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***In vivo* Diffusion Tensor Imaging of Amyloid- β -Induced White Matter Damage in Mice**

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Abstract

Background—Diffusion tensor imaging (DTI) suggests the presence of white matter abnormality at the prodromal stage in human Alzheimer's disease (AD).

Objective—To use a mouse model of AD to determine whether the white matter abnormality detected by *in vivo* DTI is associated with functional deficits and axon damage.

Methods—Amyloid- β_{1-42} ($A\beta_{1-42}$) was injected into the left lateral ventricle in mice. Two months after the injection, *in vivo* DTI and visual evoked potential (VEP) recordings were performed, followed by immunohistochemistry of phosphorylated neurofilament and myelin basic protein.

Results—DTI of $A\beta_{1-42}$ -treated mice showed a significant increase of radial diffusivity in white matter including the optic nerves and tracts. The abnormality was associated with decreased amplitude and increased latency of VEP. Immunohistochemistry confirmed a significant loss of axons and myelin integrity.

Conclusion—White matter damage induced by $A\beta_{1-42}$ in mice can be detected non-invasively by DTI.

Keywords

Amyloid- β_{1-42} ; diffusion tensor imaging; mice; optic nerve; white matter diseases

INTRODUCTION

Alzheimer's disease (AD) is an age-related, neurodegenerative disease and a leading cause of dementia. AD is not usually considered a white matter disease, but recent evidence suggests a widespread white matter abnormality in patients with AD [1, 2]. More importantly, the abnormality may occur in the early stage of AD [3–9] and may therefore serve as a marker for early diagnosis and as a potential indicator for treatment efficacy. Consistent with white matter damage in AD, amyloid- β peptide ($A\beta$), known to play a major

role in the pathogenesis of the disease, is toxic to not only neurons but also oligodendrocytes [10, 11], a type of glia cells providing support and forming the myelin sheath around the axons.

Diffusion tensor imaging (DTI) measures water diffusion and has been shown to be highly sensitive to the micro-structural changes caused by white matter damage [12, 13]. The axial diffusivity ($\lambda_{||}$) and radial diffusivity ($\lambda_{\perp}$) derived from DTI are sensitive to structural changes in directions along and across the fibers, respectively [14–17]. In patients with AD, increased $\lambda_{||}$ and increased $\lambda_{\perp}$ have been observed, indicating a disruption of white matter integrity and an increased micro-structural space for water movement along and across the nerve fibers [18–20]. DTI of AD animal models would help further understand the pathology underlying the white matter abnormality revealed by DTI.

Despite the increasing number of DTI studies in AD patients, studies in AD animal models are limited; so far, only one *in vivo* [21] and a few *ex vivo* [22–24] DTI studies have been reported. In rats and mice, intracerebroventricular (i.c.v.) injection of A β produces neurological deficits and neural pathologies similar to human AD [25–31]. In this study, *in vivo* DTI was performed in mice 2 months after i.c.v. injection of A β_{1-42} . Major white matter tracts, including the corpus callosum, external capsule, optic nerves, and optic tracts, were examined. We also recorded visually evoked potentials (VEP) to determine whether changes detected by DTI are associated with changes in function. Immunohistochemistry targeting the phosphorylated neurofilament (SMI-31) and myelin basic protein (MBP) was performed to further confirm the possible axonal and myelin damage detected by DTI.

MATERIALS AND METHODS

All experimental procedures were in accordance with National Institutes of Health guidelines for the use of animals in research and were approved by the Institutional Animal Care and Use Committee of Loma Linda University.

i.c.v. injection

A β_{1-42} (A9810, Sigma Aldrich, USA) dissolved in sterile saline was incubated at 37°C for 72 h before the injection [29–31]. Eight female C57BL/6 mice at 12-weeks old were anesthetized by 1.5% isoflurane/oxygen using an isoflurane vaporizer (VetEquip, Pleasanton, CA). Body temperature was maintained using an electric heating pad. Mice were placed in a stereotactic apparatus. The mice were shaved and skin was cleaned with Povidine (Rugby Laboratories). A β_{1-42} (4 nmole in 3 μ l) [29–31] was injected slowly into the left lateral ventricle using a 5- μ l Hamilton syringe with an injection speed of 0.3 μ l/min. After the injection, the needle was kept in place for another 10 min and then slowly withdrawn. Eight age- and gender-matched mice, injected with A β_{42-1} , were used as controls. Operated animals were monitored daily, and 2 mg penicillin (Sigma) was injected subcutaneously per day for three days after surgery to prevent infections [27].

Imaging procedure

Two months after A β_{1-42} injection, mice were anesthetized with a mixture of oxygen and isoflurane. Core body temperature was maintained at $37.0 \pm 0.5^{\circ}\text{C}$ using warm water circulating in a pad. Mice were placed in a holder to immobilize the head. A 7-cm inner volume coil was used as a transmitter coil, and an 1.5-cm inner diameter surface coil was used as a receiver to collect data in a Bruker 4.7T small animal MRI instrument. Spin-echo DTI was performed with TR 3 s, TE 29 ms, duration between a diffusion gradient pair (Δ) = 20 ms, diffusion gradient duration (δ) = 3 ms, and six-direction diffusion scheme with b-values of 0 and 0.85 ms/ μm^2 . Images with slice thickness 0.5 mm, field of view of 1.5 cm $\times$

1.5 cm, and matrix 128×128 (zero filling to 256×256) was collected to cover the entire brain. Using software written in Matlab (Math-Works, Natick, MA, USA), the eigenvalues (λ_1 , λ_2 , and λ_3) derived from diffusion tensor were used to calculate axial diffusivity ($\lambda_{||}$), radial diffusivity ($\lambda_{\perp}$), relative anisotropy (RA), and trace of the diffusion tensor (Tr) defined by the following equations:

$$\text{Tr} = \lambda_1 + \lambda_2 + \lambda_3 \quad (1)$$

$$\lambda_{||} = \lambda_1 \quad (2)$$

$$\lambda_{\perp} = 0.5 \times (\lambda_2 + \lambda_3) \quad (3)$$

$$\text{RA} = \frac{\sqrt{(\lambda_1 - \text{Tr}/3)^2 + (\lambda_2 - \text{Tr}/3)^2 + (\lambda_3 - \text{Tr}/3)^2}}{\sqrt{3}(\text{Tr}/3)} \quad (4)$$

Regions of interest (ROI) were selected in the optic nerves, optic tracts, external capsule, and corpus callosum referencing to the mouse brain atlas [32]. The ROI of external capsule and corpus callosum were collected in 6 contiguous slices (Bregma 0.7 mm – Bregma –2.3 mm). On the same slices, cortical regions, the areas above external capsules, were also selected to evaluate the volume of cortex. Data were presented as mean $\pm$ standard deviation. Since no difference was found between the left and right in control mice, data from both sides were pooled to enhance the statistic power for statistical analysis. Multiple comparisons were performed using one-way analysis of variance (ANOVA) followed by the *post-hoc* Holm-Sidak test using Sigma Plot 11.0 (San Jose, CA). $p < 0.05$ was considered to be statistically significant.

Visual evoked potential (VEP)

Animals were anesthetized with a mixture of oxygen and isoflurane (1.5%). Core body temperature was maintained at $37.0 \pm 0.5^\circ\text{C}$ using a heating pad. To minimize discomfort, all incision sites and soft tissue pressure points were infiltrated with the long-acting local anesthetic bupivacaine. A midline incision was made to expose the skull. Two small holes were drilled and silver wires were placed over the visual cortex (0.5 mm anterior and 2.5 mm lateral to the Lambda point) and cerebellum (1.5 mm posterior to Lambda) for recording and reference, respectively [33]. After dark adaption for 10 min, light stimulation was produced by a LED light source (Radioshack, Fort Worth, TX), at frequency of 0.2 Hz with a duration of 5 ms [34]. Field potentials were recorded using a CyberAmp380 amplifier (Molecular Devices, Sunnyvale, CA) and a Digidata1440A interface (Molecular Devices, Sunnyvale, CA), with a sampling rate of 20 KHz and a bandpass filter of 0.1–300 Hz, controlled by Clampex 10.2 (Molecular Devices, Sunnyvale, CA). Averaged data from 100 continuous traces was used to calculate implicit time and amplitude of major negative component for each mouse.

Immunohistochemistry examination

Animals were intracardially perfused with phosphate buffered saline (PBS) followed by 4% paraformaldehyde in PBS. A 4-mm-thick coronal section (Bregma 1 to –3 mm) was obtained from each brain and embedded in paraffin. Brain tissue slices, 3 μm thickness, were cut using a microtome and visualized under a light microscope. Using 4x–10x objective lenses, brain structures including the corpus callosum, hippocampus, external

capsules, optic nerves, chiasm, and optic tracts, were identified. Tissue slices with the best approximation with MRI images were then selected and deparaffinized in xylene for immunohistochemical examinations. The integrity of axons was evaluated using a primary antibody against phosphorylated neurofilament (SMI-31, 1:1000; Covance Inc., Emeryville, CA, USA). Myelin integrity was assessed with a primary antibody against myelin basic protein (MBP, 1:250; Zymed Laboratories Inc., South San Francisco, CA) at 4°C overnight [16]. Following 15 min wash in PBS, sections were incubated with fluorescent secondary antibodies for 1 h at room temperature (1:200, anti-mouse conjugated to Alexa 488 for SMI-31, 1:200, anti-rabbit conjugated to Texas red for MBP; Molecular Probes). Histological sections were examined using an Olympus Fluoview confocal microscope equipped with a 60 × oil objective.

RESULTS

DTI showed white matter abnormalities in the optic nerves and optic tracts in A β ₁₋₄₂-treated mice (Fig. 1). The right optic nerves showed a significant 160% increase in $\lambda_{\perp}$ ($0.26 \pm 0.14 \mu\text{m}^2/\text{ms}$, compared to control $0.10 \pm 0.03 \mu\text{m}^2/\text{ms}$, $p < 0.05$) and a significant 21% decrease in RA (0.95 ± 0.19 , compared to control 1.2 ± 0.06 , $p < 0.05$), while the changes in the left optic nerves did not reach a significant level (Fig. 2). The left optic tract showed a significant 13% decreases in $\lambda_{\parallel}$ (1.46 ± 0.12 , compared to control $1.67 \pm 0.17 \mu\text{m}^2/\text{ms}$, $p < 0.05$), a significant 83% increase in $\lambda_{\perp}$ (0.38 ± 0.05 , compared to control $0.21 \pm 0.12 \mu\text{m}^2/\text{ms}$, $p < 0.05$), and a significant 31% decrease in RA (0.71 ± 0.05 , compared to control 1.0 ± 0.19 , $p < 0.05$), while the right optic tract did not show a significant change. In addition, the left external capsule showed a 31% increase in $\lambda_{\perp}$ (0.55 ± 0.12 , compared to control $0.42 \pm 0.03 \mu\text{m}^2/\text{ms}$), but the change did not reach a significant level. The right external capsule and corpus callosum appeared normal (Fig. 3). We also found a significant 9% decrease of the volume of the left cortex ($14.9 \pm 2.4 \text{ mm}^3$) compared to the right cortex ($16.3 \pm 0.9 \text{ mm}^3$).

To measure the function of the visual system, VEPs were recorded (Fig. 4). Compared to the controls, the right eye showed a significant 44% decrease in VEP amplitude (34 ± 13 , compared to control $60 \pm 27 \mu\text{V}$, $p < 0.05$) and 20% increase in VEP latency (69.6 ± 7.5 , compared to control $57.8 \pm 10.5 \text{ ms}$, $p < 0.05$). The left eye showed a 27% increase in VEP latency (73.7 ± 14.4 , compared to control $57.8 \pm 10.5 \text{ ms}$, $p < 0.05$), but the changes in the VEP amplitude did not reach a significant level.

Following VEP recordings, animals were sacrificed, and the axonal and myelin integrities were examined using immunostainings for SMI-31 and MBP, respectively. As shown in Fig. 5, the left and right optic nerves showed a loss of staining of axons and myelin in the A β ₁₋₄₂-treated mice. The density of axons was reduced 30% and 44% in left and right optic nerves, respectively, compared to controls. The left optic tracts showed a significant 29% loss of axonal counts, while the change in the right tracts did not reach a significant level. The correlation between *in vivo* DTI and axonal counts was also calculated. In the optic nerves, the axonal counts were moderately correlated with $\lambda_{\parallel}$ ($\gamma = 0.748$, $p < 0.001$), $\lambda_{\perp}$ ($\gamma = -0.598$, $p < 0.005$), and RA ($\gamma = 0.689$, $p < 0.001$). In the optic tracts, the axonal counts were modestly correlated $\lambda_{\perp}$ ($\gamma = -0.559$, $p < 0.005$), RA ($\gamma = 0.507$, $p < 0.05$), and TR ($\gamma = -0.499$, $p < 0.05$). Losses of axons were also found in the left external capsule in the A β ₁₋₄₂-treated mice, while the right external capsule did not appear different from the control mice (Fig. 6).

DISCUSSION

This study showed, for the first time, that *in vivo* DTI was sensitive enough to detect white matter changes in A β -treated mice, and the changes were associated with alterations in function and a loss of axons and myelin.

A β animal model

We tested whether DTI can detect AD-related white matter damage using a mouse model of AD in which A β was injected i.c.v. A β is toxic to both neurons [30, 31] and glia cells [10, 11], and is believed to be one of the major factors involved in the pathogenesis of AD [35–40]. A β i.c.v. injection has been widely used to study AD [25–31], because it produces a number of molecular, cellular, and behavioral changes closely resembling those seen in AD patients [30, 41–43]. Using this animal model, this study showed that A β _{1–42} also caused significant damage to white matter, and the damage can be detected by *in vivo* DTI. The exact mechanism by which A β causes axon damage is unknown. Studies showed that A β can induce tau phosphorylation [44, 45]. By disrupting axonal transport, hyperphosphorylated tau may lead to axonal degeneration [46]. In preliminary studies, we tested this possibility using immunostaining for phosphorylated tau. Our results showed, however, no significant increase in phosphorylated tau. Further studies are needed to determine whether axons with high levels of phosphorylated tau have already degenerated by the time of the experiment.

DTI changes in white matter damage

Among the white matter examined in this study, all injured regions, including the left external capsule, the left optic tract, and the left and right optic nerves, exhibited an increase of $\lambda_{\perp}$. The effect was associated with a loss of axons and myelin as determined by immunohistochemistry of SMI-31 and MBP, respectively. Our findings are in line with previous studies suggesting that a loss of myelin [15–17] and axons [47–49] can cause an increase in the extra-axonal space, leading to an increase of $\lambda_{\perp}$.

As for $\lambda_{\parallel}$, only the left optic tract showed a significant decrease in A β _{1–42}-treated mice. This result and the fact that the decrease is associated a loss of axons is consistent with previous studies showing a decrease of $\lambda_{\parallel}$ in degenerating nerves [14, 15, 17]. However, some studies in humans showed an increase in $\lambda_{\parallel}$ following various types of insults [18–20]. Animal studies have also produced controversial results [47]. We suspected that an acute microtubule disruption may reduce water diffusion, leading to a decreased $\lambda_{\parallel}$, while the clearance of cellular debris in the chronic stage may allow a less restricted diffusion, leading to an increase of $\lambda_{\parallel}$. It is also possible that different types of damage result in different changes in $\lambda_{\parallel}$. For instance, A β may increase $\lambda_{\parallel}$ [18–20], whereas Wallerian degeneration decreases $\lambda_{\parallel}$ [14, 15, 17]. More studies are needed to better understand the biophysical mechanisms leading to the changes of $\lambda_{\parallel}$ observed under different conditions.

The lateralized damage

Our results showed that the axonal damage induced by A β _{1–42} was lateralized. The right optic nerve and left optic tract showed more injury than the same regions in the opposite hemisphere. The lateralized damage is likely to be due to the adjacency of the left optic tract toward the A β injection site. A spatial relationship between the A β injection site and A β -induced pathology has been reported previously, e.g., [50]. In this study, the left optic tract was closer to the A β -injection site and may therefore be affected by A β more than the right optic tract. Because more than 90% of the axons in the left optic tract are originated from the right optic nerve, damage to the left optic tract would propagate to the right optic nerves, resulting in more damage to the right than to the left optic nerves.

VEP and DTI comparison

We measured functional changes of the visual pathway using VEP recording. Previous studies have found that VEP amplitude decreases, while its latency increases with increasing $\lambda_{\perp}$ [51, 52]. In the presented study, the right eye showed a decrease in VEP amplitude and an increase in VEP latency, while the left eye maintained normal amplitude and increased VEP latency in treated mice. These results were in line with the DTI data, suggesting a more severe damage in the pathway linking the right eye to the left cortex than that linking the left eye to the right cortex. Our results also suggest that VEP latency is more sensitive than VEP amplitude to A β -induced injury in the visual pathway since the former increased, while the latter appeared normal in the pathway connecting the left eye to the right cortex. DTI and immunostaining suggest that the pathway is also damaged, though to a lesser degree compared to the opposite side.

In conclusion, DTI can detect white matter damage induced by i.c.v. injection of A β_{1-42} in mice. Increases of $\lambda_{\perp}$ found in this animal study were in line with findings in human AD [18–20] and were associated with a loss of axons and myelin. The visual pathway showed the most significant changes possibly because of its close location to the A β injection site and the highly parallel axons in the optic nerves and tracts which would make DTI particularly sensitive to their changes. It might be important to note that visual disturbances are a common complaint in AD [54–57], which raises the possibility that optic tract and nerve degeneration occurs in parallel with memory declines in AD patients and that DTI of the visual pathway may aid early diagnosis of AD. Current methods used to evaluate degenerative process in AD mainly rely on the gray matter atrophy using the conventional MRI [58]. DTI, by detecting the microstructural changes in the white matter, would help provide a more complete evaluation of the neurodegenerative process in AD.

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Fig. 1. DTI maps of the optic nerves and optic tracts (arrows) taken 2 months after A β_{1-42} injection in left lateral ventricle. Images were from one representative control and one A β_{1-42} -treated mouse. Compared to control (ctr), the right optic nerve and left optic tract of the A β -affected mouse showed reduced RA, reduced $\lambda_{||}$, and increased $\lambda_{\perp}$.

Fig. 2.

DTI indices of the optic nerves and optic tracts in $A\beta_{1-42}$ -treated and control mice. Blue (left) bars represent the controls. Red (middle) and green (right) bars are measurements of the left and right side from the $A\beta_{1-42}$ -treated mice, respectively. The brackets indicate significant differences with $p < 0.05$. In $A\beta_{1-42}$ -treated mice, the right optic nerves showed a significant decrease of RA and a significant increase of $\lambda_{\perp}$. The left optic tracts showed a decrease in RA and $\lambda_{||}$ and an increase in $\lambda_{\perp}$.

A. DTI maps of an A β -treated mouse

B. External Capsule (EC) ■ Control ■ Left ■ Right

C. Corpus Callosum (CC) ■ Control ■ A β icv **Fig. 3.**

DTI maps of external capsules (red arrows) and corpus callosum (blue arrows) of A β_{1-42} -treated mice (A). The left external capsule showed slightly decreased RA and increased $\lambda_{\perp}$. Quantitative analysis of DTI indices of external capsules was summarized with the blue bars for controls, and the red and green bars for the left and right external capsules of the A β_{1-42} -treated mice, respectively (B). DTI indices of corpus callosum were summarized with the blue and red bars for controls and the A β_{1-42} -treated mice, respectively (C). The left external capsules of A β_{1-42} -treated mice showed an increase in $\lambda_{\perp}$, but not reaching the significant level).

Fig. 4. Visual evoked potential (VEP) in $A\beta_{1-42}$ -treated and control mice. The brackets indicate significant differences with $p < 0.05$. In treated mice, the right eyes showed a significantly reduced VEP amplitude (left). Both eyes showed an increased VEP latency (right).

Fig. 5.

Representative immunohistochemical staining for SMI-31 (green) and MBP (red) in the optic nerves (a–c) and optic tracts (e–g) of control (a and e) and the $A\beta_{1-42}$ -treated mice (b and f: left hemisphere; c and g: right hemisphere). The black bar below Graph g indicates 20 μm . Charts d and h show the SMI-31 positive axon counts in the optic nerves and optic tracts, respectively. The blue bars represent the controls. The red and green bars represent the left and right side measurement in the $A\beta_{1-42}$ -treated mice, respectively. The brackets indicate significant differences with $p < 0.05$. The loss of axons and myelin integrity caused by $A\beta_{1-42}$ toxicity were observed in both the optic nerves and tracts. Significant reductions of axonal counts were found in the left optic tracts and both sides of the optic nerves.

Fig. 6. Representative immunohistochemical staining for SMI-31 (green) and MBP (red) in the external capsules in the left (a) and right (b) hemisphere of an Aβ₁₋₄₂-treated mouse. The bar indicates 40 μm. The left external capsule (a) showed openings (white arrows), suggesting a loss of axons and myelin caused by Aβ₁₋₄₂ injection. The right external capsule appeared normal (b).