Manganese Enhanced Magnetic Resonance Imaging in a Contusion Model of Spinal Cord Injury in Rats – correlation with motor function
Objectives: Various models of spinal cord injury in rodents have been established and various techniques for lesion quantification have been implemented. Measurement of the extent of the underlying injury is essential to monitor the reproducibility of the experimental injury and for assessment of therapeutic effects. In this study we tested manganese enhanced magnetic resonance imaging (MEMRI) for post-mortem quantification of experimental SCI in rats. Materials and Methods: 12 rats were subjected to contusion injuries at the 11th thoracic vertebra, followed by MnCl₂ injections into the cisterna magna. After observation for 3 days, post-mortem MEMRI-features were correlated with values of locomotion testing and histology. Results: MnCl₂ yielded a strong contrast enhancement of the uninjured spinal cord, whereas no enhancement was observed at the injury site and caudally. MRI findings correlate closely with values of locomotor rating. Conclusions: MEMRI represents a reliable method for visualization and functional assessment of spinal cord integrity in rats.

Introduction

In past decades, various models of experimental spinal cord injury (SCI) have been implemented (e.g. contusion, hemisection) to adequately test promising therapeutic strategies and techniques to assess the neurological deficits have been applied. However, current methods like histology or locomotor rating scale have certain limitations, and a lack of standardization of clinical read-out measures hampers direct comparison of injury models and therapeutic outcomes.

Magnetic resonance imaging (MRI) of acute spinal injury provides excellent visualization of neurological and soft-tissue structures noninvasively. Manganese enhanced magnetic resonance imaging (MEMRI) has been used successfully for the visualization of neuronal circuits and activity patterns of the brain in different animal models. Mn²⁺ is a paramagnetic ion which leads to a strong contrast enhancement in T₁-weighted MRI. Its chemical properties resemble calcium, which results in an active transport of Mn²⁺ into intact active neurons via voltage-gated Ca²⁺ channels, followed by a fast, anterograde microtubuli-based transport and transport across active synapses. Thus Mn²⁺ leads to contrast enhancement of intact neurons, whereas there is a lack in Mn²⁺ uptake and contrast enhancement in injured neurons. Recently, it has been demonstrated that MEMRI represents a sensitive in vivo method for monitoring neuronal activity and functionality within the injured spinal cord of mice. Sharp trauma was used in this study for SCI. One limitation of sharp trauma is poor control of the inflicted injury.

The objective of this study was to establish MEMRI as an objective and non-invasive post-mortem method for both structural and functional assessment of spinal cord contusion injury in rat. We correlated the imaging derived parameters of manganese uptake with clinical parameters of locomotion testing as well as histology, using an impactor device that yields reproducible contusion injury.
Materials and Methods

12 male Sprague-Dawley rats, weighing 250-300 g were obtained from the Institut fuer LaborTierkunde und -genetik der Medizinischen Universitat Wien (Himberg, Austria). The protocol was in accordance with national and international guidelines on the use of laboratory rats and approved by the city government of Vienna. Surgical procedures were carried out at the Ludwig Boltzmann Institute for Experimental and Clinical Traumatology (Vienna); MRI of collected specimens was performed at the German Cancer Research Center (DKFZ, Heidelberg, Germany). Prior to surgery, rats were randomized into two groups (n=6 each).

Surgery

Twelve rats were anesthetized with intraperitoneal injections of Ketason® (Ketaminhydrochloride 100 ml/mg, Dr. E. Graeub AG, Bern, Switzerland) and Rompun® (Xylazine-Hydrochloride, γ-Hydroxybenzoicmethyl-esterate, 2%, Bayer, Leverkusen, Germany). Core body temperature was measured with a rectal probe and maintained at 37-38°C with a heating pad until recovery.

A laminectomy was carried out on each rat at the level of the 11th thoracic vertebra (TH11) and a contusion injury was evoked using the Infinite Horizon Impactor (Precision Systems and Instrumentation, LLC, Lexington, KY, USA). This device allows for application of standard-force contusion injuries to the spinal cords of mice and rats. Impact force and displacement are selectable displayed as time-dependent curves. Rats were subjected to injuries with an impact force of 50 kilodyne (kdyn or 0.05 x 103 Newton; group 1, n = 6) or 150 kdyn (or 0.15 x 103 Newton; group 2, n = 6), respectively. The wound was closed in anatomical layers.

MnCl₂ Injections

MnCl₂-injections were administered immediately after surgery. Rats were placed on a Styrofoam block, with 90° declination of the head. 80 μl of a 0.8M MnCl₂-solution was manually injected into the cisterna magna via the membrana atlanto-occipitalis using a 27-gauge needle.

Post surgery animal care

Post-operative management was identical for all rats in the study. Subcutaneous (sc.) injections of 10 ml Ringer’s solution and antibiotics (Peni-Strepto®, 50,000 iu, Virbac Lab., Carros, France) were administered promptly after surgery as well as once daily until euthanization. Rats were housed under simulated daylight conditions with alternating 12-hour light-dark cycles. Standard rat food and water was provided and rats were checked daily for signs of infection or dehydration. When necessary, manual bladder expression was carried out until spontaneous urination occurred.

Locomotion testing

Tests were performed by observers unaware of injury force on day 3 post-injury using the locomotor rating scale by Basso, Beattie and Bresnahan (BBB-score). It allows separate assessment of hind-limb function using a scale from 0 to 21, where 0 denotes total paraplegia and 21 denotes full function of the hind-limb. The rating 10 stands for “occasional weight supported plantar steps, no forelimb-hindlimb coordination”. All rats were euthanized after locomotion testing on day 3.

Euthanization

All animals were euthanized after observation for 3 days. Rats were anesthetized as previously described. After intravenous injection of 0.3 ml of Heparin (1000 I.E., Heparin Immuno®, EBEWE Pharma Ges.m.b.H Nfg KG, Unterach, Austria) rats were transcardially perfused with 50 ml of saline, followed by 50 ml of buffered formaldehyde solution (4.5%, VWR, Prolabo, Leuven, Belgium).
The vertebral column was excised from the first cervical to the second lumbar vertebra, implicating the lesion site. Samples were stored in formaldehyde solution for further proceedings. Lesion site was distinguishable by the laminectomy.

MRI

For MRI, the excised vertebral columns were stored in 15 ml polypropylene-tubes with a diameter of 23 mm, in order to avoid motion artefacts. The tubes were filled with formaldehyde solution for conservation of the samples. The position of the lesion was marked on the tubes.

MRI was performed at room temperature on a clinical 1.5-T-scanner (Siemens Symphony, Erlangen, Germany) with a dedicated custom-made animal volume resonator using a 3D-FLASH imaging pulse sequence with the following parameters: TR/TE 14.0/5.22 ms, flip angle 30°, 28 partitions, partition thickness: 0.5 mm, FOV 80 mm, matrix size 512 times 512, voxel size 0.15 times 0.15 mm, 32 averages. Imaging was performed perpendicular to the spinal cord. Imaging time was 60 minutes per sample.

Histology

After MRI the vertebral columns were opened and the lower thoracic spinal cord containing the lesion was excised from each sample. The spinal cord at TH 11 was explanted, including the site of maximum damage which was determined by the hemorrhagic contusion mark. After embedding in paraffin, transverse sections (5µm) were taken at 100 µm intervals. Serial sections were stained with hematoxylin-eosine, Cresyl echt violet stain and luxol blue. At the site of maximum injury, sections were examined for the extent of damaged neuronal tissue, hemorrhage and macrophages as a sign of inflammation. These parameters were graded from zero to three by the pathologist. Zero indicated no, 1 moderate, 2 strong and 3 maximum alteration in comparison to tissue from native rats, based on the following criteria:

Demyelination:
0 - no demyelination
1 - up to 5% of cross sectional area demyelinated
2 - 6 - 30% of cross sectional area demyelinated
3 - more than 30% of cross sectional area demyelinated

Vacuolization:
0 - no vacuolization
1 - up to 5% of cross section area vacuolized
2 - 6 - 30% of cross section area vacuolized
3 - more than 30% of cross section area vacuolized

Macrophages:
0 - no macrophages
1 - 1 - 10 macrophages per high power field of lesion (HPF, 400 x magnification)
2 - 11 - 50 macrophages per high power field of lesion (HPF, 400 x magnification)
3 - more than 50 macrophages per high power field of lesion (HPF, 400 x magnification)

Hemorrhage:
0 - no extravasation of erythrocytes in cross section area
1 - extravasation of erythrocytes in up to 5% of cross section area
2 - extravasation of erythrocytes in 6 - 30% of cross section area
3 - extravasation of erythrocytes in more than 30% of cross section area

Data processing and statistical analysis

Images were evaluated using the scanner software package (Syngo, Siemens Erlangen, Germany). The spinal cord was outlined on axial slices and the mean signal was calculated. A region of interest was placed outside the animal contours for noise measurement. SNR in the most proximal slice was set to 100% and SNRs in consecutive slices were scaled accordingly expressing relative
SNRS (%SNR) as previously described. Statistics were performed using SAS/STAT (SAS Institute GmbH, Heidelberg, Germany). Mean and standard deviation (SD) were calculated for %SNR and values of locomotor rating of each group. Differences in %SNR and mean values of locomotor rating between both groups were assessed with T-test (99% confidence). Relation between %SNRs at the lesion site and locomotor rating was determined using Pearson’s correlation.

Results

MRI

The described sequence yielded good image quality for postmortem imaging of the spinal cord. In all rats, injection of MnCl2 into the cisterna magna yielded a consistently strong contrast enhancement of the uninjured spinal cord in $T_1$-weighted MRI (figure 1b). In SCI, manganese uptake by active neuronal tissue resulted in signal enhancement, reflected by a high SNR in the neuronal tissue cranial to the injury site, followed by a decrease in SNR both at and caudal to the lesion (figure 1a). The decline of SNR first seemed slightly cranial to the defined lesion localization at TH11. Contrast enhancement in the intact spinal cord yielded a clear depiction of the gray matter in the typical butterfly form (figure 1b). Gray matter could not be delineated either at or caudal to injury site (figure 1c, d). %SNR results of group 1 (50 kDyn) showed a mean of 60.9 and an SD of 2.5. No application failure occurred in group 1. SNR results of group 2 (150 kDyn) showed a mean of 39.8 and an SD of 5.5. No application failure occurred in group 2. The mean %SNR was significantly lower in the 150 kDyn group than in the 50 kDyn group ($P < 0.0002$).

Locomotor rating

Locomotor rating results of group 1 (50 kDyn) showed a mean of 19.7 and an SD of 1.0; results of group 2 (150 kDyn) showed a mean of 6.5 and an SD of 3.2. The mean locomotor rating for the 150 kDyn group was significantly lower than for the 50 kDyn group ($P < 0.0003$). Comparison of locomotor rating values of both groups on day 3 postinjury is depicted in figure 2. Group 1 (50 kdyn) showed explicitly higher values than group 2 (150 kdyn), inferring that a lower injury force results in improved motor function of the hind limbs. Group 1 values ranged from 18 to 21, denoting consistent plantar stepping with forelimb-hindlimb coordination.
during gait, and permanent toe clearance and parallel paw position at initial contact and lift-off of the hindlimb, varying in points of trunk instability and ability to keep the tail up. Group 2 values ranged from 1 to 11, with 1 denoting slight movement of 1 or 2 joints and 11 denoting frequent to consistent weight-supported plantar steps without forelimb-hindlimb coordination. A score of 7, which is approximately the mean in this group, denotes extensive movement of all 3 joints.

**Correlation of MRI and locomotor rating**

Comparison of %SNR at lesion sites of both groups is shown in figure 3. According to the locomotor rating results, group 1 showed higher values of %SNR than group 2. In figure 4, %SNRs of all rats are depicted depending on average locomotor rating values, showing an almost linear correlation of these 2 parameters. Calculation of Pearson correlation yielded a value of 0.99 (p = 0.001).

**Histology**

Histologic evaluation of the lesions supports MRI findings. Group 1 samples indicated a higher degree of tissue integrity. Semiquantitative parameters of neuronal damage, such as demyelination and vacuolization, were minor when compared with samples of group 2, and signs of hemorrhage and inflammation, characterized by invasion of macrophages. No parameter was graded 3 (maximum alteration) in group 1 or group 2. Results of the histologic grading are listed in Table 1.

Figure 5 shows spinal cord slices and semiquantitative evaluation of an animal of group 1; figure 6 shows corresponding slices and evaluation of an animal of group 2.
Discussion

This study demonstrates for the first time that MEMRI is an objective method for both structural depiction and functional assessment of spinal cord contusion injury in rats, as the MRI features of spinal cord impairment correlate with clinically derived values and are also in accordance with histology findings. Both MEMRI and locomotor rating show that the applied contusion model for SCI yields reproducible SCI with minimal variance in each group. Variations within the groups are most likely due to parameters that influence injury outcome, especially in the early phase (i.e., immunologic condition, slight age differences). The MEMRI method is minimally invasive, preventing additional damage to the neuronal tissue and allows for in vivo measurements in rats in the future. Thus, MnCl2 injection into the cisterna magna allows objective quantification of the functional status of the spinal cord in vivo, providing the possibility for kinetic follow-ups of injury development and therapeutic outcome. One potential disadvantage of Mn2+ is its neurotoxicity at higher concentrations that can lead to manganism, marked by symptoms of tremors, gait disorders, compromised motor skills, and abnormal balance. In accordance with previous reports, we did not observe any of these symptoms of extrapyramidal motor system dysfunction at the concentrations used. One reason may be that MnCl2 dilutes quickly when injected into cerebrospinal fluid (CSF). Also, manganism described in mine workers is due to chronic exposure to manganese that may have different toxicologic effects when compared with a single exposure.

Table 1 Histological evaluation of group 1 and group 2.

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<tr>
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<th>Group 1 (50 kdyn)</th>
<th>Group 2 (150 kdyn)</th>
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<tr>
<td>Demyelination</td>
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<tr>
<td>Grade 0</td>
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<td>0</td>
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<td>Grade 1</td>
<td>3</td>
<td>3</td>
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<tr>
<td>Grade 2</td>
<td>0</td>
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<td>Vacuolization</td>
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<td>Grade 0</td>
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<td>Grade 1</td>
<td>6</td>
<td>4</td>
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<td>Grade 2</td>
<td>0</td>
<td>2</td>
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<tr>
<td>Macrophages</td>
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<td>Grade 0</td>
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<td>Grade 1</td>
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<td>Grade 2</td>
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<td>Grade 2</td>
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Figure 5 Typical histology example from group 1. No demyelination or occurrence of macrophages could be observed. Vacuolization (arrow v) and hemorrhage (arrow h) were mild and correspondingly graded 1. Parameters were generally graded minor in this group when compared with group 2. Only 1 sample from this group was graded 2 in terms of macrophages.

Figure 6 Typical histology example from group 2. Demyelination (arrow d) and vacuolization (arrow v) were moderate and therefore graded 2. The parameter “macrophages” (arrow m) was graded 2; only hemorrhage (arrow h) was reported as mild and therefore graded 1. All parameters in this group were graded 1 or 2 (moderate or strong alteration); no parameter was graded 0. Gradings of demyelination and macrophages were especially higher than group 1, representing higher degree of injury and neuronal damage.
Currently, various techniques are used to evaluate SCI, but all techniques have their limitations. Common procedures of lesion depiction, like histology and anterograde or retrograde tracing techniques, are limited to the structural assessment of the damage and require invasive preparation of the spinal cord, risking secondary damage to neuronal tissue. Behavioral tests, such as the locomotor rating scale, allow functional in vivo assessment of impairment but do not allow visualization of the neuronal tissue and the underlying mechanisms of SCI. Well-trained observers are required to ensure objective results.

With the variety of procedures to assess spinal cord integrity and recovery, a general problem is the lack of standardization among different laboratories, complicating the comparison of successful therapies.

One clinical application of MRI to study the condition of neuronal white matter is diffusion tensor imaging (DTI). It has recently been shown that DTI can be used as a stable and observer-independent in vivo measure for the comparative assessment of white matter integrity. The method allows imaging of fiber tracts by measuring water diffusion and anisotropy as markers of neuronal tissue integrity. Commonly used for the depiction of neurologic diseases concerning the brain, ie, Alzheimer disease, microangiopathy and paraneoplastic diseases, the reproducibility of this method was recently validated, also for higher-field MRI techniques. DTI continues to gain acceptance in spinal cord imaging and it was recently demonstrated that in vivo derived DTI parameters are sensitive and specific biomarkers for spinal cord white matter damage in mice. In comparison to MEMRI, DTI provides information about the structural integrity of the tissue but cannot assess tissue viability, it mainly detects changes in fiber integrity when secondary loss of axons leads to a reduction in axon count.

Mn²⁺ is a divalent ion which mimics Ca²⁺ and is therefore actively transported into intact neurons via voltage-gated calcium channels; its uptake is reduced in injured, disconnected neurons. Uptake is followed by axonal transport at a rate that depends on injection volume and functional status of the neuronal tissue. After cisterna magna injection of MnCl₂, Mn²⁺ is directly distributed via the CSF and leads to strong contrast enhancement of intact neuronal tissue in T₁-weighted MRI, whereas there is a lack of enhancement in injured neurons. Thus, MEMRI offers both structural and functional information about neuronal tissue. Pautler et al were the first to use Mn²⁺ for the depiction of neuronal pathways in the olfactory system of mouse brains.

Since then, various tract-tracing studies have been performed on different species, laying the foundation of Mn²⁺-enhanced functional studies in the central nervous system. A recent publication describes the use of Mn²⁺ for depiction of SCI in a rat hemisection model via direct injection of MnCl₂ into the white matter of the spinal cord. Stieltjes et al described the use of MEMRI for functional depiction of SCI in the mouse in vivo. As in our study, MnCl₂ was injected into the CSF, without requiring an additional laminectomy. That minimizes injection-induced trauma. One major disadvantage of the used transection model carried out at the level of T₇/₈ in that study is poor control by nature of sharp trauma models.

In our study, the decrease of SNR first seems slightly cranial to the defined lesion location, surely due to the spreading of apoptosis and inflammatory response after spinal cord injury. The lack in SNR caudal to the lesion could partly be connected with this spreading of injury. Stieltjes et al observed a similar behavior in enhancement. They investigated if a block in CSF circulation after SCI could be a reason for signal decrease in the caudal part of the spinal cord, as Mn²⁺ is distributed via the CSF in the uninjured spinal canal. But gadolinium-diethylenetriamine penta-acetic acid (Gd-DTPA) injection into the cisterna magna showed that the injury had not led to a complete disruption of the CSF circulation.

The excellent correlation we observed in this study between 2 distinct in vivo techniques of SCI quantification is very promising. Further research is warranted to evaluate the precise relationship between applied contusion pressure and final clinical outcome.
Conclusions

MnCl₂ enhanced MRI represents a standardizable and reliable method for postmortem depiction and quantification of spinal cord contusion injuries in rats. As MRI features of lesion quantification correlate closely with results of clinical assessment of motor function, MEMRI yields a measure of functional postmortem imaging of spinal cord integrity and severity of SCI, additionally providing a structural depiction of the spinal cord. Our method can be used in laboratories without an on-site imaging facility that otherwise prevents in vivo imaging. Also, our results indicate that in vivo MEMRI of rat spinal cord seems feasible using the procedure described.

References

15. Deppe M, Duning T, Mohammadi S, et al. Diffusion-tensor imaging at 3 T: detection of white matter alterations in neurological patients on the basis of
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