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Visualisation of the extent of damage in a rat spinal cord injury model using MR Microscopy of the water diffusion tensor

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Abstract. Magnetic Resonance Diffusion Tensor Imaging (DTI) of the control and traumatic injured spinal cord of a rat *in vitro* is reported. Experiments were performed on excised spinal cords from 10 Wistar rats, using a home-built 6.4 T MR microscope. MRI and histopathological results were compared. Presented results show that DTI of the spinal cord, perfused with formalin 10 minutes after the injury, can detect changes in water diffusion in white matter (WM) and in gray matter (GM), in areas extending well beyond the region of direct impact. Histology of neurons of the GM shows changes that can be attributed to ischemia. This is in agreement with the observed decrease of diffusion in the injured regions, which may be attributed to the cytotoxic edema due to ischemia. However, the diffusion changes in highly anisotropic WM seem to be caused by a direct action of mechanical force of impact, which significantly distorts the nerve fibers.

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Key words: magnetic resonance microscopy, diffusion tensor imaging (DTI), diffusion anisotropy, spinal cord injury

INTRODUCTION

Diffusion MRI became one of well established techniques in early detection diagnosis of brain ischemia (Minematsu et al. 1992). Attenuation of the NMR signal due to diffusion has been extensively used for investigations of water dynamics since introduction of pulsed field gradient techniques (Stejskal 1965). Introduction of diffusion gradients into MR imaging sequences allowed visualization of water diffusion and thus, insight into tissue microstructure and microdynamics (Le Bihan 1991, Taylor et al. 1985). Biological materials usually have anisotropic structure, therefore water diffusion has to be described by a tensor. Values of the apparent diffusion coefficient (ADC) in the laboratory frame depend upon the orientation of the sample. Local properties of diffusion related to the tissue structure can be obtained by diagonalizing the diffusion tensor. Its principal components (TPC) and principal axes (TPA) determine the state of diffusion independent of the sample orientation. Any changes in tissue structure and water dynamics due to different processes developing after contusion reflect in values of diffusion tensor components (DTC) (Basser et al. 1994a,b).

The spinal cord consists of white matter (WM) and gray matter (GM). The white matter is composed of highly oriented axons parallel to each other surrounded by myelin sheaths relatively impermeable to water, neighbouring oligodendrocytes, astrocytes and blood vessels. The gray matter contains perikarial parts of neurons, glial cells, capillary blood vessels and a meshwork of cell processes (neuropile) creating more isotropic structure. Due to the tissue structure, diffusion in the white matter is highly anisotropic - fairly free along tubules of axons, while restricted across them. Diffusion in the gray matter usually reveals some degree of anisotropy. During injury the structure of nervous tissue becomes disordered or damaged.

Permanent damage of the spinal cord tissue may cause paralysis of major parts of the human body leading to permanent disability. Therefore, early diagnosis and assessment of the extent and character of the nervous tissue damage is an important factor in the therapeutic decision making process. Diffusion weighted magnetic resonance imaging (DWI) became a promising technique in studying spinal cord when it was found that changes in ADC may be observed very early after ischemic injury of the brain tissue, earlier than changes in other contrast parameters (Moseley et al. 1990). Since then DWI has been used to investigate injury induced damage in the spinal cord tissue (Ford et al. 1994, Moseley et al. 1990, Nakada et al. 1994). Attention was focused on the implementation and development of DTI (Basser et al 1994a, b) in investigations of the brain (Pierpaoli et al. 1995, Ulug et al. 1995) and the spinal cord (Inglis et al. 1997, Nevo et al. 2001).

The aim of this work was to correlate the presence and extent of spinal cord tissue damage occurring within first 10 min after contusion with changes in the apparent diffusion tensor (ADT) components of water in order to find a parameter, which is useful in the visualization of the injury. We report ADT weighted data measured *in vitro* on excised, formalin fixed samples of control and injured rat spinal cord in the sagittal and transverse planes and analysis of the diffusion anisotropy as a function of distance from the center of the injury, which allows estimation of the extent of the damage. Throughout this paper we assume that the fixation process preserves the tissue structure although altering the absolute values of ADT. Thus these values still reflect relative differences between normal and traumatic spinal cord tissues (Ford et al. 1994, Krzyżak et al. 1999, Nevo et al. 2001, Węglarz et al. 2001).

METHODS

Spinal cord injury model

We used a well-characterized “dynamic load” rat spinal cord injury model (Black et al. 1988) with minor modifications. Ten male Wistar rats of 280 g to 300 g were used divided into two groups of control and injured animals. Under general anesthesia (4% chloral hydrate 0.9 ml/100 g of body mass given intraperitoneally) a laminectomy was performed at the Th9 level. A 2.8 g weight was dropped from a height of 8 cm onto the impounder of 2 mm diameter positioned on the exposed *dura mater*. The force was chosen to induce damage to dorsal parts of the spinal cord and not to crush it. Animals were sacrificed 10 min after injury. Spinal cords were fixed *in situ* by intracardiac perfusion of 4% buffered formalin solution. After initial fixation, they were excised en-block with adjacent bone structures and placed in the formalin solution. The samples were placed in sample tubes filled with 4% formalin solution. The control group used in this

work consisted of 5 healthy rats whose spinal cords were fixed and excised in the same way as injured rats. Animal procedures were approved by the Jagiellonian University Committee of Bioethics.

NMR measurements

The samples in test tubes were put into a home-built MR microscope, with a 6.4 T narrow vertical bore superconducting magnet. A home-built probe was equipped with a set of actively shielded gradient coils capable of generating gradients of up to 1 T/m in less then 100 ms when driven from Techron 5760 amplifiers. A conventional Spin-Echo imaging sequence (T_1 and T_2 weighted) modified by adding diffusion gradient pulses on both sides of the 180°rf pulse was used to measure the effect of diffusion on MR images. To measure the diffusion tensor (Basser et al. 1994b), diffusion gradients were applied in the following directions: (1,0,0), (0,1,0), (0,0,1), (1,1,0), (1,0,1) and (0,1,1). MR images (256 × 256) were acquired at room temperature (21°C) with an in-plane resolution varying from 30 µm × 30 µm up to 40 µm × 40 µm and a slice thickness of 400 µm–800 µm. The following acquisition parameters were used: repetition time (TR) = 0.8 s, echo time (TE) = 47 ms, diffusion time Δ= 9 ms, diffusion gradient length δ = 3 ms and amplitude G_d up to 280 mT/m.

Data analysis

Determination of the diffusion tensor components is based on the solution of the Bloch equations for the Spin Echo MR experiment with the diffusion gradients. The signal attenuation is given by

$$\ln\left(\frac{A(b)}{A(0)}\right) = -\gamma^2 \sum_{\alpha,\beta=1}^{3} b_{\alpha\beta} D_{\alpha\beta} \qquad (1)$$

where: A(0) is the echo intensity without diffusion gradients, A(b) is the echo intensity for a given diffusion gradient, $b_{\alpha\beta}$ is the component of the diffusion gradient matrix, and $D_{\alpha\beta}$ is the component of the diffusion tensor (Basser et al. 1994b).

To measure the diffusion tensor, accurate determination of the b matrix is required. In our experiment, the b matrix values were calculated, taking into account all gradients in the imaging sequence including the "cross terms" (Matiello et al. 1994). To check the calculation, elements of the b matrix were calibrated by measuring

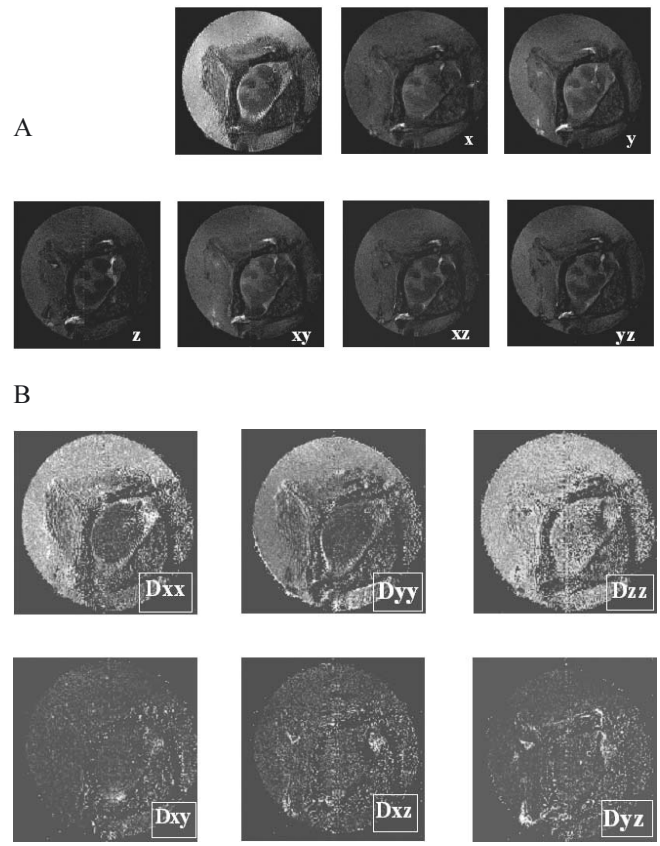


Fig. 1. Transversal diffusion weighted images (A) and corresponding images of water diffusion tensor components (B) of the control spinal cord of a rat. Experimental data consists of an image without diffusion gradient and images with diffusion gradients applied in 6 directions: (1,0,0), (0,1,0), (0,0,1), (1,1,0), (1,0,1) and (0,1,1). Images of water diffusion tensor show diagonal components (Dxx, Dyy, Dzz) and off-diagonal components (Dxy, Dxz, Dyz).

the diffusion in a water phantom. At the largest diffusion gradient of 280 mT/m maximum values for b_{xx}, b_{yy}, b_{zz} of 626 s/mm^2, 647 s/mm^2, 596 s/mm^2, were obtained for read, phase and slice selection directions, respectively. Corresponding off diagonal elements b_{xy}, b_{xz}, b_{yz} were equal to 600 s/mm^2, 595 s/mm^2, 625 s/mm^2, respectively. Elements of the diffusion tensor were calculated by minimization of the χ^2 function of ln[A(b)] (1) for every pixel in the image.

Principal diffusivities (λ_1, λ_2, λ_3) were determined after diagonalizing diffusion tensor for every pixel, after that, they were used for calculation following parameters: longitudinal diffusion tensor component (DL) = λ_3; transverse diffusion tensor component (DT) = $(\lambda_1+\lambda_2)/2$; isotropy index ID = DT/ DL.

Statistical analysis

An alpha level (α) of significance at 0.1 was used for all statistical tests. To determine adhesion to the same statistical population Snedecor's test of variances was performed. To examine differences between control and injured rats *t*-student's test was used for samples comparison. The data are presented as means (x) $\pm$ SEM $\times$ $t_{n,\alpha}$, where (SEM) standard error of the mean x, $t_{n,\alpha}$ critical *t*-value was assumed $t_{5,0.1}$=2.01.

Histopathology

After performing the DTI experiment, the spinal cord was retrieved from the test tube and sectioned. Portions that corresponded to the site of direct injury and adjacent regions caudally and rostrally were embedded in paraffin. Slides were stained with hematoxylin-eosin and the Klüver-Barrera method for myelin and examined with an optical microscope.

RESULTS

A set of transverse diffusion weighted images of the injured spinal cord of a rat without and with diffusion gradients applied in 6 different directions, listed above, and the corresponding images of the water diffusion tensor components are shown in Figs 1A, B respectively. The first T_1/T_2 weighted image in Fig. 1A is the most bright due to absence of diffusion gradients. The next diffusion weighted images show very good contrast and differentiation between GM and WM, indicating dependence of signal intensity on the orientation of the diffusion gradient. The off-diagonal components of the diffusion tensor are much smaller than the diagonal ones since the predominantly longitudinal orientation of the spinal cord structure matches the z-axis of the laboratory frame.

A sagittal T_1, T_2 weighted pilot image of the injured spinal cord of a rat is shown in Fig. 2. Four different arbitrarily selected paths (white dots) running parallel to the spinal cord but situated in different anatomical regions of the cord were chosen, with the level of injury at their center. Paths no. 1 and no. 2 are placed predominantly in the dorsal white matter. In histological slide (Fig. 4) path no. 1 roughly corresponds to the region marked with continuous circle, while path no. 2 corresponds to region encircled with dashed line. The superficial part of white matter (continuous circle) is much less compact and with numerous "vacuoles" as compared with the deeper white matter. Path no. 3 follows the gray matter in the central part of the spinal cord and path no. 4 is placed in the ventral white matter, in the region opposite to the injury. The values of the longitudinal component of the water diffusion tensor (DL=λ_3), average transverse component of the water diffusion tensor (DT = (λ_1+λ_2)/2) and diffusion isotropy index (ID = DT/DL) as a function of position along the chosen paths, are shown as bar graphs in Fig. 3. Each value of the water diffusion tensor component shown in these graphs represents an average over 0.2 mm in diameter, marked as a dot in Fig. 2, and averaged over the group of 5 rats.

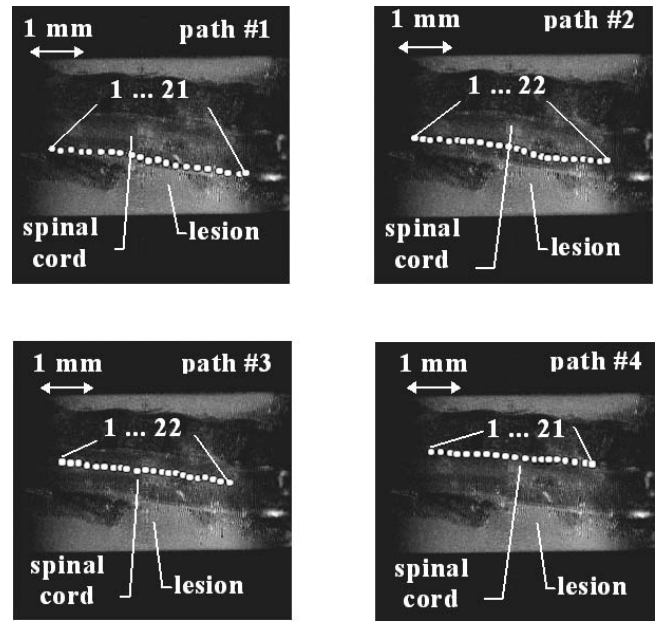


Fig. 2. A sagittal pilot T_1, T_2 weighted image of the injured spinal cord of a rat. Position dependence of diffusion isotropy index (ID), longitudinal (DL) and average transversal (DT) components of the water diffusion tensor for the injured spinal cord of a rat.

Following the longitudinal axis of the spinal cord from a point caudal to the lesion through the lesion center and then rostrally, we observe a decrease in DL with its minimum matching the center of the lesion. On the other hand, changes in DT are not readily observable. This results in the elevation of the isotropy index ID (Figs 3 A-B) over the region of the lesion.

Along path no. 1 in sagittal images, corresponding to a superficial layer of white matter directly exposed to injury (Fig. 3A), the elevation of the isotropy index is

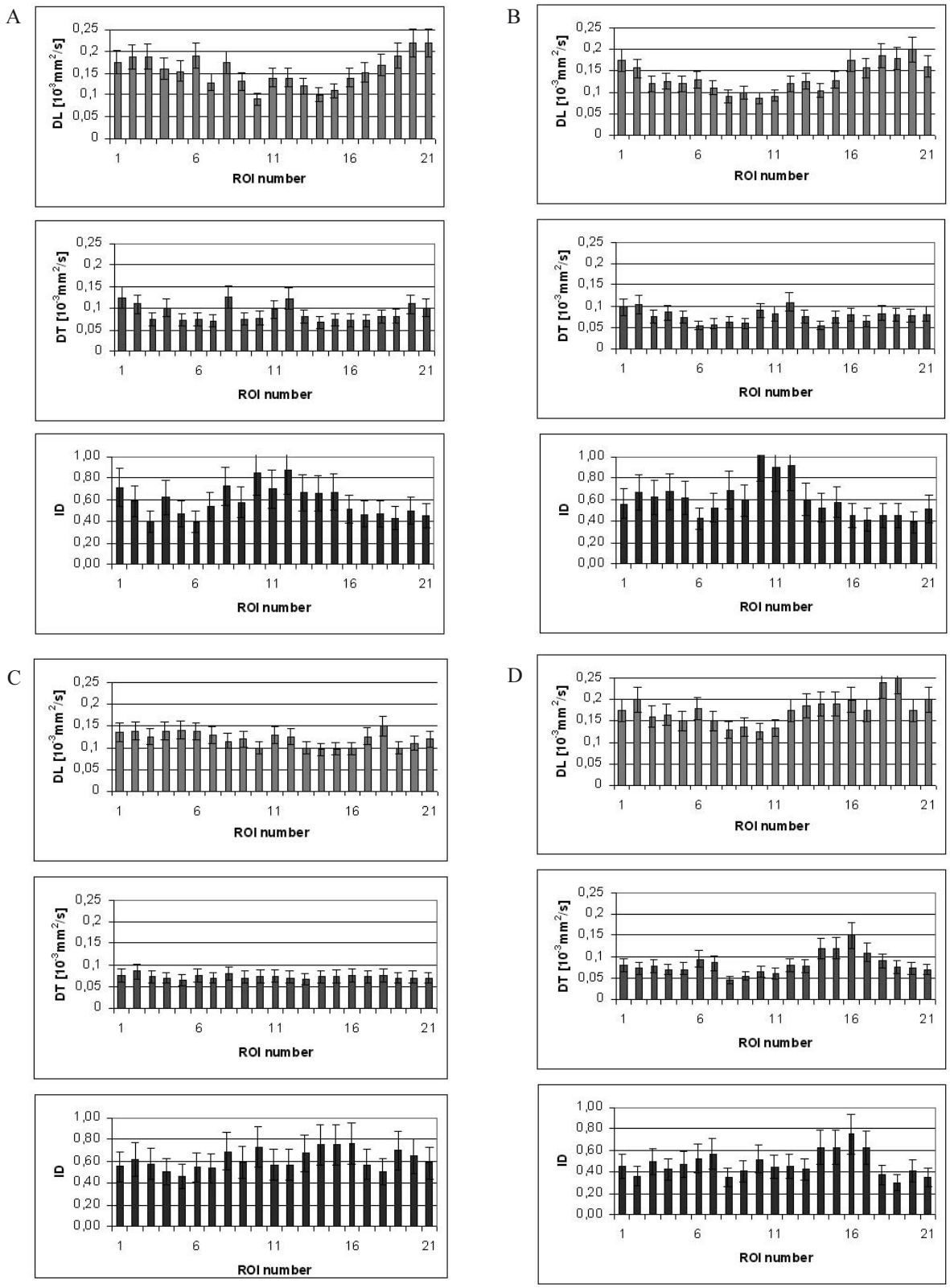


Fig. 3. (A) Path no. 1 running predominantly through dorsal WM. Increase of ID from ROI no. 8 to ROI no. 15 (~2.5 mm); (B) path no. 2 running predominantly through dorsal WM. Increase of ID from ROI no. 10 to ROI no. 12 (~1 mm); (C) path no. 3 running predominantly within GM. Increase of ID from ROI no. 8 to ROI no. 20 (~4 mm). (D) Path no. 4 running through central WM.

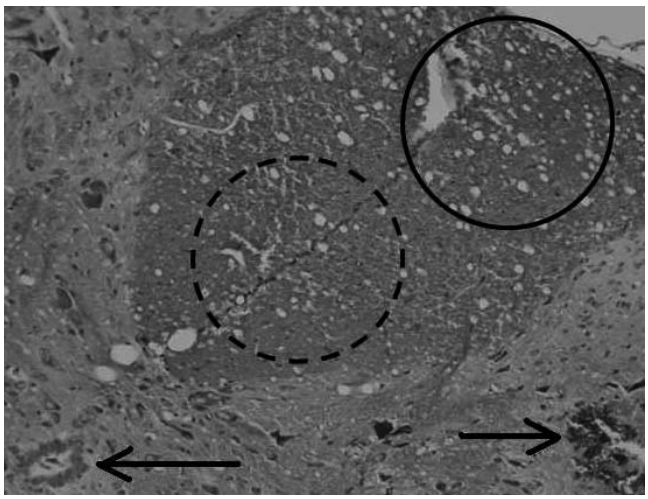


Fig. 4. Dorsal white matter of the spinal cord with directly adjacent regions of gray matter at the level of injury. The continuous circle marks more superficial part of white matter while the dashed circle marks inner part of white matter (much more compact and less vacuolated). Arrow to the right points out the small haemorrhage in gray matter. Arrow to the left shows the central canal of spinal cord. Tansverse section with relation to the long axis of spinal cord. Klüver-Barrera method for myelin.

observed over a broad region (~2.5 mm). Along path no. 2, corresponding to the pyramidal tract, the ID is elevated over a narrower region (~ 1 mm). This difference can be explained by spatial changes or distortion of the myelin fibers in the white matter (Fig. 5). The extent of this tortuosity is broader in the superficial layers of WM than in WM deeper in the spinal cord (Moseley and Kucharczyk 1995). We studied histological changes on cross sections of the spinal cord (in relation to the long axis) therefore the elevation of tortuosity of myelinated fibers cannot be directly documented. However, as shown in Fig. 4, the superficial part of dorsal white matter was evidently much more loosened and edematous and hence one can conclude, that the running nerve fibers had much more free space to acquire an undulated (more tortuous) shape instead of a straight one. An even broader region of elevation of the ID (~4 mm) is seen in path no. 3, which corresponds to the central part of the GM and the central part of the spinal cord (Fig. 3.).

Figure 6 shows a transverse T_1, T_2 weighted pilot image of the control spinal cord of a rat with dots indicating Regions of Interest (ROIs), positioned in different anatomical regions of the cord.

Values of DL, DT and ID for different anatomical regions within the WM and the GM, obtained from transverse images of the control and injured spinal cord, averaged over groups of rats are presented in Fig 6B. Results reported here, averaged over a voxel of 0.2 mm in diameter, represent differences among anatomical structures of the spinal cord.

In transverse slices, the ID rises significantly in the white matter (dorsal to the pyramidal tract – ID up to ~0.7) and in the gray matter (dorsal horns – ID up to ~0.9) directly subjected to injury. Elevation of the isotropy index is observed in all other regions, except in the ventral horns, which show only minimal changes (Fig. 6B).

In the control spinal cord (Fig. 6B), longitudinal diffusion (DL) in the WM is higher than in anterior horns of the GM. GM of posterior horns do not substantially differ from that of WM because in our analysis they are not well separated from white matter, which contains axial (longitudinal) fibers and shows significantly higher DL (Inglis et al. 1997). The lowest isotropy index ID ~0.2 was found in the pyramidal tract in WM, while the highest index (~0.7) was found in the anterior horns in GM. These findings are consistent with the microscopic picture of these structures and with literature (Inglis et al. 1997). The pyramidal tract in the rat is located between the posterior horns and the central gray matter and is characterized by particularly densely packed nerve fibers.

Statistical analysis

To confirm the observations of changes of DTI parameters the statistical analysis of data registered for control and injured rats was performed. The results of

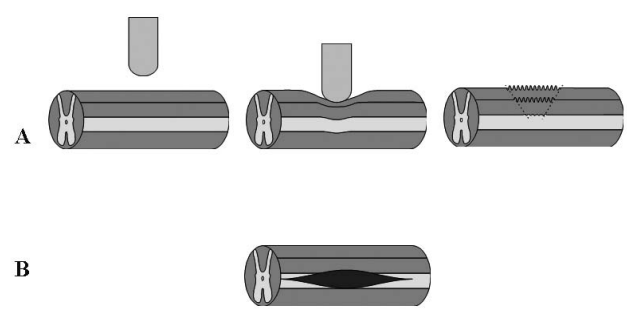
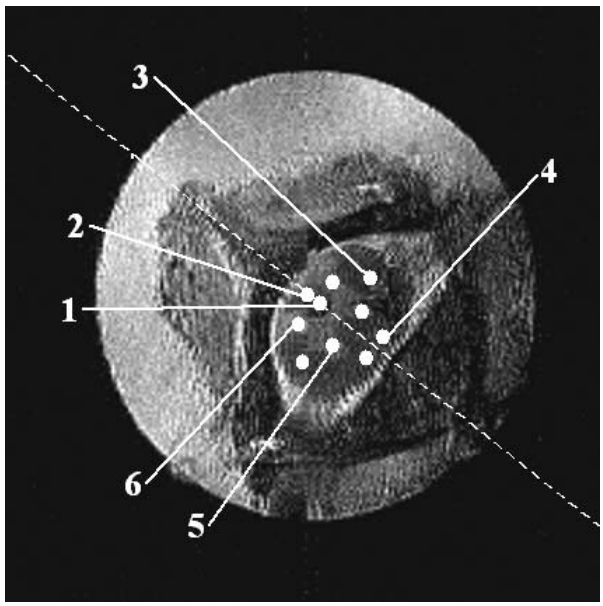


Fig. 5. Scheme of injury invoked damage in the spinal cord tissue. (A) The extent of damage in the white matter is wedge shaped with the base directed towards the dorsal aspect of the spinal cord; (B) the region of damage in gray matter is fusiform in shape and extents both rostrally and caudally beyond the place of primary injury.

A



B

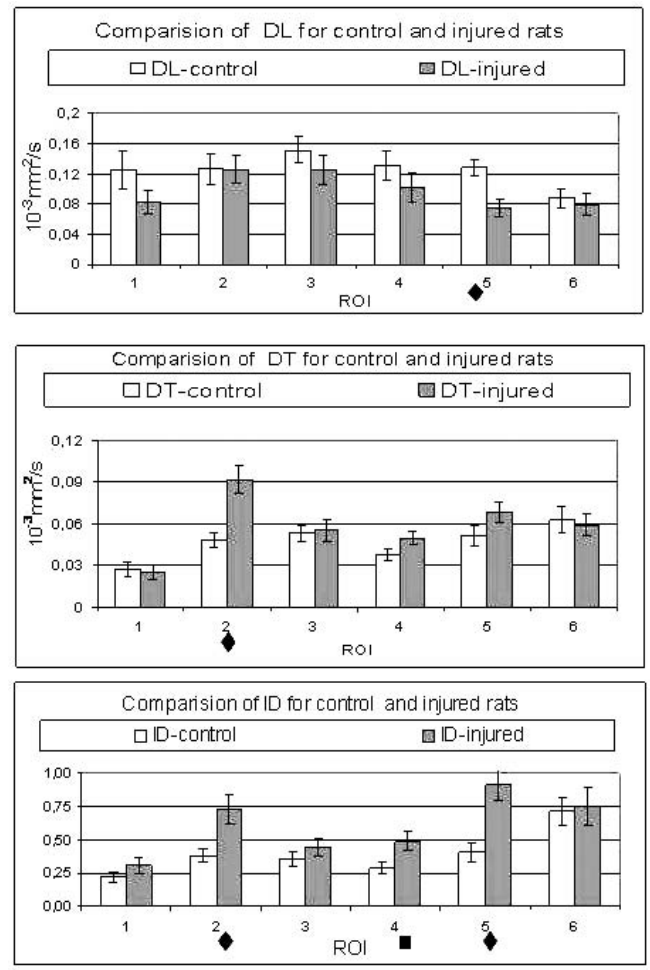


Fig. 6. (A) Transverse slice through the control spinal cord shows positions of anatomical details within white matter (1–4) and gray matter (5, 6): (1) dorsal funicle (pyramidal tract); (2) dorsal funicle (marginal region – dorsally from pyramidal tract); (3) lateral funicle (right and left); (4) ventral funicle (right and left); (5) ventral horn (right and left); (6) dorsal horn (right and left). (B) Comparison of ID, DL and DT for control and injured spinal cord of a rat. An "♦" indicates a statistically significant differences (P<0.1) for ROI no. 5 (ID, DL) and ROI no. 2 in (ID, DT). An "■" indicates ROIs for which parameters were not analyzed due to statistically different variances.

statistical analysis are presented in Fig. 6B. The analysis of variances (Snedecor's test) realized for ROIs presented in Fig. 6A showed that except for ID for ROI no. 4 there is a statistical identity of variances of DTI parameters for remaining ROIs. The comparison of DTI parameters performed by *t*-student's test show existence of statistically significant differences (P<0.1) for ROI no. 2 (analysis of ID and DT) and no. 5 (analysis of ID and DL).

DISCUSSION

In the transverse and sagittal planes, the longitudinal and transversal diffusion coefficients change in areas placed next to the level of the injury (Figs 3 and 6). The alterations of DL, which show direction of maximum diffusivity (in WM mostly in parallel to axons) and DT (direction perpendicular to the maximum diffusivity) yield information exhibiting the extent of injury. This observation found confirmation in statistical analysis for dorsal funicle region in WM and ventral horn in GM (Fig. 6B). In GM, the decrease is probably due to cytotoxic edema, similar to early ischemic changes in a stroke (Krzyżak et al. 1997, Moseley and Kucharczyk 1995). In the white matter this phenomenon can be attributed to the distortion or convolution of longitudinally oriented neural fibers. Diffusion in the transverse plane in the region of the white matter dorsal to the pyramidal tract is significantly elevated (Fig. 6B). The elevation of diffusion (dorsal to the pyramidal tract) is surely related to the proximity of this region to the direct impact which resulted in "incohesitivity" (rarefaction) of the injured tissue. This rarefaction was readily observed in histological slides, however in this case we are aware that at least partially it may be due to tissue processing artifacts.

Since there is no anatomical alignment of neural fibers in the central region of GM, which can be dis-

torted by a mechanical force, changes of the ID should be explained in a different manner. From literature (Balentine et al. 1978) and well established experimental data and clinical and neurotraumatological experience, it is well known that the extent of tissue damage in GM substantially exceeds the size of the direct action of the traumatic force both caudally and rostrally (Fig. 5). This is due to secondary events, especially ischemia and hemorrhage. Our observation of changes in diffusion in a very short time (about 10 minutes) after the trauma demonstrates existence of a very large area of tissue, which is probably under the influence of ischemia and/or hemorrhage. This hypothesis is supported by histopathology, because degenerative changes in neurons shown in Fig. 7B, resemble the so called „dark neurons" known from brain ischemia experiments (Eke et al. 1990), as markers of hypoxia.

All our experiments discussed in this work were performed on formalin fixed spinal cords. There is still an open question of the relevance of these results to the situation *in vivo*, as the effect of fixation on the diffusion is not well understood (Ford et al. 1994, Inglis et al. 1997, Pindel et al. 2001). We obtained much lower values of DTCs measured for excised rat spinal cords in formalin solution when compared to measurements in saline solution (Elshafiey et al. 2002, Krzyżak et al. 1999, Węglarz et al. 2001, 2002, 2004). Our results, corresponding to the same or close anatomical structures, have numerical values similar to the results of (Inglis et al. 1997), who used comparable method of tissue fixation, but are much lower than the values reported by Ford and coauthors (1994) and Gulani and coauthors (1997). In spite of much lower values of DTC in formalin we assumed that the fixed spinal cord tissues preserve at least basic anisotropic properties of the spinal cord *in vivo*. Our *in vivo* results reported in (Krzyżak et al. 1999) indicate that especially longitudinal diffusion component is significantly higher (up to 2 times) as compared to the results obtained in saline *in vitro* experiment.

It is known that formaldehyde penetrates tissues swiftly but fixes much more slowly (Fox et al. 1985, Puchtler et al. 1985). The chemistry of formalin fixation is based on forming the cross-links between proteins leading to apparent stiffening of tissue structures. This process reaches the equilibrium after 24 hours. The formalin fixation shortens T_2 relaxation time and decreases values of diffusion by changing permeability of cell membranes. These processes can be partially reversed by washing up the spinal cord with saline (Pindel et al. 2001). However, formalin fixation while changing absolute values of diffusion does not significantly influence the anatomical anisotropy of tissue and hence the anisotropy of water diffusion. The rinsing of the formalin with saline, increases MR signal to noise ratio and allows to obtain better quality images, but increases also measured diffusion. However, the relative differences between WM and GM as well as between normal and injured tissue are essentially preserved (Krzyżak et al. 1999, Mills et al. 2002, Węglarz et al. 2001, 2002). Our results emphasize the potential of DTI to detect the earliest changes in spinal cord structure and pathophysiology after a traumatic event. The histology of neurons of gray matter shows changes that can be attributed to ischemia, which are in agreement with observed decreases in diffusion in the injured regions. This decrease in diffusion can be attributed to cytotoxic edema due to ischemia. The diffusion changes in highly anisotropic white matter, however, appear to result from a direct mechanical impact resulting in the distortion of fibers. The extent of the changes, which were observed in the GM, likely corresponds to the region of prospected tissue damage.

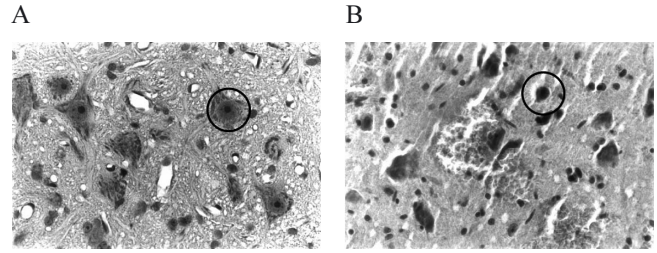


Fig. 7. Ventral horn of spinal cord (gray matter) of a rat. Hematoxylin-eosine method. Magnication × 400. (A) Control: well preserved motoneurons with distinct Nissl's substance. (B) Injured (10 min after trauma): severely damaged motoneurons with blurred outlines of nuclei and darker, shrinked cytoplasm ("dark neurons"). Petecchial haemorrhages. Relatively well preserved neuropil.

CONCLUSION

It was shown that DTI in the spinal cord even 10 minutes after injury shows changes in water diffusion in areas, which in superficial layer of dorsal WM and GM extend far beyond the region of direct impact. This finding is in agreement with the pathophysiology of spinal cord trauma.

ACKNOWLEDGEMENTS

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