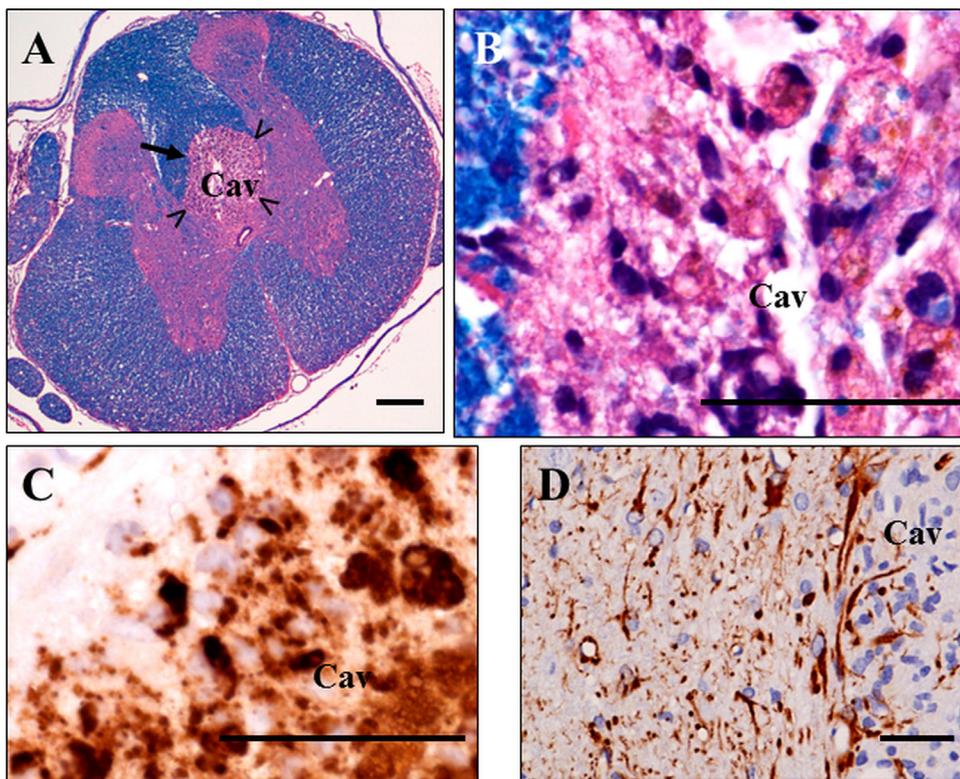


Fig. 4 – The injury caudal to the balloon crush spinal cord injury in a rat treated with subdural infusion of 0.5 mg dexamethasone for 2 weeks. Deep in the dorsal column, there is a limited area of obliteration of the white matter tissue by a dense infiltration of large cells (arrow heads in A). The large arrow in (A) indicates the area of magnification in (B–D). Some of the cells in area of infiltration contain LFB-positive granules of myelin debris (B) and similar numbers of cells are positive for the CD68 antigen (C). There is remarkable astrogliosis reaction in the tissue surrounding the area of infiltration (D). LFB+H&E – A and B; anti-CD68 antibody – C; anti-GFAP antibody – D. Size bars; A – 500 μm , B–D – 50 μm .

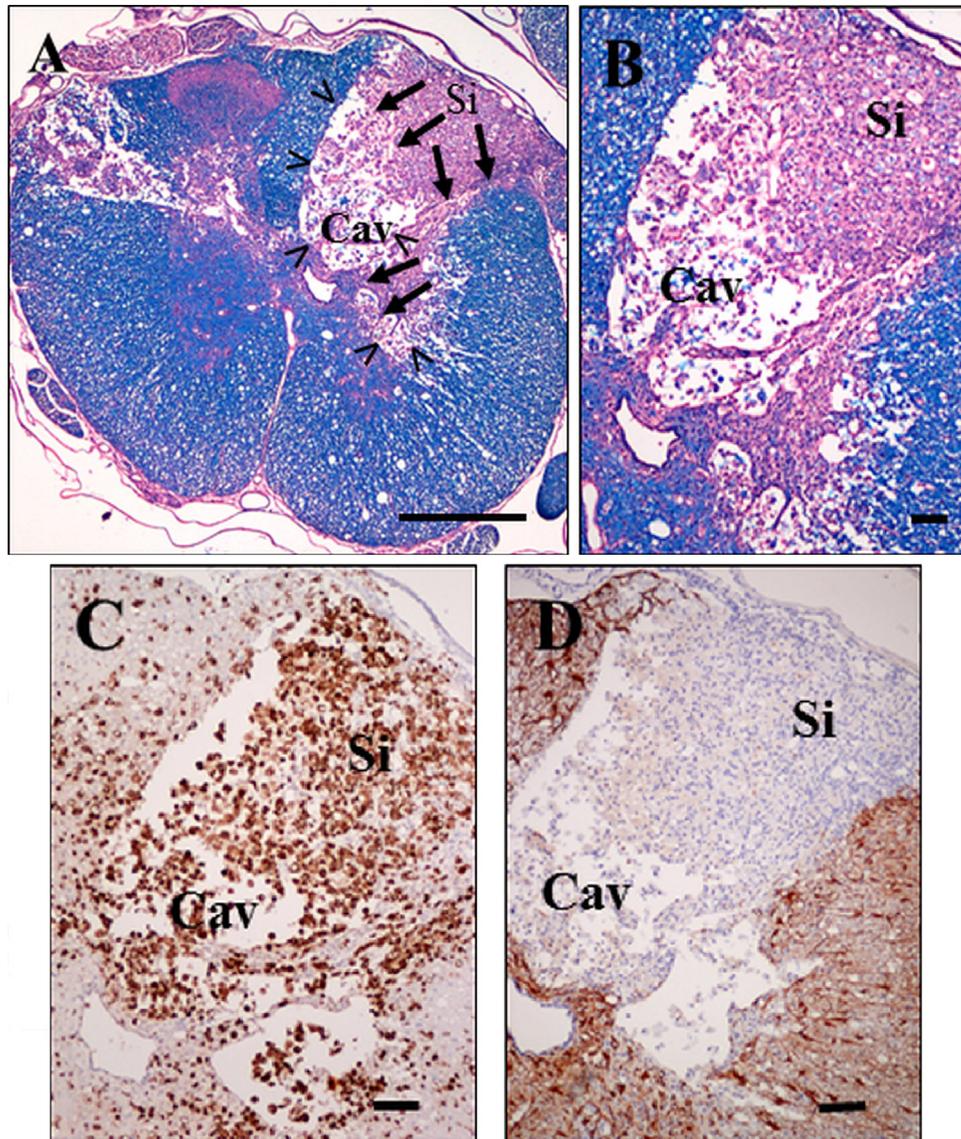


Fig. 5 – The inflammatory process following the spinal cord trauma resolves in two types of response in the dorsal column of the rat treated with 1 mg dexamethasone for 2 weeks. The large proportion of the crush is obliterated by a solid area (Si in A–D), delineated by solid arrows in (A), that is connected to the meninges and to the subdural space, and is diffusely infiltrated by CD68-positive macrophages (C) but has little intracellular myelin debris and extracellular myelin debris (B), and no astrocytes (D). The solid area borders with the cavity of the crush injury (Cav in A–D) that is delineated by arrowheads in (A). LFB+H&E – A and B; anti-CD68 antibody – C; anti-GFAP antibody – D. Size bars; A – 500 μm , B–D – 100 μm .

LFB-positive granules interpreted as phagocytized myelin debris and implying that damaged myelin is a potent immunogenic factor stimulating macrophage-rich inflammation [2]. This notion is supported by the lack of free, non-phagocytized myelin debris in control rat spinal cords where macrophages internalized all damaged myelin probably in an attempt to neutralize its immunogenic action.

Subdural administration of dexamethasone resulted in the observation of extracellular myelin debris at all concentrations used indicating that this powerful synthetic glucocorticoid interferes with the process of phagocytosis [reviewed in [2]] of myelin debris and therefore it suppresses the inflammatory response following the spinal cord trauma. Since the systematic pathological studies of the natural progression of the

spinal cord injury from its onset to resolution implied as the formation of the CSF-filled cavity with no evidence of an inflammatory infiltrate or syringomyelia [19] are lacking, we cannot at this time interpret the full therapeutic benefit of the dexamethasone treatment in the spinal cord injury. However, it appears that the severity of the macrophage-rich inflammation is directed against the damaged myelin resulting from the initial crush injury. The sustained nature of the severity of inflammation post-injury indicates that the further myelin damage in the white matter surrounding the initial lesion occurs that results in sustained macrophage chemotaxis and active myelin phagocytosis. This destructive process presumably leads to the loss of the neural tissue and to enlargement of the cavity. Volumetric measurements of the lesion cavities

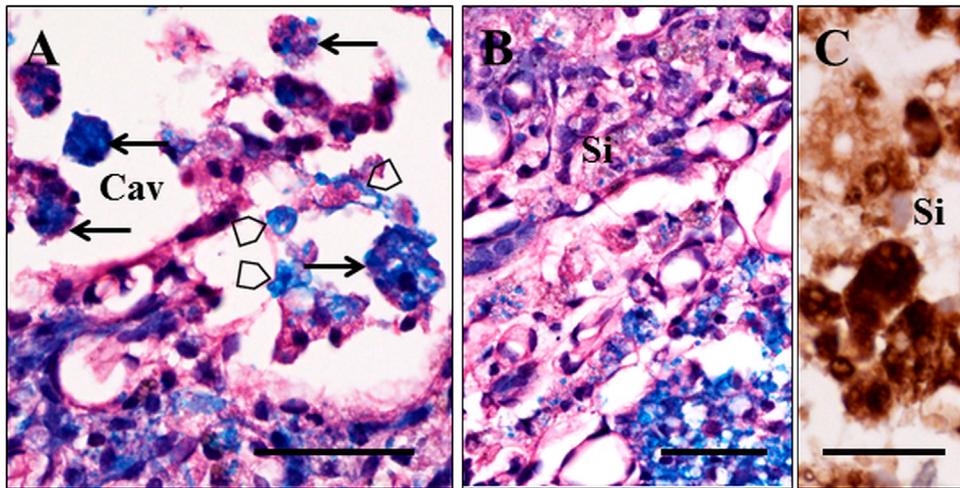


Fig. 6 – Higher magnification of the histological detail from the Fig. 5. The cavity of the crush injury (Cav in A) contains scattered large macrophages laden with LFB-positive granules of myelin debris (arrows in A) and scattered extracellular LFB-positive myelin debris (open arrows in A). The solid area of inflammation (Si in B and C) borders with myelin-rich intact spinal cord tissue (lower right corner in B), is diffusely infiltrated by CD68-positive macrophages (C) but has little intracellular myelin debris and extracellular myelin debris (B). LFB+H&E – A and B; anti-CD68 antibody – C. Size bars; A and B – 50 μm ; C – 10 μm .

was not attempted in this study for two main reasons: (1) tissue of the spinal cord encompassing the cavity is notoriously difficult to cut without causing the wall of the cavity to collapse and thus cause an artefactual change in the volume; and (2) the severe inflammatory process in the lesion cavity far exceeds the period of 2 weeks post-op in treated rats, therefore the measurement of the volume at 2 weeks alone would not provide a sufficient indication on the neuroprotective effect of administered dexamethasone. Although, effective inhibition of the macrophage-rich inflammation should lead to reduction of the tissue destruction, a smaller cavity and more tissue preservation. We had previously inhibited the macrophage-rich inflammation after the spinal cord injury by a subdural infusion of a high dose, 4 mg/week, of dexamethasone which however has proven to be unduly toxic [2]. In the present study, lower doses, ranging from 0.125 to 2 mg were administered for 2 weeks and were tolerated well by the recipient rats. However, a longer subdural administration of dexamethasone or other potentially anti-inflammatory drugs, still to be tested in this model, will be required to achieve elimination of the inflammatory response, presumably a limited cavity devoid of inflammatory exudate and of cell-free myelin debris that would be capable to re-ignite the inflammation.

At two weeks after the injury the site of the injury became obliterated by a liquid filled cavity with macrophage exudate surrounded by a 150–250 μm thick area of tissue with prominent astrogliosis. The astrogliosis surrounding the site of the recent injury, presumably with an active inflammation has been described previously in animal models [8,9] and it was associated with remarkable increase of expression of an aquaporin 4 (AQP4) water channel on hypertrophied astrocytic processes [8,9] that can contribute to removing water from the interstitial space of the spinal cord tissue [10] into the area of damage converting it into a cavity. The syringomyelia or

formation of the water filled space, syrinx, after the spinal cord trauma is a common chronic outcome in patients [11]. It has been proposed to form due to an increase in the hydrostatic pressure in the confines of the subdural space due to inflammatory changes including scarring of the leptomeninges and of the adjacent spinal cord tissue [11–13]. Although a systematic pathologic analysis of the progression of the spinal cord injury from its initiation to its resolution into an inflammation-free state has not been performed to date, we think that in many cases the cavitation filled with the CSF forms in the area of injury as the result of the destructive inflammatory process involving a severe macrophage-rich infiltration [2]. The severe inflammatory process is maintained in the resulting cavity with continuous and progressive destruction to the surrounding tissues, particularly of damaged myelin, a potentially immunogenic material [14–18]. The astrogliosis surrounding the cavity is a tissue response unique to the CNS, no other tissue reacts to injury, necrosis and inflammation by forming a fluid-filled cavity, instead, a scar rich in collagen is a more likely outcome. The astroglial hypertrophy coincides with the increase of the expression of AQP4 channels and their water exuding activity into the cavity [8,9]. In time the cavity is cleared of the inflammatory cells and the cavity becomes a quiescent syrinx [19]. The entire process of the formation of the syrinx takes more than 8 weeks in the rat with balloon crush (Kwiecien, unpublished) but it may be completed by 4 months post injury in a minipig model [19] and it may be suggestive of a natural anti-inflammatory process that is water soluble and generated by the CNS tissue surrounding the cavity and perhaps related to astrogliosis [1]. In a recent study, a macrophage-rich inflammation in the crush injury in the dysmyelinated Long Evans Shaker rat was completely eliminated from the cavity of injury before the day 7 post-injury and the cavity apparently did not increase in size

[1]. Since the large size of the syrinx correlates with the severity of the tissue loss and thus with the severity of neurologic deficits, the smaller the syrinx the better neurologic outcome. Therefore, the inhibition of the destructive inflammatory process by a powerful anti-inflammatory compound such as dexamethasone or methylprednisolone administered directly into the cavity appears as an attractive therapeutic strategy. In the current study, subdural administration of dexamethasone apparently coincided with the reduction in the prominence of astrogliosis but it remains unknown whether this effect altered apparent functions of astroglial response to injury including increased expression of AQP4.

The epidural balloon crush injury resulted in perforation of the external glia limitans and formation of an area of solid fibrovascular tissue that was diffusely infiltrated by CD68-positive macrophages but it was devoid of astrocytes. This type of inflammatory response was distinct from a cavity-forming CNS inflammation. It is considered an extra-neural process with involvement of cellular elements derived from leptomeninges and is probably similar to arachnoiditis described previously [20]. Formation of the arachnoiditis in our model of injury clearly resulted in irreversible loss of the tissue of the spinal cord since it was devoid of astrocytes and it was delineated from the neural tissue by a wall of astrogliosis. Therefore we think that the spinal cord trauma initiates 2 distinct inflammatory processes; the first, results in the fluid-filled cavity with a macrophage-rich infiltration directed against damaged myelin, isolated by the neural tissue with astrogliosis; the second, results in the solid area of severe, macrophage-rich area of inflammation containing fibrovascular elements originating from leptomeninges and devoid of astrocytes, called arachnoiditis. Both inflammatory processes lead to irreversible loss of the neural tissue and thus worsen the clinical outcome. Subdural administration of dexamethasone a powerful anti-inflammatory drug appears to inhibit the inflammation in the cavity but apparently not in arachnoiditis.

5. Conclusions

1. Subdural infusion of 0.125–2.0 mg dexamethasone over the period of 2 weeks attenuated phagocytosis of damaged myelin and was presumably neuroprotective in a model of spinal cord injury.
2. Inflammatory process induced by the spinal cord injury has a chronic duration well beyond 2 weeks post-injury and will require a potent anti-inflammatory therapy of considerable duration to be effectively inhibited.
3. The spinal cord injury site can be converted into an area of inflammatory reaction of one of the two distinct types: (1) a fluid-filled cavity surrounded by a wall of astrogliosis, containing a pure population of macrophages internalizing myelin debris and contributing to the tissue destruction; and (2) a solid, granulosomatous tissue connected to the leptomeninges, containing small blood vessels, fibroplasia, scattered macrophages with internalized myelin debris and no astrocytes. Both types of inflammation irreversibly replace the tissue of the spinal cord contributing to the loss of neurological function.

Conflict of interest

The authors declare no conflict of interest.

Financial disclosure

Funding provided by the Medical University of Lublin, Poland.

Ethics

The work described in this article has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans; Uniform Requirements for manuscripts submitted to Biomedical journals.

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