Characterization of White Matter Injury in a Hypoxic-Ischemic Neonatal Rat Model by Diffusion Tensor MRI

Silun Wang, MPhil; Ed X. Wu, PhD; Chung Nga Tam, MPhil; Ho-Fai Lau, MPhil; Pik-To Cheung, FRCR; Pek-Lan Khong, FRCR, MD

Background and Purpose—We evaluate white matter (WM) injury after hypoxic-ischemic (HI) insult in a neonatal rat model using diffusion tensor imaging (DTI) to determine whether $\lambda_s$ and $\lambda_c$ are able to characterize type and severity of brain damage.

Methods—Eighteen 7-day-old Sprague-Dawley rats underwent unilateral ligation of left common carotid artery followed by 50 minutes (n=9) or 90 minutes (n=9) of hypoxia at 37°C. Rats were divided into 2 groups, according to absence (group A, n=11) or presence (group B, n=7) of cystic lesions on D7 post-HI T2-weighted imaging. DTI was performed for all rats at D1 and for group A rats at D7 post-HI. Signal intensity of ipsilateral and contralateral external capsule (EC) on D1 was compared by paired $t$ test, with histological correlation.

Results—Group A rats had significantly reduced FA, elevated trace, elevated $\lambda_c$ and similar $\lambda_s$ on D1 in the ipsilateral compared to contralateral EC, whereas group B rats had significant reduction in all parameters in the ipsilateral EC. Elevated trace normalized on D7 in group A rats. Histopathologic results demonstrated reduced myelination in group A noncystic HI and severe necrosis in group B cystic HI.

Conclusions—Increased $\lambda_c$ with no significant change in $\lambda_s$ appears to characterize noncystic WM injury with reduced myelination, whereas reduction in both $\lambda_s$ and $\lambda_c$ characterize severe damage with loss of structural integrity and necrosis. Combining with FA and trace, $\lambda_s$ and $\lambda_c$ provide additional information which reflects type and severity of HI injury. (Stroke. 2008;38:2348-2353.)

Key Words: MRI ■ diffusion tensor imaging ■ white matter ■ neonatal rat ■ hypoxic-ischemic

It is well recognized that the white matter (WM) is susceptible to perinatal hypoxic-ischemic (HI) injury, especially at the preterm period.1 In mild neonatal HI, WM injury is diffuse and noncystic, whereas in severe neonatal HI focal cystic lesions in the WM often ensue. By varying the duration of hypoxia in the well established model of neonatal HI using a D7 rat, the severity of brain injury may be modified2–4 leading to selective WM injury with relative sparing of gray matter (GM)2 in the mild HI model and global necrosis and infarction in both WM and GM in the severe HI model.4 The histological findings in the WM of mild HI injury include gliosis and reduced myelination,5 and in severe HI injury, there is neuronal necrosis and infarction.4

Studies have demonstrated that severity of HI injury is an important criteria for treatment selection. In animal studies6–7 hypothermia has been demonstrated to have protective effect in mild/moderate HI but not in severe HI, as animals with severe initial injury developed infarctions despite hypothermic treatment. Recently, the results of a randomized prospective study using head cooling and systemic hypothermia in the treatment of human neonatal hypoxic-ischemic encephalopathy found hypothermia to be beneficial in neurodevelopmental outcome determined at 18 months of age.8 In addition, this study compared the electroencephalogram changes between moderate HI group and the severe HI group and found that treatment was protective only selectively in newborns with moderate but not severe hypoxic-ischemic encephalopathy.8 Thus, it is imperative that noninvasive markers that may characterize the type and severity of HI brain injury are identified so that accurate and timely diagnosis can be made, with important implications for treatment selection.

MRI is a widely used noninvasive method to evaluate central nervous system injury. Previous animal studies2–5,9,10 have demonstrated that conventional MRI sequences such as T1-weighted imaging (T1WI) and T2-weighted imaging (T2WI), and diffusion-weighted imaging (DWI) measuring
trace, could detect HI-induced brain damage. Diffusion tensor MR imaging (DTI) allows the measurement of the directional diffusivities of water and is more sensitive than conventional MRI sequences in detecting WM injury. Furthermore, associations between directional diffusivities (λ₁ and λ₂) derived from DTI indices and neuropathological processes have been demonstrated. Currently, there is limited information about the changes in DTI parameters after HI injury and whether directional diffusivities can be used to characterize the type and severity of HI-induced brain damage.

In this study, we aim to evaluate WM injury after HI using DTI in 2 different sequelae of WM injury after HI: noncystic and cystic injury. Our hypothesis is that directional diffusivities λ₁ and λ₂ can provide additional information to FA and trace, and can be used to characterize the severity and type of injury after HI, and that the histopathologic processes of HI injury in the WM can be reflected by the pattern of change in λ₁ and λ₂.

### Materials and Methods

#### Animal Preparation

The experiment was approved by the University Animal Ethics Committee, according to the local Government Legislation. The method of animal surgery and HI injury was similar to our previous studies but with variation in duration of HI. Briefly, 7-day-old female Sprague-Dawley rats (n=18, 12 g to 14 g weight) underwent unilateral ligation of the left common carotid artery via a midline neck incision after anesthesia with 0.2 mL of inhalational isoflurane in an air tight box for 2 minutes. After that, they were returned to their mother for nursing for 2 hours until they regained normal movement. Rats were subsequently placed in a hypoxic chamber of 8% O₂/92% N₂ maintained at 37°C for 50 minutes (n=9) and 90 minutes (n=9) to create HI injury of different severity.

#### MRI Scanning

MRI was performed using a 7 T animal MRI scanner (Bruker BioSpin MRI PharmaScan) and microimaging mouse brain coil. The rats were restrained on a custom made holder with restraining to minimize head motion while respiration was monitored. Rats were anesthetized using isoflurane/O₂ (3% for induction and 1.5% for maintenance). All 18 rats were imaged at D1 (approximately 24 hours post-HI) using DTI and T2WI and on D7 using T2WI plus DTI for rats with noncystic injury and T2WI only for rats with cystic injury (see below). Time interval between the onset of HI and MR imaging at D1 ranged between 23 hours 30 minutes and 24 hours 15 minutes. Coronal MRI sections were performed from 2 mm anterior to the corpus callosum to the end of the cerebrum. T2WI were acquired with the following parameters: field of view=2.5 cm², matrix size=128×128, repetition time (TR)=11189 ms and echo time (TE)=20 ms, slice thickness=1.0 mm. DTI were acquired with a respiration-gated spin echo 4-shot EPI readout sequence. An encoding scheme of 30 gradient directions which are homogenously distributed on the unit sphere was used to acquire diffusion tensor images. The imaging parameters were: TR=3000 ms, TE=32 ms, Δ=20 ms (diffusion gradient duration), δ=4 ms (diffusion gradient separation), field of view=3.2 cm², slice thickness=0.5 mm, matrix size=128×128 (zero filled to 256×256), image resolution=250×250 μm², acquisition time=8 minutes, b value=0 and 1000 s/mm².

Fractional anisotropy (FA), trace of the diffusion tensor, λ₁ and λ₂, maps were derived using DtiStudio software v2.4 (Johns Hopkins University, Baltimore, Md) by the following equations:

\[
(1) \quad FA = \sqrt{\frac{3(λ_1^2 + λ_2^2 + λ_3^2)}{λ_1^2 + λ_2^2 + λ_3^2}}
\]

\[
(2) \quad Trace = λ_1 + λ_2 + λ_3
\]

\[
(3) \quad λ_1 = λ_2
\]

\[
(4) \quad λ_3 = 0.5(λ_1 + λ_2)
\]

#### Image Analysis

As it is well-recognized that there is high interindividual variability in extent and severity of injury in the neonatal rat HI model, we used the outcome seen on D7 T2WI to divide the rats into the noncystic group (Group A) and cystic group (Group B) for analysis. Rats without cystic lesions were included in group A (n=11, comprising 9 rats with 50 minutes hypoxia and 2 rats with 90 minutes hypoxia), while rats with cystic lesions were included in group B (n=7, all rats with 90 minutes hypoxia). Cystic lesions were defined as lesions of signal intensity similar to cerebro-spinal fluid on T2WI. The area of the cystic lesion and total brain area in every slice was manually measured by ImageJ 1.34 (National Institutes of Health, Bethesda, Md). Percentage volume of cystic lesions on the D7 T2WI was calculated as the sum of the lesion area across all slices, multiplied by the total slice thickness, then dividing by the total hemispheric volume. After generation of the parameter maps, region-of-interest (ROI) was manually drawn over the external capsule (EC) of each hemisphere on the FA maps on 5 consecutive slices. Area of ROI was approximately 1 mm². ROI was firstly defined in the FA map on the contralateral and the corresponding ipsilateral EC because of its relatively clear WM/GM boundary (Figure 1a). Then, the ROIs were placed on identical sites on the trace, λ₁ and λ₂ maps (Figure 1b, 1c, 1d). Because of the severe cystic changes at D7 in Group B rats, DTI was not obtained for these D7 Group B rats ie, ROI analysis of DTI parameters was obtained for Group A rats at D1 and D7 and Group B rats at D1 only. Images were excluded from analysis if the boundary of EC was not clearly demarcated because of motion artifact. ImageJ was also used to evaluate the quantitative indices in all image maps. Percentage value changes of FA, trace, λ₁ and λ₂, on the ipsilateral hemisphere compared to the contralateral hemisphere were calculated.

#### Histopathology Evaluation

Eight rats (n=5 in group A, n=3 in group B) were randomly selected for histological evaluation of brain injury after the second scan. Rats were perfusion fixed through the left cardiac ventricle with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in PBS. The specimens were fixed in 4% PFA in PBS (pH 7.4) at 4°C overnight. The brains were put into 30% sucrose solution in PBS for a few days. Coronal sections were obtained from each brain by frozen sectioning. Brain specimen was cut in 10-μm-thick coronal slices between the locations corresponding to the most posterior and most anterior ROI slices.

Hematoxylin and eosin (H&E) stain was used to detect morphological characteristics of brain tissue in each section of Group A and B rats. Luxol fast blue (LFB) stain (Sigma) was performed to detect the myelin in the WM. All slices were examined using a light microscope (Axioplan 2 Imaging system, Carl Zeiss MicroImaging GmbH, Germany) under 25× to ×400 magnification. Histological images were recorded by digital photomicrography (Spot advanced, Diagnostic instruments). Optical densities (OD) of LFB stained images were measured in both symmetrical EC at the region corresponding to ROI analysis of DTI images at 200× histological digital images by ImageJ for semiquantification of the intensity of myelin in group A rats. The higher the OD means the lower the transmittance and is represented by higher LFB staining intensity.

#### Statistical Analysis

All results were expressed as mean±SD. Paired t-test was used to detect statistical differences in the DTI quantitative indices and OD of LFB between the ipsilateral and contralateral EC. To evaluate the intraobserver reliability of ROI measurement of DTI parameters, 8 rats (n=5 in group A and n=3 in group B) were randomly selected for remeasurement of DTI parameters 2 weeks later. Intraobserver reliability was assessed by calculating 1-way random intraclass correlation coefficients (ICC) for repeated measurements of each parameter.
correlation coefficients (ICCs). All statistical analyses were performed using the statistical package SPSS for Windows (Version 15, SPSS). A probability value of $<0.05$ was considered to indicate statistical significance.

**Results**

**Magnetic Resonance Imaging**

In group A rats at D1 post-HI, an area of hyperintensity was detected on T2WI in the ipsilateral cortex in 8 rats and along the WM in 10 rats (Figure 2a). No signal change was found in 1 rat. Subsequently at D7, T2WI showed persistent high signal at the cortex in only 1 rat, whereas no signal change was found in the other 10 rats (Figure 2b). In group B rats at D1 post-HI, an area of high signal occupying a large portion of the ipsilateral hemisphere including WM and cortex was found on T2WI in all rats, and this subsequently developed into cystic lesions on D7 (Figure 2c and 2d). Percentage volume of the cystic lesion was $24\%\pm5\%$ (mean$\pm$SD).

**Diffusion Tensor Imaging**

Of a total of 580 analyzed DTI slices, 98.6% ($n=572$) slices were found to have satisfactory image quality and were included into image analysis. Two slices belonging to 2 rats were excluded because of unsatisfactory image quality from motion artifact. Therefore, only 4 slices of 5 were analyzed for 2 rats. None of the animals were excluded from image analysis. Intraobserver reliability for the ROI measurement of DTI parameters was found to be in good agreement (ICC ranged from 0.90 to 0.96, Table 1).

Values of FA, trace, $\lambda_1$, and $\lambda_2$ of all rats on D1 and group A rats on D7 are shown in Table 2. Significant reductions of FA were demonstrated in the ipsilateral EC compared to the contralateral EC in both group A and B, with a decrease of 10.1% (D1) and 4.5% (D7) in group A and 13.5% (D1) in group B. On D1, trace of ipsilateral EC was found to be significantly higher than contralateral EC in group A.

![Figure 1. ROI placement for measurement of signal intensity in the coronal fractional anisotropy (FA) (a), trace (b), $\lambda_1$ (c), and $\lambda_2$ (d) maps. ROI (circled area) was delineated in the contralateral (yellow line circled area) and ipsilateral (red line circled area) external capsule (EC) in FA map as it best demarcates the EC. Then the ROIs were placed on identical sites on the trace, $\lambda_1$ (c) and $\lambda_2$ (d) maps in both sides.](http://stroke.ahajournals.org/)

![Figure 2. Coronal T2WI of group A (a, b) and B (c, d) on D1 and D7. In group A, high signal intensity could be detected in the cortex (a, closed arrow) and white matter (a, open arrow) on D1, and these high signal intensity areas were not seen at D7 (b). In group B, a large area of high signal intensity was noted in ipsilateral hemisphere including cortex and white matter (c), and this subsequently developed into cystic lesions in D7 (d).](http://stroke.ahajournals.org/)
Table 1. Quantification of Intraobserver Reliability by Intraclass Correlation Coefficient (ICC)

<table>
<thead>
<tr>
<th>Intraobserver ICC</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>FA</td>
<td>0.92</td>
</tr>
<tr>
<td>Trace</td>
<td>0.92</td>
</tr>
<tr>
<td>λ₁</td>
<td>0.96</td>
</tr>
<tr>
<td>λ₂</td>
<td>0.90</td>
</tr>
</tbody>
</table>

(11.0%), and significantly lower in group B (-28.8%). The elevated ipsilateral trace of group A normalized on D7. Different patterns of directional diffusivities were detected in group A and group B on D1. In group A, significantly increased ipsilateral $\lambda_1$ (13.5%, $P<0.001$) was demonstrated compared to the contralateral side but no significant difference was found in $\lambda_2$ between both hemispheres. In group B, significantly decreased $\lambda_1$ (-30.9%, $P<0.001$) and $\lambda_2$ (-27.2%, $P<0.001$) were found in the ipsilateral EC compared to the contralateral EC. In group A on D7, ipsilateral $\lambda_2$ remained significantly elevated albeit to the lesser degree (3.0%, $P=0.004$) and $\lambda_1$ remained similar in both hemispheres.

Histopathologic Evaluation

Histological evaluation by H&E and LFB are shown in Figure 3 (3a to 3h).

Group A Rats

Tissues in the ipsilateral cortex and WM appeared normal in group A rats. On H&E staining (Figure 3a and 3b), both hemispheres appeared symmetrical and no necrosis was found. On LFB staining, both hemispheres were found to have similar staining pattern when observed under 25× magnification. The corpus callosum and the EC were stained with abundant LFB, indicating the presence of myelin (Figure 3e and 3f). The mean (±SD) OD of EC for the ipsilateral and contralateral hemispheres were 0.32±0.15 versus 0.41±0.17 respectively, and the difference was statistically significant ($P=0.005$).

Group B Rats

The morphological differences between the ipsilateral and the contralateral hemispheres were profound. On H&E staining, 1 of the rats had an atrophic ipsilateral hemisphere and the other 2 rats had severe vacuolated neuron cells and brain tissue loss in the cortex. Cystic necrosis in the ipsilateral hemisphere caused loss of brain tissue including almost all the EC (Figure 3c and 3d). Much weaker LFB staining in the remaining portion of the WM (corpus callosum) was visualized compared to the contralateral EC, which indicates reduced myelination in the ipsilateral WM (Figure 3g and 3h).

Discussion

We compared DTI indices of WM including FA, trace, directional diffusivities $\lambda_1$ and $\lambda_2$ at D1 post-HI between noncystic and cystic HI groups of rats to determine whether the indices could reflect outcome in terms of the type and severity of HI injury, as determined on the D7 T2WI scan and histopathologic studies. We found that mild HI leading to noncystic WM injury was characterized by reduced FA, transiently elevated trace, no change in $\lambda_2$, and elevated $\lambda_1$ in EC whereas severe HI leading to cystic WM injury was characterized by reduction in all DTI indices, ie, FA, trace, $\lambda_1$, and $\lambda_2$. Histopathologic results demonstrate reduced myelination in mild HI and necrosis in severe HI. Thus, the indices at D1 are able to reflect the severity and underlying histopathology of HI injury.

MR imaging findings in the neonatal rat HI model using conventional T1WI and T2WI and DWI have been described.4,5 Our findings of both increased and decreased trace at D1 in group A and B, respectively, are in keeping with the published literature.4,5 Dynamic distribution of water between the extracellular space and intracellular space is a key factor which affects the value of trace. Early reduction of trace in either mild or severe HI insult in WM is generally considered to correspond to cell swelling and reductions in extracellular space,14 namely attributable to cytotoxic edema in HI injury, which will decrease water diffusion. Subsequently, increased trace may be associated with water influx from vessels to brain tissue, namely vasogenic edema or reduced cell volumes with apoptotic cell death, which will both increase extracellular space and therefore increase the water diffusion. At 24 hours post-HI, it is likely that a combination of cytotoxic and vasogenic edema coexist, but for group A rats, vasogenic edema, reflected by increased trace, is dominant whereas for group B rats, cytotoxic edema, reflected by reduced trace is dominant. Although demyelination5/3/the lack

Table 2. DTI Quantitative Indices of Ipsilateral and Contralateral External Capsule (EC) on D1 and D7 After Hypoxic-Ischemic Injury in Mild HI (group A) and Severe HI (group B) Cohorts

<table>
<thead>
<tr>
<th></th>
<th>Group A (n=11)</th>
<th></th>
<th>Group B (n=7)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D1</td>
<td>D7</td>
<td>D1</td>
<td>D7</td>
</tr>
<tr>
<td></td>
<td>Ipsilateral EC</td>
<td>Contralateral EC</td>
<td>Δ %</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>0.286±0.044</td>
<td>0.318±0.042</td>
<td>-10.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FA</td>
<td>2.724±0.186</td>
<td>2.453±0.150</td>
<td>11.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Trace</td>
<td>1.137±0.096</td>
<td>1.126±0.094</td>
<td>0.98</td>
<td>0.125</td>
</tr>
<tr>
<td></td>
<td>0.765±0.049</td>
<td>0.674±0.041</td>
<td>13.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FA</td>
<td>0.199±0.030</td>
<td>0.230±0.036</td>
<td>-13.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Trace</td>
<td>7.575±0.012</td>
<td>1.096±0.007</td>
<td>30.9</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values are shown in mean±SD Trace, $\lambda_1$ and $\lambda_2$ are in units of $\mu$m²/ms. Δ % = Percentage of variations in DTI indices of ipsilateral compared with contralateral EC.
of myelination contributes to increase in trace, this is unlikely the case in group A rats because the increase was transient with normalization of trace at D7. Although the pattern of trace differed between group A and B at D1, this parameter may not be a reliable indicator of WM damage attributable to the dynamic nature of the evolution of brain insult in the early stages of injury with rapid changes in extracellular and intracellular fluid. Also, it has been shown that normalization of apparent diffusion coefficient (ADC) does not equate to histological normalization and on the other hand, areas of reduced ADC, even when severe, may be reversible.

DTI is increasingly used in animal and clinical studies to evaluate brain damage, especially in WM, because of its ability to measure the directional diffusivities of water. The degree of directionality is described by FA. Reduction in FA has been found in the mouse optic nerve after retinal ischemia injury and in moderate brain injury after hypoxic-ischemic insult in the early stages of injury with rapid changes in extracellular and intracellular fluid. Also, it has been shown that normalization of apparent diffusion coefficient (ADC) does not equate to histological normalization and on the other hand, areas of reduced ADC, even when severe, may be reversible.

Recent studies have shown that axial diffusivity (\(\lambda_a\), presumed to be diffusivity along the axon) and radial diffusivity (\(\lambda_r\), presumed to be diffusivity perpendicular to the axon) are more specific to underlying biological processes such as myelin and axonal change especially in WM. Song et al. found a significant increase of \(\lambda_a\) without change of \(\lambda_r\) reflected dysmyelination in a Shiver mouse model and demyelination in a cuprizone induced WM demyelination model (including the processes of demyelination and remyelination). In another study of mouse optic nerve ischemia, a significant decrease of \(\lambda_r\) reflected the pathological process of axonal damage without demyelination. Hence, directional diffusivities may be regarded as biological markers of WM damage. In our mild HI cohort, the pattern of change in \(\lambda_a\) and \(\lambda_r\) was consistent with the dysmyelination/demyelination models. Dysmyelination was confirmed to be a major neuropathological process in this group of rats at the subsequent histopathologic studies. Dysmyelination is attributable to dysfunction of immature oligodendrocytes, which are highly vulnerable to HI injury, and has been found in other studies of both mild and severe HI. It has been found that acute disruption of myelin gene expression and death of oligodendroglial precursors may occur as early as 3 hours after HI insult leading to damage of myelin, after which marked reduction of myelin basic protein (MBP), a marker of the amount of myelin, was found in the WM at 24 hours post-HI. In a longitudinal histological study of HI rats, reduction of MBP was found ipsilaterally on D14 post-HI (earlier time-points were not evaluated), with restoration in myelination 2 weeks later in the mild HI group, but not in the severe HI group. Thus, it was hypothesized that in severe HI, breakdown of structural integrity and severe axonal damage and neuron death lead to loss of the ability of recovery from HI. On the other hand, in mild HI-induced injury, there is the potential to generate new cells such as oligodendrocytes. In our cohort of group A rats, the persistent elevation of \(\lambda_r\) at D7 corresponds to the finding of relative lack of myelin in the white matter. However, although \(\lambda_r\) remained significantly elevated at D7, it was to a lesser degree compared to D1. This may be partly attributable to the reduction in vasogenic edema as reflected by normalization of trace, but partly attributable to an element of recovery in myelination. It would be interesting to evaluate whether directional diffusivities will be sensitive enough to reflect this reversibility in a mild HI model and therefore be used to monitor injury and recovery of myelin after HI induced injury. We are currently conducting a longitudinal study to evaluate this.
transport may also contribute to the reduction of $\lambda_2$. Thus, a significant decrease of $\lambda_1$ and $\lambda_2$ may reflect an irreversible histological damage in severe HI. Indeed, histological findings in this group were predominantly infarction and necrosis.

In conclusion, we found that DTI analysis of directional diffusivities could provide additional information to FA and trace, and may reflect the outcome in terms of the severity and type of HI induced WM damage. Our findings suggest that the pattern of directional diffusivities reflected more specifically the histological changes of reduced myelination and necrosis and may be a potentially useful biomarker in treatment selection and monitoring in HI injury.

Acknowledgments

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Disclosures

None.

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