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High-Field Proton Magnetic Resonance Spectroscopy of a Swine Model for Axonal Injury

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Abstract: A miniature swine model for diffuse brain injury has recently been developed that replicates the inertial loading conditions associated with rotational acceleration during automotive accidents. The swine model induces diffuse axonal pathology without macroscopic injury such as contusions and hematomas, thus affording a unique opportunity to study axonal injury with noninvasive techniques such as magnetic resonance imaging (MRI) and spectroscopy (MRS). In the present study, we evaluated this diffuse injury model with proton MRS, in vivo, using a high-field (4.0-T) MR scanner, since MRS has been demonstrated as a sensitive probe for detecting neurochemical abnormalities. Our study examined a region of the swine brain at timepoints before and after brain injury. Spectroscopic results indicate that *N*-acetylaspartate/creatine is diminished by at least 20% in regions of confirmed axonal pathology, whereas conventional MRI did not detect any abnormalities. These findings suggest that MRS has high sensitivity in diagnosing microscopic pathology following diffuse brain injury. **Key Words:** Diffuse axonal injury—*N*-Acetylaspartate—Spectroscopy.
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Traumatic brain injury is a leading cause of disability and death in children and young adults, affecting millions of people in the United States each year (Sosin, 1989, 1995; Kraus et al., 1994). However, the understanding of the neurochemical processes resulting from brain injury remains limited. Two major classifications of brain injury have developed in the literature: focal brain injury representing pathologies such as contusions and hematomas that typically result from impact forces such as blows to the head (Gennarelli, 1993), and diffuse brain injury typically resulting from inertial loading conditions commonly associated with motor vehicle accidents (Adams et al., 1989).

Although diffuse brain injury accounts for a high percentage of mortality due to brain trauma (Adams et al., 1989), most experimental brain trauma models are designed to induce focal brain injury. This may reflect the difficulty in replicating the dynamics of diffuse injury, such as nonimpact inertial loading of the brain that causes high strain between brain regions and selective injury of white matter tracts (Adams et al., 1982; Graham et al., 1988). Recently, a swine model for diffuse brain injury has been developed that replicates the inertial loading conditions associated with rotational acceleration during automotive accidents (Meaney et al., 1993; Smith et al., 1997). This nonimpact model proceeds from the research on nonhuman primates initiated by Gennarelli et al. (1982). Loading conditions for this model can generate axonal injury in isolation without the complication of contusions and hematomas. Thus, this model affords a unique opportunity to study axonal injury with magnetic resonance imaging (MRI) techniques. In the present study, we utilized proton MR spectroscopy (MRS) using a high-field (4.0-T) MR scanner to evaluate diffuse brain injury in swine. MRS has been shown to be a sensitive probe for detecting neurochemical abnormalities. *N*-Acetylaspartate (NAA), a chemical produced exclusively in neurons, is readily measured with proton MRS. Preliminary studies using this injury model suggested a decline in NAA levels in conjunction with axonal pathology (Smith et al., 1994). In the present study, we examined a region of the swine brain for the detection of axonal injury with proton MRS at several timepoints. Prior histologic analysis of the model guided MRS acquisition.

MATERIALS AND METHODS

The protocols described in this section were approved by the University of Pennsylvania Institutional Animal Care

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Abbreviations used: Cr, creatine; CT, computerized tomography; MRI, magnetic resonance imaging; MRS, magnetic resonance spectroscopy; NAA, *N*-acetylaspartate.

and Use Committee and were conducted in full compliance of the accepted standards of research involving animals.

Eight miniature swine (Hanford strain), young adult (4 months of age) males and females, weighing 17–20 kg, were included in the proton MRS study. Five animals were studied with MR techniques at three timepoints (preinjury, ~1 h postinjury, and 1 week postinjury). One animal was studied at preinjury and 1 h postinjury. One animal was studied only as a preinjury control. One animal was studied as an acute postinjury control. After the MR study at 1 week postinjury, the animals were killed and histologic analysis performed.

Animal protocol

In preparation for injury, the animals were fasted for 12 h and water was restricted for 2 h prior to brain injury surgical preparation or MRI/MRS preparation. The animals were sedated with an initial injection of midazolam (400–600 mg/kg). Once sedated, animals received 2–4% isoflurane via snout mask until they reached a plane of surgical anesthesia, at which time a venous catheter was inserted in the ear and the animals were endotracheally intubated and maintained on 1.5–2% isoflurane. Additional monitoring apparatus included noninvasive electrocardiogram electrode leads affixed to the chest and extremities, a pulse oximeter placed on the skin of the tail, a rectal thermometer, and sampling tubes for end-tidal CO_2 measurement attached to the endotracheal tube. The animals were continuously monitored, and all data from physiologic monitoring were collected on a computer-driven storage system.

To induce brain injury, the heads were secured to the rotational acceleration injury apparatus, the HYGE pneumatic impactor, via a padded snout clamp. The injury parameters have been previously described in detail (Meaney et al., 1993; Smith et al., 1997). Briefly, the snout clamp is directly mounted to the linkage assembly of the HYGE device that converts the linear motion to an angular motion. For these experiments, the linkage was adjusted to produce a pure impulsive head rotation of 110° in the coronal plane, with the center of rotation close to the brain center of mass. Head rotational acceleration was biphasic with a predominant deceleration phase. Once the animals were securely fastened to the HYGE device, inhalation anesthesia was withdrawn 10 s prior to triggered release of pressurized nitrogen from a storage chamber, initiating firing of the device. Once fired, the HYGE device rapidly rotated the miniature swine heads in the coronal plane over 20 ms. To measure the mechanical injury parameters, an uniaxial accelerometer (Endevco Instruments, San Juan Capistrano, CA, U.S.A.) mounted to the linkage sidearm was used to determine linear acceleration. Brain mass was determined when the brain was removed for histological analysis and was used to normalize the loading parameters to a 70- to 80-g brain mass.

Following injury, the swine's heads were released from the clamps. Upon stabilization of vital signs, animals were extubated and continuously monitored until awake and ambulatory, which always occurred within 8 h of injury. The animals received buprenorphine (0.1 mg/kg i.m., q 12 h, p.r.n.) for postoperative analgesia.

At 7 days following brain injury, the animals received an overdose of sodium pentobarbital (150 mg/kg i.v.) and were transcardially perfused with 4 L of saline following by 10 L of 4% paraformaldehyde. The brains were removed and postfixed in 4% paraformaldehyde for 2 h, stored in phosphate buffer solution, cryoprotected with sucrose, and blocked into 0.5-cm coronal section intervals for gross examinations and photography.

Histologic techniques

A series of 40-μm frozen sections were cut from the front face of each block and mounted on microscopic slides. One set of sections was mounted and stained with Nissl stain, cresyl violet, hematoxylin and eosin, and acid fuchsin. Additional sets were evaluated with immunohistochemical techniques using the following primary antibodies: Monoclonal antibodies used included the NR4 antibody, targeting the 68-kDa neurofilament subunit (1:4; Boehringer Mannheim); NN18 antibody, targeting the 160-kDa neurofilament subunit (1:40; Sigma); N52 antibody, targeting the 200-kDa neurofilament subunit (1:400; Sigma); and SMI-31 antibody, targeting selected epitopes primarily on the 170- to 200-kDa neurofilament subunits and reacting with extensively phosphorylated neurofilament and to a lesser extent with neurofilament M (1:5,000; Sternberger and Meyer monoclonals). The sections were incubated with primary antibody overnight at 4°C and then incubated at room temperature for 1 h, each with the appropriate secondary antibodies and ABC (avidin-biotin complex) solution (1:1,000). Peroxidase activity was revealed with 0.025% 3,3′-diaminobenzidine, 300 mg imadizole, and 0.25% H_2O_2 for 10 min.

All sections were examined under light microscopy, and a semiquantitative analysis was performed to determine the extent and distribution of axonal damage throughout the brain. Two sections from each block of tissue were examined under 200× magnification, and the following brain regions were evaluated: frontal, parietal, temporal, and occipital lobes, lateral ventricle region, external capsule, cerebral peduncles, thalamus, and brainstem. Scoring of axonal pathology (terminal clubbing or substantial swelling of axons) demonstrated by immunohistochemical staining was performed by rating 1–5 damaged axons/section/anatomic region as "1+," 6–15 damaged axons as "2+," and >15 damaged axons as "3+." The total number of injured axons and the relative severity of injury per brain region were tallied for comparison. The MR study was blinded to the severity of the injuries generated in each animal.

MRI and MRS evaluation

All MR studies were performed on a 4.0-T whole-body clinical Signa MR scanner equipped with a spectroscopy software package (GE Medical Systems, Milwaukee, WI, U.S.A.). The animals remained sedated with 1.5–2% isoflurane. Animals were positioned in the magnet for each study with a vacuum-evacuated foam head holder. Each animal was examined before injury when the foam head holder was evacuated, and the center of the swine head was marked on the animal and set as the MRI scan landmark. Subsequent examinations postinjury used these markers for reproducing the head position. A modified quadrature birdcage head coil was employed for the imaging sequence and image-guided single-voxel spectroscopic acquisition. Voxel selection was based upon the axial T_2- and proton density-weighted images. Voxels were positioned in predominately white matter along with the junctions of gray and white matter at the hippocampal–caudal slice. Localization was achieved using a stimulated echo acquisition mode (STEAM) technique. The voxel dimensions routinely prescribed from the axial image approximated 15 × 15 × 15 mm for the single volume

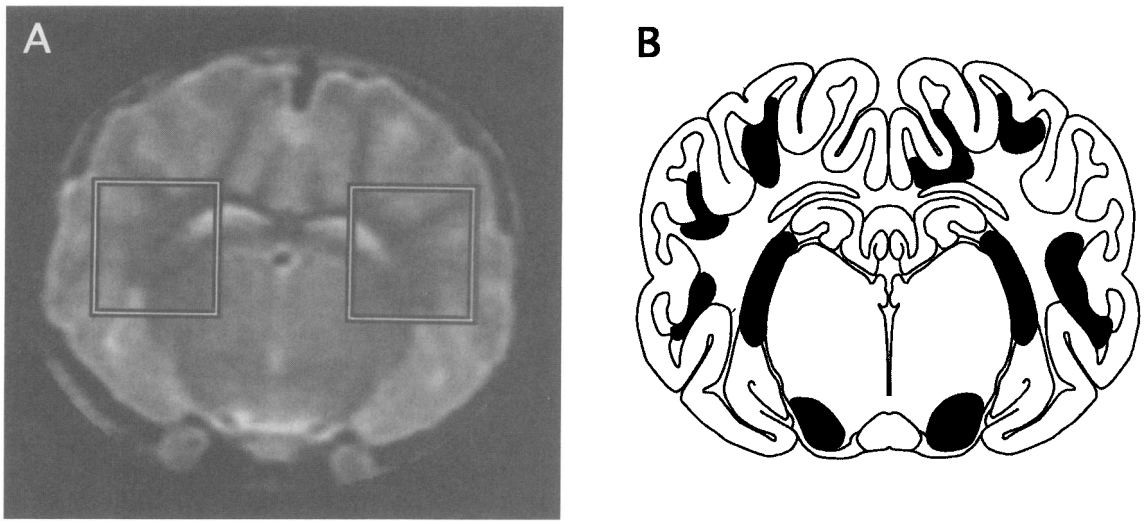

FIG. 1. A: Voxel placement representation upon a T_2-weighted axial image acquired at 7 days postinjury. **B:** Schematic representation of the distribution of axonal injury at the caudal–hippocampal level. This represents a compilation of all seven injured animals.

of interest. Water suppression was achieved by using three chemical shift-selective radiofrequency pulses (CHESS) followed by a dephasing gradient applied on each of the three axes. The sequence parameters included the following: spectral bandwidth of 2,500 Hz, repetition time (*TR*) of 2,000 ms, echo time (*TE*) of 31 ms, mixing time of 13.7 ms, 4,096 complex points, eight-step phase cycling, and 128 acquisitions. Gradient shimming on the voxel and optimization of the water suppression were performed before the start of the acquisition. The spectral acquisition time per voxel was <6 min.

The spectral processing was performed with ProNMR (Softpulse Software, Guelph, Ontario, Canada) using zero filling to 8K data points, 4-Hz line broadening applied in the time domain, Fourier transformation, and zero-order phase correction. Areas under the peaks were estimated using a Marquardt fitting routine to Lorentzian lineshapes in the frequency domain. From this method, metabolite ratios were calculated. The metabolite ratios were compared for significant differences using the Student's *t* test, assuming unequal variances for a two-tail measurement. Values of $p < 0.05$ were considered significant. Values of $p < 0.01$ were considered highly significant.

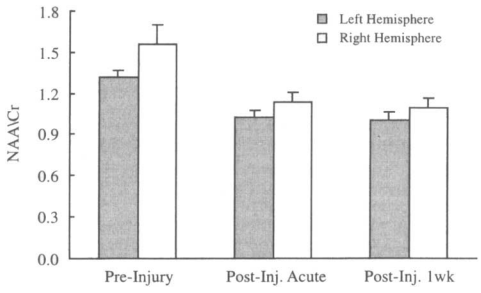

FIG. 2. The NAA/Cr ratios of the injured swine over the period of measurements. Results are expressed as means and SD. A Student's *t* test indicates highly significant difference for the left hemisphere among preinjury and postinjury acute animals as well as preinjury and postinjury 1 week animals, $p < 0.01$. A Student's *t* test indicates significant difference for the right hemisphere among preinjury and postinjury acute animals as well as preinjury and postinjury 1 week animals, $p < 0.05$.

RESULTS

No substantial physiologic changes were observed following injury in any of the animals.

Volumetric regions selected for MRS acquisition were graphically prescribed from an axial T_2-weighted image at the hippocampal–caudal level of a miniature swine (Fig. 1A). MRS voxels positioned in the left hemisphere demonstrated a mean decline in NAA/cre-

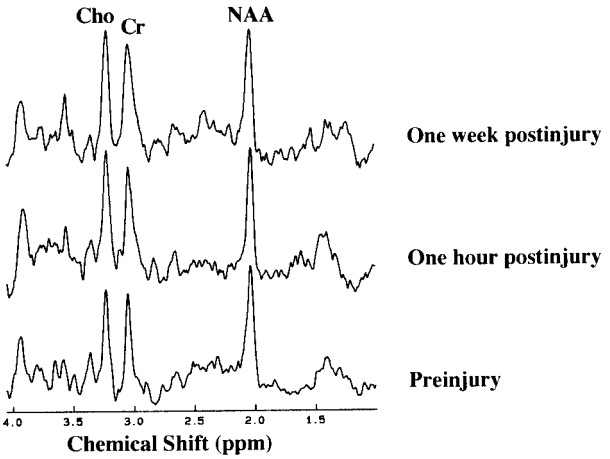

FIG. 3. Characteristic single-voxel ^{1}H MR spectra of a swine at multiple timepoints. Cr, creatine and phosphocreatine; Cho, composite of compounds with choline moiety.

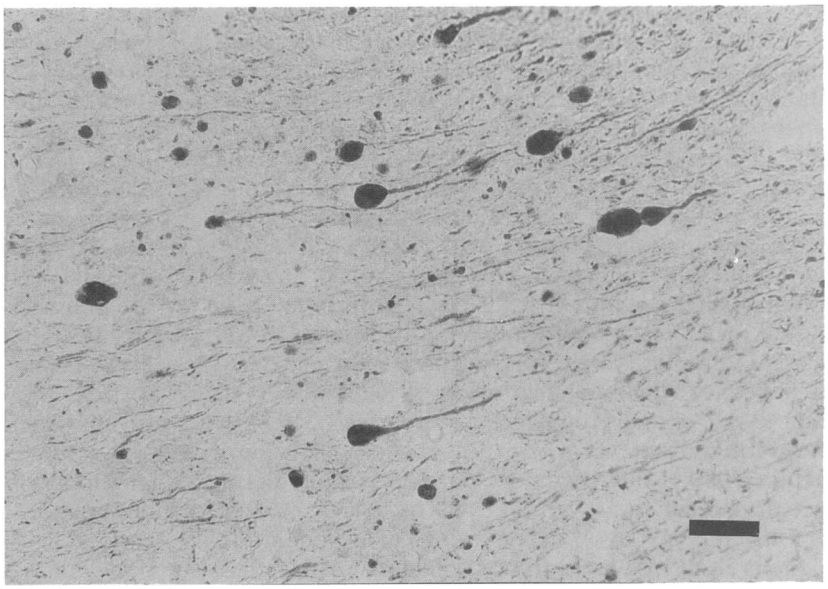

FIG. 4. Representive photomicrographs of SMI-31-immunostained white matter. Dark bulb formation represents axonal pathology. Bar = 50 μm.

atine (Cr) of 22% between MRS studies at preinjury and ~1 h postinjury that was highly significant (Fig. 2). The mean NAA/Cr decline between preinjury and 1 week postinjury (23%) was also highly significant. Proton MRS measurement of the right hemisphere was performed in five animals. The mean NAA/Cr decline between preinjury and 1 h postinjury was 21%; between preinjury and 1 week following injury, it was 30% (Fig. 2). The decline of NAA/Cr in the right hemisphere after injury was significant. Resonances attributable to lactate were not observed in the spectra (Fig. 3). Preinjury scans show baseline lipid content that may be sufficient to interfere with the detection of lactate. The choline/Cr and the *myo*-inositol/Cr values did not have statistically significant changes. There were alterations in the composite areas and lineshapes of resonances between 2.1 and 2.6 ppm. However, no consistent pattern of change was observed upon analysis.

Semiquantitative histological analysis showed diffuse axonal damage in the seven animals injured by rotational acceleration brain injury (Fig. 1B), whereas no axonal pathology was noted in the sham animal. The axonal pathology included bulbs and axonal swellings (Fig. 4). The most common regions demonstrating extensive axonal injury included the deep white matter at the root of the gyri and at the junction of white and gray matter in the frontal, parietal, temporal, and occipital lobes. In addition, axonal pathology was routinely observed at the margin of lateral ventricles, external capsule, cerebral peduncle, and basal ganglia. These regions demonstrated relatively equal proportions of the injury severity grades (i.e., one-third "1+," one-third "2+," one-third "3+"). Figure 1B displays a compilation of axonal pathology for all seven animals. There is a high degree of uniformity and symmetry to the injury. This is attributed to the reproducibility of the HYGE pneumatic impactor in replicating the loading parameters for a rotational acceleration injury.

DISCUSSION

Proton MRS of diffuse brain injury in swine demonstrated a significant and persistent decline in the NAA/Cr levels of >20% in both hemispheres, specifically in regions with histologically confirmed axonal pathology. The analysis of the spectroscopic data shows only significant changes in NAA/Cr of the proton spectra between the multiple study timepoints for both hemispheres of the swine brain. The T_2- and proton density-weighted MR images failed to present abnormalities in regions histologically identified with axonal pathology. Thus, proton MRS provides a sensitive, noninvasive measure of metabolite alterations resulting from brain injury.

The animal model of diffuse axonal pathology used for this MRS study generates nonimpact inertial loading conditions in the relatively large gyrencephalic brain of miniature swine (Meaney et al., 1993; Smith et al., 1997). This model reproducibly induces diffuse damage to axons throughout the white matter, with scattered and selective damage to neurons in the hippocampus. The axonal pathology consists of axonal bulbs and swellings. Previous studies of axonal pathology in humans and nonhuman primates report the same pathologic characteristics (Gennarelli et al., 1982). The total amount of axonal pathology in all injured animals appeared consistent, with mild to moderate diffuse axonal injury seen in humans, but without any overt macroscopic damage.

Detection of axonal injury is of great relevance in

understanding traumatic brain injury, since axonal damage is the principal pathology in many head trauma patients. However, detection of axonal pathology in vivo is difficult. Although gross pathologic changes can be detected with computerized tomography (CT) and demonstrated with selected MRI techniques, overall the presence of diffuse, microscopic axonal pathology is severely underestimated in the radiologic literature (Gentry et al., 1988). One study indicated that as many as 30% of mildly injured trauma patients with normal CT evaluations had evidence of diffuse axonal injury (Mittl et al., 1994). Therefore, microscopic injury is currently undetectable with conventional neuroimaging techniques (CT and T_1-, T_2-, and proton density-weighted MRI) in head-injured patients (Gennarelli et al., 1982; Adams et al., 1989; Mittl et al., 1994). Moreover, microscopic axonal injury is believed to be responsible for the clinically observed neurological and cognitive deficits in mildly brain-injured patients (Alexander, 1995). Conventional MRI of the miniature swine found no focal pathology in the study animals. However, the metabolic alterations detected with proton MRS likely reflect the microscopic changes found with histopathologic analysis.

The most routine protocols for proton MRS of the CNS in vivo can readily detect NAA, Cr, and choline. These metabolites have sufficient concentration levels for MRS measurements. In brief, MRS detection of these metabolites provides a measure of component viability upon comparison with normal controls. Although its role has yet to be elucidated, NAA has been suggested as a marker for neuronal viability as it is believed to be synthesized exclusively in neurons (Clarke et al., 1975; Miyake et al., 1981; Koller et al., 1984; Birken and Oldendorf, 1989; Frahm et al., 1991; Moffett et al., 1991; Urenjak et al., 1992). In brain cortex, NAA is located in neuronal cell bodies, whereas in the white matter, it is located largely in axons. In addition, NAA is second only to glutamate in concentration levels of amino acids in the brain (Perry et al., 1971; Birken and Oldendorf, 1989). The Cr resonance detected with MRS is an energy marker composed of Cr and phosphocreatine. Because the total Cr concentration generally remains constant (Lewis et al., 1974), Cr is routinely used as a reference for reporting spectroscopic findings. Detection of elevated choline serves as a marker for myelin breakdown in certain circumstances. Alteration of these metabolite levels reveals characteristics of a disease or injury state.

Whereas a decrease of NAA may reflect axonal and neuronal damage following brain trauma, an alternative explanation may be that NAA was used for repair. NAA is a source of acetate groups in lipid synthesis (D'Adamo et al., 1973; Burri et al., 1991), with an estimated turnover in whole brain of 1 h, with that of white matter being on the order of minutes. Our observation of NAA decline within 1 h postinjury is consistent with these turnover rates. Hochachka (1980) has suggested that the rate of acetate incorporation into fatty acids is ~20 times faster under hypoxic conditions than under normoxic conditions. The increase in acetate incorporation may be a result of a favorable bioenergetic pathway for fatty acid chain elongation (Whereat et al., 1967; Seubert and Podack, 1973; Rabinowitz et al., 1976). This discussion suggests that NAA is at steady state in the brain. Under conditions of injury or trauma, this steady state may be disrupted. The rate of NAA hydrolysis may be accelerated to provide acetate groups to meet the demand for lipid synthesis involved in myelin repair or to provide a temporary source of cellular energy locally at the site of axonal injury. In support of this hypothesis, we recently found a decrease in the concentration of NAA occurring as early as 1 h following brain trauma in a high-resolution proton MRS study of rat brain following parasagittal fluid percussion injury (Rubin et al., 1997). However, a reciprocal rise in acetate concentration was not detected, suggesting that it was rapidly incorporated into macromolecules. In either case, the level of NAA might drop acutely. Therefore, the acute decrease in NAA may signal repair, but the persistent loss may be a result of neuronal or axonal death.

Our preliminary studies performed at 1.5 T examined diffuse brain injury in swine with imaging and spectroscopy techniques (Smith et al., 1995*a,b*; Kimura et al., 1996). Phosphorus spectroscopy showed no acute changes in phosphocreatine/P_i, pH, or loss of ATP, whereas proton spectroscopy indicated no change in lactate for animals injured by rotational acceleration (Smith et al., 1994). These findings suggest that axonal injury in this model does not appear to result from ischemic injury. Five miniature swine were studied at 1.5 T with an experimental protocol analogous to the one detailed in this report. We observed an ~20% decline in NAA/Cr values upon axonal brain injury. The similarities in values for this decline suggest that it is not due to a disproportional change in the T_1 relaxation rates of NAA and Cr. Since relaxation effects are dependent upon field strength, our result appears consistent with the assertion that the change is a result of the injury.

We have also examined traumatically brain-injured patients with a bilateral, single-voxel, proton MRS protocol. Human patients enrolled in our spectroscopic research protocol have demonstrated analogous findings to this swine model. Conventional MRI (T_1-, T_2-, and proton density-weighted imaging) shows no significant pathology in mildly injured human patients. Our studies with proton MRS find a majority of mildly brain-injured patients with regions having lowered NAA/Cr compared with normal control subjects (Cecil et al., 1997, 1998). Choe et al. (1995) evaluated neuronal and axonal dysfunction

in closed head injury patients with in vivo proton MRS. Left frontoparietal white matter was studied in 10 patients using proton MRS. The results of this study suggested that the observed reduction of NAA/Cr ratio may be an indicator of axonal loss in closed head injury patients. The present animal model study affords not only corroboration of results but also the gold standard of histopathologic verification of the MRS results. Although MRS consistently demonstrated declines in NAA/Cr in regions of axonal pathology, ranking of the severity level based upon scoring of the semiquantitative histopathologic analysis did not correlate significantly to the measured declines in NAA/Cr levels.

The strength of the swine model is its focused approached to the axonal injury created upon rotational acceleration. Detection of axonal injury in this model requires the development of ultrasensitive techniques. Thus, in the effort to expand to novel techniques, implementing these studies on a high-field scanner provides an inherent sensitivity advantage compared with routine clinical 1.5-T MR scanners. However, the findings presented in this report suggest MRS at any field strength to be of exceptional utility for revealing diffuse brain injury. It is also important to note limitations of this study. We report the data in the form of metabolite ratios rather than absolute concentrations. Additionally, we have employed Cr as an internal reference. At present, the invariance of Cr in this model is a hypothesis that remains to be verified.

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