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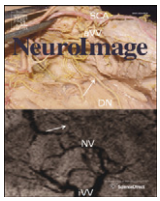

Radial diffusivity predicts demyelination in *ex vivo* multiple sclerosis spinal cords ☆,☆☆

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ABSTRACT

Objective: Correlation of diffusion tensor imaging (DTI) with histochemical staining for demyelination and axonal damage in multiple sclerosis (MS) *ex vivo* human cervical spinal cords.
Background: In MS, demyelination, axonal degeneration, and inflammation contribute to disease pathogenesis to variable degrees. Based upon *in vivo* animal studies with acute injury and histopathologic correlation, we hypothesized that DTI can differentiate between axonal and myelin pathologies within humans.
Methods: DTI was performed at 4.7 T on 9 MS and 5 normal control fixed cervical spinal cord blocks following autopsy. Sections were then stained for Luxol fast blue (LFB), Bielschowsky silver, and hematoxylin and eosin (H&E). Regions of interest (ROIs) were graded semi-quantitatively as normal myelination, mild (<50%) demyelination, or moderate–severe (>50%) demyelination. Corresponding axonal counts were manually determined on Bielschowsky silver. ROIs were mapped to co-registered DTI parameter slices. DTI parameters evaluated included standard quantitative assessments of apparent diffusion coefficient (ADC), relative anisotropy (RA), axial diffusivity and radial diffusivity. Statistical correlations were made between histochemical gradings and DTI parameters using linear mixed models.
Results: Within ROIs in MS subjects, increased radial diffusivity distinguished worsening severities of demyelination. Relative anisotropy was decreased in the setting of moderate–severe demyelination compared to normal areas and areas of mild demyelination. Radial diffusivity, ADC, and RA became increasingly altered within quartiles of worsening axonal counts. Axial diffusivity did not correlate with axonal density ($p = 0.091$).
Conclusions: Increased radial diffusivity can serve as a surrogate for demyelination. However, radial diffusivity was also altered with axon injury, suggesting that this measure is not pathologically specific within chronic human MS tissue. We propose that radial diffusivity can serve as a marker of overall tissue integrity within chronic MS lesions. This study provides pathologic foundation for on-going *in vivo* DTI studies in MS.

Introduction

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system (CNS) that commonly results in disability (Noseworthy et al., 2000). Spinal cord involvement is common in MS, even early in disease (Bot et al., 2004a). Multiple sclerosis plaques contain marked pathologic heterogeneity, with variable demyelination, axonal damage, gliosis, remyelination and inflammation (Barnes et al., 1991). While demyelination may be considered the hallmark of MS pathology, axonal damage is hypothesized to be the histologic substrate for disability (Trapp et al., 1998). Axonal loss can occur early in active disease (Ferguson et al., 1997; Ghosh et al., 2004) and is commonly observed later in the disease subtypes of primary progressive MS (PPMS) and secondary progressive MS (SPMS) (Tallantyre et al., 2009).

Brain magnetic resonance imaging (MRI) lesion load on T2 weighted (T2W) images correlates only modestly with physical disability in MS

Abbreviations: MS, multiple sclerosis; CNS, central nervous system; PPMS, primary progressive multiple sclerosis; SPMS, secondary progressive multiple sclerosis; MRI, magnetic resonance imaging; T2W, T2 weighted; DTI, diffusion tensor imaging; ADC, apparent diffusion coefficient; RA, relative anisotropy; ROI, region of interest; WMT, white matter tract; LFB, Luxol fast blue; H&E, hematoxylin and eosin; NAWM, normal-appearing white matter; ICC, intraclass correlation coefficient; ANOVA, one-way analysis of variance; SPSS, Statistical Package for the Social Sciences; FA, fractional anisotropy; DAWM, diffusely abnormal white matter.

☆ The corresponding author takes full responsibility for the data, the analyses and interpretation, and the conduct of the research. The corresponding author guarantees the accuracy of the references. The corresponding author has full access to all the data and has the right to publish any and all data, separate and apart from the attitudes of the sponsor.

☆☆ The Methods section includes a statement of compliance with institutional standards for the use of human subjects and tissue for this study.

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(Barkhof, 1999; Brex et al., 2002). In addition, correlation of conventional spinal cord parameters with disability is weak at both 1.5 and 3.0 T MR field strengths (Nijeholt et al., 1998; Stankiewicz et al., 2009). While conventional MRI, including T2W, is relatively sensitive to spinal cord abnormalities, it lacks specificity to the underlying pathologic correlate. In addition, spinal cord axonal loss occurs largely independent of T2 changes (Bergers et al., 2002). Advanced imaging techniques to improve pathologic specificity and correlate with physical disability would be clinically valuable.

Diffusion tensor imaging (DTI) measures microscopic movement of water in tissues. Diffusion tensor imaging provides information on CNS tissue integrity and structure (Filippi et al., 2001). The directional diffusivity derived from DTI measurements describes microscopic water movement parallel to ($\lambda_{||}$, axial diffusivity) and perpendicular to ($\lambda_{\perp}$, radial diffusivity) axonal tracts. We have previously shown in mouse models of white matter injury that *decreased* $\lambda_{||}$ is associated with acute axonal injury, and *increased* $\lambda_{\perp}$ is associated with myelin injury (Budde et al., 2009; Song et al., 2002, 2003, 2005). Additionally, in a rat spinal cord model, DTI detects axonal damage distal to inflammatory lesions, perhaps secondary to Wallerian degeneration (DeBoy et al., 2007). In humans, $\lambda_{||}$ has been demonstrated to decrease with acute optic neuritis (Naismith et al., 2009). Also, $\lambda_{\perp}$ increases with clinical disability and severe tissue injury (Naismith et al., 2010a, 2010c).

This study sought to determine the relationship between DTI parameters and histopathology in human *ex vivo* spinal cord in the setting of chronic MS. The spinal cord provides an excellent model to study directional diffusion because axons within white matter tracts run in parallel, without significant crossing. As *in vivo* spinal cord DTI studies are explored (Agosta et al., 2007; Benedetti et al., 2010; van Hecke et al., 2009), it is important to understand the pathologic correlate of DTI parameters. A validated histopathology correlation study is a necessary prerequisite to the interpretation of *in vivo* spinal cord DTI studies. Our hypothesis was that $\lambda_{\perp}$ would differentiate among degrees of demyelination, whereas $\lambda_{||}$ would provide information about axonal damage.

Methods

Specimen preparation

Institutional standards were followed for the use of human subjects and tissue for this study. Cervical spinal cords were obtained following autopsy from 13 clinically definite MS subjects and eight normal control subjects. Four MS subjects and three controls were excluded due to T2* artifact on MRI. Cervical spinal cord blocks were fixed in 10% formalin in PBS at room temperature. Time from expiration to beginning fixation ranged from 4 to 16 h. Specimens remained in fixative for 7–10 days before being cut into blocks.

Magnetic resonance imaging

MRI was performed using an Oxford Instruments 200/330 magnet (4.7 T, 33-cm clear bore) equipped with a 15-cm inner diameter, actively shielded Oxford gradient coil (18 G/cm, 200-μs rise time). The magnet, gradient coil, and Techron gradient power supply were interfaced with a Varian UNITY-INOVA console controlled by a Sun Microsystems Ultra-60 Sparc workstation. A custom-made sample holder and a custom-made 8 mm solenoid surface coil were used for transmission and reception.

A conventional multislice spin echo imaging sequence modified by adding the Stejskal–Tanner diffusion sensitizing gradient pair was employed for acquisition of the required series of diffusion weighted images. The diffusion weighted images were acquired with repetition period (TR) 3 s, spin echo time (TE) 43 ms, time between application of gradient pulses 25 ms, diffusion gradient on time 10 m, slice thickness 0.25 mm with 0.25 mm isotropic voxels. Diffusion sensitizing gradients were applied along six directions with *b*-values of 0 and 1.813 ms/μm^2 (Basser and Pierpaoli, 1998). After imaging processing and averaging, the diffusion tensor was diagonalized to derive directional diffusivity on a voxel-by-voxel basis. Diffusion tensor imaging parameter maps were calculated for apparent diffusion coefficient (ADC), relative anisotropy (RA), $\lambda_{||}$, and $\lambda_{\perp}$ as previously described (Song et al., 2002). In addition, conventional T2W images were obtained at the same resolution.

ROI identification

A neuroradiologist (T.L.B.) blinded to pathologic and clinical data independently identified regions of interest (ROIs) within lesions from the T2W and DTI parameter maps. Additionally, standard ROIs were created for white matter tracts (WMTs) including the dorsal white matter, right and left lateral white matter, and right and left ventral WMTs (Fig. 1). These standard column ROIs were applied to all slices of control subjects to derive normal values for DTI parameters in human cervical spinal cords *ex vivo*.

Histological staining

Following DTI, the formalin fixed MS and control spinal cords were embedded in paraffin and cut on a sliding microtome at a thickness of 3 μm. Sections were stained with Luxol fast blue (LFB) for myelin quantification, Bielschowsky silver for axonal counts, and hematoxylin and eosin (H&E) for inflammatory infiltrates. Methods of grading myelin, counting axons and determining presence of inflammation were pre-determined by an experienced neuropathologist (R.E.S.). A single investigator (E.C.K.) blinded to MRI and clinical data independently identified ROIs from LFB stained sections using a Nikon 80i microscope (Nikon USA). Histologic white matter ROIs were identified and graded as: 1) normal myelination, 2) mild demyelination (>50% myelin staining preserved), or 3) moderate to severe demyelination (<50% myelin staining preserved) (Fig. 2) similar to previous grading systems (Bot et al., 2004b; Cook et al., 2004). An attempt to identify a group of regions characterized by severe demyelination (>90%) was made. However, the paucity of this observation in only a few samples precluded valid statistical analysis. Regions most representative of normal myelination in MS samples were selected to represent MRI-described normal-appearing white matter (NAWM). Myelin scoring was determined by one investigator (E.C.K.). A second investigator (T.L.B.) reviewed the myelin scores for each ROI and agreed with the first investigator's determination in all cases. Next, ROIs as identified by LFB staining were co-registered on immediately adjacent sections stained with Bielschowsky silver stain. Axons within ROIs were counted manually at magnified 40x high power fields. The H&E stained slides were evaluated for evidence of active inflammation, marked by inflammatory infiltrates. Specimens were graded as inflammation present or absent. Normal control

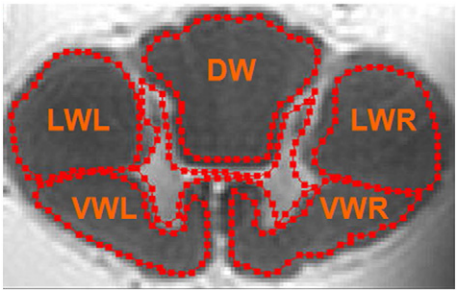

Fig. 1. White matter tract ROIs. Regions of interest are drawn to incorporate spinal cord white matter WMTs including dorsal white matter, left and right lateral white matter, and left and right ventral white matter WMTs.