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Cellular correlates of longitudinal diffusion tensor imaging of axonal degeneration following hypoxic–ischemic cerebral infarction in neonatal rats

Ursula I. Tuor[a,b,c,d,*], Melissa Morgunov[b,c], Manasi Sule[b,c], Min Qiao[b,c], Darren Clark[a,d,e], David Rushforth[b], Tadeusz Foniok[b], Adam Kirton[a,d,f]

[a] Hotchkiss Brain Institute, Faculty of Medicine, University of Calgary, Calgary, T2N 4N1, Canada
[b] Experimental Imaging Centre, Faculty of Medicine, University of Calgary, Calgary, AB T2N 4N1, Canada
[c] Departments of Physiology and Pharmacology, Faculty of Medicine, University of Calgary, Calgary, AB T2N 4N1, Canada
[d] Department of Clinical Neurosciences, Faculty of Medicine, University of Calgary, Calgary, AB T2N 4N1, Canada
[e] Department of Medical Physics and Informatics, School of Medicine, University of Szeged, Szeged, Hungary
[f] Department of Pediatrics, Alberta Children's Hospital Research Institute, Faculty of Medicine, University of Calgary, Calgary, AB T2N 4N1, Canada

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ABSTRACT

Ischemically damaged brain can be accompanied by secondary degeneration of associated axonal connections e.g. Wallerian degeneration. Diffusion tensor imaging (DTI) is widely used to investigate axonal injury but the cellular correlates of many of the degenerative changes remain speculative. We investigated the relationship of DTI of directly damaged cerebral cortex and secondary axonal degeneration in the cerebral peduncle with cellular alterations in pan-axonal neurofilament staining, myelination, reactive astrocytes, activation of microglia/macrophages and neuronal cell death. DTI measures (axial, radial and mean diffusivity, and fractional anisotropy (FA)) were acquired at hyperacute (3 h), acute (1 and 2 d) and chronic (1 and 4 week) times after transient cerebral hypoxia with unilateral ischemia in neonatal rats. The tissue pathology underlying ischemic and degenerative responses had a complex relationship with DTI parameters. DTI changes at hyperacute and subacute times were smaller in magnitude and tended to be transient and/or delayed in cerebral peduncle compared to cerebral cortex. In cerebral peduncle by 1 d post-insult, there were reductions in neurofilament staining corresponding with decreases in parallel diffusivity which were more sensitive than mean diffusivity in detecting axonal changes. Ipsilesional reductions in FA within cerebral peduncle were robust in detecting both early and chronic degenerative responses. At one or four weeks post-insult, radial diffusivity was increased ipsilaterally in the cerebral peduncle corresponding to pathological evidence of a lack of ontogenic myelination in this region. The detailed differences in progression and magnitude of DTI and histological changes reported provide a reference for identifying the potential contribution of various cellular responses to FA, and, parallel, radial, and mean diffusivity.

1. Introduction

Inadequate blood flow to the brain, termed cerebral ischemia, will produce brain injury and a progression of pathophysiological responses associated with energy depletion that include cell swelling, cytotoxic edema, vasogenic edema and if sufficiently severe or prolonged, neuronal cell death. When brain regions are directly damaged by ischemia this can lead to secondary injury or degeneration in associated axonal connections such as the corticospinal tracts. Detection of such changes on early subacute diffusion MRI have been described as "early" or "pre" Wallerian degeneration following perinatal stroke in neonates (De Vries et al., 2005; Kirton et al., 2007). Such early signs of Wallerian degenerative injury are predictive of specific functional outcomes and may improve prognostication, guide rehabilitation, and could represent both a selection criteria and potential novel target for clinical trials. In particular, if there is diagnostic evidence for early degenerative injury, such as increases in the intensity of the corticospinal tract in diffusion weighted magnetic resonance (MR) images, then prognosis for a good recovery in such infants has consistently been poor (Domi et al., 2009; Kirton et al., 2007; van der Aa et al., 2011). Understanding better the cellular responses that evolve with axonal degeneration following

Abbreviations: MR, magnetic resonance; DTI, diffusion tensor imaging; ADC, apparent diffusion coefficient of water; FA, fractional anisotropy; GFAP, glial fibrillary acidic protein; MBP, myelin basic protein.

* Corresponding author at: Depts of Physiology and Pharmacology, Clinical Neurosciences, and, Radiology, Univ. of Calgary, Teaching, Research and Wellness Building, Room P2E36, 3280 Hospital Drive N.W., Calgary, AB T2N 2T8, Canada.

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stroke are important considering both their potential contributions to diagnosis and the potential for providing novel targets for therapeutic intervention. Currently, the tissue alterations underlying these MR biomarkers are unknown and speculative because histopathology, if available, is usually performed at single and relatively chronic time points many weeks to months after an ischemic insult.

Studies of the evolution of ischemic brain damage or infarction at subacute and chronic times has been assessed using standard MR imaging sequences such as T_2 or diffusion weighted imaging (Latchaw et al., 2009). Detecting secondary axonal or Wallerian degeneration is also possible with standard MR sequences. We recently demonstrated that following neonatal hypoxia with unilateral transient ischemia there is evidence for early Wallerian degeneration visible as either decreases in the apparent diffusion coefficient (ADC) and magnetization transfer ratio or increases in DW or T2 (Lama et al., 2011; Tuor et al., 2013) which pseudonormalize transiently. In these studies, the early corticospinal axonal changes (e.g. in the cerebral peduncle) reflected several cellular alterations such as a reduced staining for phosphorylated neurofilament H and increased vacuolation in hematoxylin and eosin stained sections.

DTI holds additional promise for detecting specific degenerative responses because of its potential sensitivity to certain ultrastructural cellular changes depending on the measured DTI parameter — i.e. fractional anisotropy, mean diffusivity and parallel or radial diffusivity. Of the extensive cellular responses produced by ischemia, those considered most likely to affect the diffusion properties of water are those that involve morphological tissue changes such as necrosis, astrogliosis, loss of myelin, loss of axonal neurofilaments or cellular inflammation (i.e. microglial/macrophage activation) (Nucifora et al., 2007; Zhang et al., 2012). Our group and others have shown that corticospinal tract DTI alterations are strongly correlated with motor outcome in children with perinatal stroke (Hodge, 2013; Roze et al., 2012; van der Aa et al., 2011). However the progression of DTI changes and whether there are differences between the evolution of cellular responses in direct ischemically injured brain versus associated connected tracts is not known. Differences are expected considering the different cellular compositions, for example neuronal rich cerebral cortex compared to axonal bundles within the descending corticospinal tract. A differential timing of DTI alterations is also expected considering direct ischemic injury is thought to be followed by a delayed Wallerian degeneration (Lama et al., 2011; Tuor et al., 2013). In order to better understand the unique cellular and DTI imaging alterations associated with secondary Wallerian degeneration relative to the onset and progression of direct ischemically damaged brain, it is necessary to compare their progression directly at multiple time points.

In the current study we hypothesized that DTI imaging would provide measures of progressive tissue changes within axonal tracts distal to the ischemic injury distinct from those in directly injured brain (e.g. cerebral cortex) and these differences would correspond with specific tissue morphological alterations in response to ischemia. This was investigated by using a neonatal rat model of unilateral transient cerebral hypoxia–ischemia and measuring DTI changes in the parietal cerebral cortex compared with its associated descending corticospinal tract axon fibers within the cerebral peduncle. DTI measurements were made at acute, subacute and chronic times after hypoxia–ischemia along with processing of brains at each of these time points to assess immunohistochemically potential contributions of morphological modifications. We focused on using markers for detecting cell death in neurons, loss of myelin and loss of neurofilaments in axons, and, activation of astrocytes and microglia/macrophages.

2. Material and methods

2.1. Model of hypoxia–ischemia

Fifty one pups delivered from 9 different pregnant Wistar female rats (Charles River Laboratories, Montreal, Canada) were used in the study. Experiments followed the Canadian Council on Animal Care guidelines and were approved by a University of Calgary Animal Care Committee. A moderate unilateral ischemic lesion with hypoxia (Vannucci and Vannucci, 2005) was produced as described previously (Lama et al., 2011; Qiao et al., 2009; Tuor et al., 2013). Briefly, pups ($n = 38$) on their 7th day of life had their right common carotid artery ligated under isoflurane anesthesia followed by a 60 minute exposure to hypoxia in a chamber containing 8% O_2 and 92% N_2 at 35.5 °C. Sham control animals ($n = 13$) experienced a similar surgical procedure without carotid artery ligation and hypoxia exposure. The Vannucci model of neonatal cerebral hypoxia–ischemia (Vannucci and Vannucci, 2005) produces ischemic damage and infarction within the distribution of the middle cerebral artery territory. Thus, the parietal *cortex* was selected as a representative brain region of *direct* hypoxic–ischemic damage or infarction. The posterior *cerebral peduncle* supplied by the posterior circulation was selected as a region of *secondary injury* remote to the hypoxia–ischemia but with axonal connections to directly damaged regions. Brain near the aqueduct in the posterior *pons* was selected as a control region generally unaffected by the hypoxia–ischemia.

2.2. Acquisition of MR images

Sham animals or animals subjected to cerebral hypoxia–ischemia were anesthetized (1.5–2% isoflurane) and DTI images in addition to anatomical scans were acquired at 3 h, 1 d, 2 d, 1 w or 4 w post-insult. Anatomical images were also acquired at 1 d post insult in the chronic animals (1 or 4 w) to confirm the extent of ischemic damage. MR images were acquired using a 9.4 T Bruker Biospin MR imaging system and Paravision 5.1 software. Throughout the scanning, respiration was monitored and maintained by adjustments in anesthesia and body temperature was maintained using a feedback heated air system (Small Instruments Inc., Stony Brook, NY). Images were acquired using a 3.5 cm diameter quadrature volume coil for radiofrequency transmission and reception. The head and body was restrained using custom designed swaddling and a head band or ear pins. Depending on the age of the animal, each MR imaging scan consisted of 25–30 slices of 0.5–0.55 mm thick covering the cerebrum and medulla, a 2×2 cm^2 or 2.5×2.5 cm^2 field of view and a data matrix size of 128×128. Anatomical T_2 maps were first generated using a T_2 imaging sequence consisting of a set of T_2 weighted spin echo images with 32 echoes, repetition time of 10 s and echo time of 10 ms between echoes. For DTI, a four-shot echo-planar imaging sequence was used to acquire four averages of sets of diffusion weighted images. These were acquired with b values of 0 (5 images) and 1000 s/mm^2 (30 images in non-collinear directions) using a repetition time of 6500 ms and an echo time of 35 ms. Artifacts associated with imperfections in the radio frequency pulse, gradient stability, and gradient echo currents were removed using a navigator-echo phase correction. Nyquist ghost artifacts were suppressed in the image reconstruction using information acquired during the initial automatic receiver gain adjustment. DTI Image acquisition time was approximately 1 h.

2.3. Analysis of MR images

T2 weighted images were visualized using local MR analysis software (Marevisi, National Research Council of Canada, Winnipeg, Canada). These images were used to assess the extent of ischemic damage and measure brain volumes (atrophy). Inspection of T2 images was also used to exclude from further analysis animals with no cortical T2 lesions or very large hyperintense lesions extending into the pons. They were also used to identify anatomical landmarks for selection of the regions of interest in subsequent DTI analysis and histological analysis as identified using a rat brain atlas (Paxinos and Watson, 1998). These regions of interest were manually defined in the MR images and histological sections and included the cerebral peduncle (0.3–0.4 mm^2), pons (within the central gray and mesencephalic trigeminal nucleus regions, 0.6–0.9 mm^2) and parietal cortex (0.7–1.0 mm^2) for areas both ipsilateral and contralateral to the lesion containing hemisphere. Once the ischemic infarct had