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The Effect of Iron in MR Imaging and Transverse Relaxation of Amyloid-Beta Plaques in Alzheimer's Disease

Mark D. Meadowcroft, Ph.D.^{1,2}, Douglas G. Peters, M.S.^{1,3}, Rahul Dewal, B.S.², James R. Connor, Ph.D.¹, and Qing X. Yang, Ph.D.^{1,2}

¹Department of Neurosurgery, The Pennsylvania State University College of Medicine, Milton S. Hershey Medical Center, Hershey, Pennsylvania

²Department of Radiology (Center for NMR Research), The Pennsylvania State University College of Medicine, Milton S. Hershey Medical Center, Hershey, Pennsylvania

³Department of Neural and Behavioral Sciences, The Pennsylvania State University College of Medicine, Milton S. Hershey Medical Center, Hershey, Pennsylvania

Abstract

Dysregulation of neural iron is known to occur during the progression of Alzheimer's disease. Visualization of amyloid-beta (A β) plaques with magnetic resonance imaging (MRI) has largely been credited to rapid proton relaxation in the vicinity of plaques due to focal iron deposition. The goal of this work was to determine the relationship between local relaxation and related focal iron content associated with A β plaques. Alzheimer's disease (N=5) and control tissue (N=3) sample slices from the entorhinal cortex were treated overnight with the iron chelator deferoxamine or saline and had microscopic gradient-echo MRI datasets taken. Subsequent to imaging, the same slices were stained for A β and iron then compared to parametric R₂* relaxation maps and gradient-echo weighted MR images. A β plaques in both chelated and un-chelated tissue generated MR hypo-intensities and had relaxation rates significantly greater than the surrounding tissue. The transverse relaxation rate associated with amyloid plaques was determined not to be solely due to iron load, as much of the relaxation associated with A β plaques remained following iron chelation. The data indicate a dual relaxation mechanism associated with A β plaques such that iron and plaque composition synergistically produce transverse relaxation.

Keywords

Alzheimer's disease; Iron; Amyloid-beta; A β ; Plaques; MRI; Deferoxamine; Chelation

Introduction

Iron is the most abundant transition metal present in the human brain. This is in most part due to the essential reduction-oxidation role iron plays in numerous biological processes. The high level of oxygen consumption in the brain results in the production of required

reactive oxygen species as the driving force for multiple redox reactions. It is central to multiple cellular respiration pathways, electron transport for ATP and cytochrome-C, as a cofactor in enzyme catalysis, and oxygen transport in hemoglobin. Iron uptake in the brain and storage is tightly regulated through coordinated homeostatic transcriptional and translational regulation of proteins such as divalent metal transporter 1, ferroportin, transferrin, transferrin receptor, iron regulatory protein, and ferritin (1,2). However, the necessity for iron is tempered by its deleterious effect as a source of oxidative stress. Iron stores increase as the body ages (3), resulting in accumulative homeostatic pressure on the body's ability to maintain iron storage regulation. In addition to the natural age induced increase in body iron, there has been a longstanding link established between the accumulation of iron in the brain and numerous neurodegenerative disorders (4).

The increased concentration of iron in the brain tissue of Alzheimer's disease (AD) patients has been well demonstrated (5,6), and a close association of iron with amyloid-beta (A β) plaques has been established in AD tissue (7,8). Understanding the relationship between iron and amyloid is evolving, with focus on the role of amyloid precursor protein (APP) and its subsequent non-amyloidogenic cleavage as moderators of cellular iron transport (9,10). When APP is cleaved along an amyloidogenic pathway, the resulting fibrils that comprise A β plaques have been characterized as metalloproteins which bind ferric iron (Fe⁺³) (11,12). In addition, the incorporation of the A β fibrils into plaque assemblies is accelerated in an iron-enriched environment (13). Oxidative free radical damage is theorized to play an important role in AD pathogenesis as well as other age-related neurodegenerative disorders (14,15). Damage surrounding A β plaques has been postulated to be due to the iron induced generation of reactive oxygen species in the Fenton - Haber-Weiss reactions (16-19). Specifically, the catalytic reduction of iron to Fe⁺² and oxidization to Fe⁺³ in these cyclical reactions produce highly reactive hydroxyl radicals (20,21). Iron (Fe⁺² and Fe⁺³) in the redox-active biomineral forms of magnetite (22) and ferrihydrite (23) are found in greater quantities within the Alzheimer's brain and are speculated to catalyze these reactions. Oxidative stress eventually causes characteristic nuclear changes of neuronal apoptosis and the activation of apoptotic cell kinases, leading to cell death and atrophy (24). Within the hippocampus and entorhinal cortex, regions known to be involved in early AD pathology, there is regional overlap between iron accumulation, A β plaque formation, localized neurodegeneration, neurofibrillary tangle deposition, and glial localization (25). These data indicate that A β plaques are major sites for catalytic redox activity when combined with high concentrations of iron (14,26-29).

Imaging of amyloid burden in the brain has been realized with positron emission tomography (PET) A β radionuclide tracers. However, visualization of individual A β plaques with PET is currently out of reach due to spatial resolution constraints and potentially beyond the fundamental limits of radionuclide detection (30). High-field MRI permits spatial resolution increases beyond PET by orders of magnitude, theoretically allowing the non-invasive *in vivo* visualization of individual plaques. As such, understanding the transverse relaxation and image contrast associated with A β plaques in Alzheimer's disease is of great interest for magnetic resonance imaging (MRI) research. Recognizing how microscopic MRI metrics relate to individual amyloid plaques will translate to and assist in

our understanding of regional plaque load at resolutions currently obtained in the clinical setting. It has conventionally been believed that MR image contrast and the ability to discern A β plaques is due to focal iron associated with plaque deposition (31,32). Our previous research (7,33) has helped to illustrate that both iron and plaque morphology are synergistic in their increasing of transverse relaxation rates in Alzheimer's and the A β generating APP/PS1 transgenic model plaques. Alzheimer's A β plaques containing and devoid of iron are both observable on high-resolution MR images. This trend is furthered when imaging transgenic mouse A β plaques lacking iron as they also are discernible on gradient-echo (GRE) weighted MR images. The goal of this research is to further understand the intricacies of how plaque iron content and morphology is related to transverse relaxation of Alzheimer's disease A β plaques. A detailed histological MR examination of deferoxamine treated AD and control tissue samples in comparison to traditional histology methods is described.

Materials & Methods

Human Alzheimer's and Control Brain Samples

Brain tissue samples from the parahippocampal gyrus from clinically and histologically determined AD subjects (n=5) and age-matched controls (n=3) were obtained with consent following The Pennsylvania State University's institutional review board guidelines (Harvard Brain Tissue Resource Center, McLean Hospital, Belmont, MA). Upon sample receipt, a section of the entorhinal cortex was separated from the larger mass of approximately 1 $\times$ 1 cm in the coronal dimension. The location of the entorhinal cortex was determined medial-laterally as between the perirhinal cortex and the hippocampal formation and caudal to the amygdala based on unstained tissue anatomy within the parahippocampal gyrus. While the methods aimed to utilize the entorhinal cortex, inclusion of the perirhinal cortex boundary is possible. Analysis of the tissue obtained from the brain bank indicated that there was not a significant difference ($p = 0.66$) between the age of the subjects at the time of death; AD = 73.6 ± 2.9 , CT = 75.6 ± 2.9 years. The postmortem index (length of time between death and tissue harvesting) was significantly longer for controls (29.0 ± 1.3 hours) compared to Alzheimer's patients (17.3 ± 2.0 hours), $p < 0.01$. Tissue samples were fully fixed in 4% paraformaldehyde for 48 hours in pH 7.3 phosphate buffered saline (PBS) after which blocks of tissue were sequentially cryogenically protected in 10, 20, and 30% sucrose solutions for 48 hours each. Tissue samples were sectioned at 60 μ m on a cryostat and rinsed in PBS for thirty minutes to eliminate any residual fixative and sucrose. Two tissue slices sequentially cut from the same sample were used for each MR and histological experiment.

Iron Chelation

Sequential slices were placed on standard microscope slides, circled with a hydrophobic barrier pen, and placed in a humidity chamber. For iron (Fe⁺³) chelation, deferoxamine mesylate (DFO) (#D9533, Sigma Aldrich, St. Louis, MO) was prepared at its maximum solubility of 76 mM/L (50 mg/ml) in PBS to optimize iron chelation. DFO clinically has been used in chelation therapy to facilitate the elimination of excess iron from the body and is routinely used in the research setting to chelate iron from biological systems (34,35). For

each imaging experiment, one of the pair of sequentially sectioned tissue samples was treated with a 500 μ l of the DFO solution and the other sample was treated with 500 μ l of PBS under the same conditions for 24 hours. Samples were then rinsed in PBS and placed adjacent to one another for micro-imaging according to previous methods, briefly explained below (7,33).

Histological slice MRI Scanning Protocols

Histological MRI was performed on a 7.0 T Bruker Biospec 70/30 system (Bruker BioSpin MRI GmbH, Ettlingen, Germany) with an in-house developed histological RF coil (7,33). The 60 μ m DFO treated and negative sham tissue samples were floated in 250 ml of PBS for 60 minutes to allow any residual DFO, formalin, and/or sucrose in the tissue to leach out. The tissue samples were placed adjacent to one another and encased between two coverslips with a hydrophobic barrier around the edges to maintain hydration. For T_2^* imaging, gradient-echo data were acquired in 6h 32m with a TR / TE = 718 / 6.48 ms, FA = 30°, eight echoes with 9ms echo spacing (6.48 – 69.48 ms), 64 averages, FOV=23mm $\times$ 23mm, and a matrix of 512 $\times$ 512 resulting in a final in-plane resolution of 45 μ m $\times$ 45 μ m. A coronal slice section with a left-right readout direction was set to 6 cm in thickness with the 60 μ m tissue sample in the center of this selection; resulting in a nonselective whole volume excitation and an effective 60 μ m slice thickness based on tissue dimensions. Each tissue block (AD N = 5, CT N = 3) had at least two imaging sessions obtained from recently cut tissue sections.

Iron and Amyloid Staining

After histological MR imaging, the 60 μ m tissue sections were free floated and rinsed in deionized water (dH₂O) for 15 minutes. Tissue sections were co-stained for both iron and amyloid-beta with a Perl's stain followed by an aqueous thioflavin-S stain, respectively. The sections were placed in equal volumes of freshly prepared 4% potassium ferrocyanide (#P236, Fisher Scientific, Waltham, MA) and 4% hydrochloric acid (final 2% combined concentration for each) for 30 minutes under gentle rocking followed by two rinses in dH₂O. Intensification of the iron stain was performed with five minutes of 3,3'-diaminobenzidine tetrahydrochloride (DAB) staining (#D5637, Sigma, St. Louis, MO) (10 mg dissolved in 15ml of PBS with 16 μ l of 30% H₂O₂) followed by two five-minute rinses in dH₂O. Tissue samples were then free floated in 20 ml of 1% thioflavin-S (#T1892, Sigma, St. Louis, MO) aqueous solution for five minutes, followed by differentiation in 70% ethanol for five minutes and two five-minute washes in dH₂O. Sections were mounted on histological slides with FluorSave Aqueous mounting media (#345789, Calbiochem, San Diego, CA). To test for the possibility of confounding interactions between the two staining methods during co-staining, each was tested individually on separate tissue samples to determine their individual efficacy and compared to the co-stained sections. DAB counterstaining was also done independently to test the compounds affinity for amyloid-beta alone.

Microscopy

Low resolution microscopy was performed using a Nikon SMZ-745T dissecting scope at the same optical magnification and light intensity for all tissue sections. Microscopic images were taken using the same camera settings across all fluorescent images and across all bright

field images to standardize image contrast. High resolution microscopy of the tissue sections was performed using a Nikon OptiPhot microscope and Nikon Digital Sight camera using NIS-Elements software. Bright field microscopy under the visible light spectra was used to view iron stains. A FITC fluorescence cube at 430 nm excitation and 550 nm emission was used to visualize thioflavin-S positive A β deposition. Whole tissue image mosaics were created with the 40 $\times$ magnification bright field and fluorescent images using the photomerge option in Adobe Photoshop CS4 (Adobe Systems Incorporated, San Jose, CA, USA). The final images had their color profile matched to the CMYK color model and contrast slightly enhanced for prepress proofing; the same settings for chelated and non-chelated images were used.

MRI and Histology Post Processing

Post processing was performed using in-house software developed with the interactive data language (IDL) 8.3 framework (Exelis Visual Information Solutions, Boulder, CO, USA). The software allows for the creation of $R_2^*(1/T_2^*)$ relaxation rate parameter maps from GRE data with a linear least squares curve fitting method (36). T_2^* -weighted images were generated by the summation of the magnitude images from all of the echoes and were resampled to a higher image resolution with bicubic interpolation within Photoshop CS4. Manual visual co-registration of the thioflavin microscopy images to the summated MRI datasets within Photoshop helped in the localization of the A β plaques within the MRI datasets. Microscopy images were rotated to align with the orientation of the imaging data to aid in tissue registration and minimize inaccuracies due to tissue manipulation during staining. Relaxation measurements from individual A β plaques in Alzheimer's tissue samples were obtained by manually selecting plaque regions of interest (ROIs) and averaging the relaxation value of the selected voxels within each measured plaque. A β plaque ROI voxels were limited to the homogeneous hypo-intense dark region centrally within the plaques to minimize partial volume effects. The in-plane resolution allowed the inclusion of two to four voxels of interest from A β plaques of approximately 40 – 100 μ m in diameter. In total, 255 plaque ROIs were obtained (DFO – = 124, DFO + = 131) over multiple imaging sessions from the five cortical AD specimens. The presence or absence of iron within the A β plaques was determined through visualization of the co-registered Perl's iron and thioflavin stains. Manually selected regions of interest were drawn in AD and control white and gray matter. The number of ROIs per specimen was evenly distributed across all tissue sample MR datasets. White and gray matter regions were parcellated into 3 – 5 non-overlapping ROIs per acquired specimen image. Alzheimer's disease gray matter had ROIs manually selected such that no hypo-intense positions (amyloid-beta plaques and iron) were included in the selection. A total of 108 white matter (30 AD DFO –, 30 DFO +, 24 CT DFO –, and 24 CT DFO +) and 123 gray matter ROIs (30 AD DFO –, 45 DFO +, 24 CT DFO –, and 24 CT DFO +) were selected. All ROIs were selected using the GRE magnitude images and were subsequently applied to the R_2^* parametric maps calculated from the GRE data.

Susceptibility Weighted Image Processing

All image processing was performed using MATLAB R2012b (The Mathworks, Inc., Natick, MA) with an in-house written script following a multi-echo SWI processing

methodology (37). A symmetrically centered 2D Hanning low pass frequency filter was applied to the 2D k -space data for each echo (TE). As the contrast in the phase image is linearly proportional to TE and field strength (38), the filter-width (FW) was adjusted for each echo incorporating the 7 T B_0 field strength. Filter-width increments of 0.05 for every 8ms of echo time were utilized using the first echo as a reference such that $\text{Filter-width} = 0.05 / 8 * \text{TE} + 0.107$. The resulting phase wrap of each echo was assessed such that they were comparable to the short TE times. The low pass filtered datasets were subtracted from the original phase images to produce high pass filtered datasets. The FW was further assessed via visual inspection of the resulting high pass phase images for each echo. The corrected high pass filtered phase images were then converted into a linear mask sensitive to $-\pi$ to 0 phase values. The negative mask for each echo was then multiplied by the corresponding magnitude image by a power of four. A final susceptibility weighted image was created by summing the susceptibility weighted images for each echo of the GRE dataset.

Statistical analysis

For statistical data analysis, the SPSS 22 program package was utilized (IBM Corporation, Armonk, New York). Within and between group MRI R_2^* relaxation rate measures were analyzed with an analysis of variance (ANOVA), followed by a Tukey–Kramer post-hoc analysis. Further analysis utilized a Fisher test (F-test) to determine if a significance in variance existed between all measures prior to individual equal or unequal variance T-test analyses. A p value of less than $\alpha = 0.05$ was utilized as a determinant of statistical significance.

Results

Gradient-echo T_2^* -weighted MR and low magnification microscopic images of AD tissue samples treated with saline (Fig. 1, a and c, respectively) exhibit MR image hypo-intensities in tissue regions known to contain high iron concentrations, such as white matter tracks. Punctate regions of focal iron and individual $A\beta$ plaques appear as dark hypo-insensitive spots within the cortical gray matter on the GRE datasets. The DFO treated tissue samples (Fig. 1, b and d, respectively) exhibit less iron in white matter tracks on microscope images resulting in less contrast loss on the MR images. The deferoxamine does not visually appear to chelate all iron from white matter as it still stains for iron, although to a lesser degree than the saline treated tissue. Hypo-intense spots, similar in location to those found in saline treated tissue, are visible in cortical gray matter of DFO iron chelated AD tissue.

Microscopic evaluation of MR hypo-intensities in the saline treated - unchelated tissue (Fig. 2a, red arrows) show a precise relationship to locations with focal iron content (b, Perl's) and $A\beta$ plaques (c, thioflavin-S) within the same AD tissue sample. Regions highlighted with red boxes in the gross anatomical images are shown right at 40x magnification. Samples treated with DFO exhibit hypo-intensities in the gray matter (Fig. 2d, and 40x enlargements) that are associated with $A\beta$ plaque deposition (f) but stain negatively for iron (e). The focal hypo-intensities related to plaque deposition in the DFO treated tissue visibly appear slightly less hypo-intense than those found in DFO negative tissue. A small dark

precipitate ($< 10\mu\text{m}$) in the Perl's – DAB stain remains following chelation and visually appears to be minute focal intracellular iron deposition. The morphology and visible pattern of punctate staining observed closely matches previous Perl's staining of AD tissue and is speculated to be lipofuscin (39). These lipid containing pigment granules are known to bind iron (40) and are found with increased prevalence in AD brain tissue (41).

A β plaques (Fig. 3a, red arrows, 40 $\times$ magnification) in unchelated AD tissue contain notable amounts of focal iron (b) and present as punctate hypo-intensities on gradient-echo datasets (c). Inclusion of phase perturbation in susceptibility weighted imaging (Fig. 3d) exacerbates these hypo-intensities to enhance plaque contrast. The iron chelated tissue samples have little visible iron associated with the A β plaques (Fig. 3e) on the Perl's stain images (f) and exhibit hypo-intensities produced by A β plaques on the gradient-echo (g) datasets which are amplified on the susceptibility weighted images (h). Hypo-intense regions on the GRE datasets not associated with amyloid plaques are highlighted with blue arrows. The histological data demonstrates that these regions are associated with vasculature and blood products based on morphology as viewed with the Perl's/DAB and thioflavin stains.

Transverse R_2^* relaxation rates for AD and control tissue samples exhibit marked differences due to iron chelation (Fig. 4). Chelation of iron from A β plaques results in a decrease in R_2^* rate with a comparable reduction in AD white matter (Table 1). The decrease in A β plaque relaxation rate following DFO treatment is visibly supported by the slightly less dark A β plaque hypo-intensities observed in chelated tissue (Figs. 2 and 3). Control tissue also exhibited a reduction in R_2^* rate within white matter as a result of DFO treatment, indicative of reduced iron. Alzheimer's and control gray matter had small reductions in R_2^* as a result of iron chelation; although only significant for the AD tissue, the percent reduction (effect size) in the control tissue was slightly increased. The R_2^* rate of A β plaques remained higher than the surrounding gray matter in both unchelated and chelated conditions (Table 2). The similarity in the percent decrease of AD A β and white matter upon chelation is speculated to be due to the similar reduction in iron content upon chelation. This observation is histologically observed as iron staining within the core of the plaques and diffusely within the halo region is quenched upon iron chelation. The quantitative observation that iron chelated A β plaques have a reduced R_2^* rate that is greater than that of the surrounding gray matter demonstrates that proton relaxation is occurring in relation to A β plaque deposition.

Comparison of chelated and unchelated AD and control tissue gray matter R_2^* relaxation rates demonstrate a similar trend following DFO treatment with the difference between unchelated AD and control gray matter R_2^* rate reaching significance (Table 1). White matter measures reveal that AD tissue has a consistently higher R_2^* than control tissue when treated with saline or DFO. The similar percent decrease in white matter R_2^* rate in AD and control tissue suggest a similar decrease in iron following chelation. The R_2^* relaxation rate of iron chelated AD white matter is maintained above control tissue suggesting multiple proton relaxation mechanisms are occurring in AD white matter.

Discussion

The relationship between proton relaxation, iron deposition, and plaque composition has remained unclear within AD tissue. Increased transverse relaxation associated with A β plaques has largely been credited to focal iron deposition within the plaque mass (42,43). In utilizing the current chelation approach we have diminished the iron associated with AD A β plaques while showing that plaques are still discernable on the transverse MR image sets. The data presented here confirms that the ability to distinguish AD A β plaques with MR imaging is not exclusively dependent upon the amount of iron incorporated into the plaque mass. Chelation of iron from A β plaques in AD tissue samples results in hypo-intensities on GRE data sets similar to A β plaques associated with iron. MR proton phase perturbations are associated with A β plaque localization in addition to iron concentration, as visualized in the susceptibility-weighted datasets. The data support the hypothesis that local magnetic field perturbations effecting transverse relaxation are synergistically due to both iron concentration and focal fibrillar amyloid deposition.

A β plaques in transgenic APP/PS1 mice also exhibit hypo-intensities in GRE datasets with a reduced amount of iron associated with these plaques (7). Similar to transgenic APP/PS1 A β plaques, the AD A β plaques fall under a proposed dual relaxation mechanism caused by both iron and plaque composition. The data indicate that iron content, while a factor in the MR imaging of plaques, is not solely responsible for the hypo-intensities seen on the MR images and that the dense fibrillar nature of the A β plaques has a major role in shortening of the transverse relaxation in AD tissue.

The reduction of magnetization along the transverse plane is primarily a factor of two phenomena; proton dephasing due to interactions with neighboring protons (spin-spin) and local B₀ magnetic field inhomogeneities (44). While gradient-echo R₂^{*} (1/T₂^{*}) relaxation has an R₂ (1/T₂) component, it is primarily dominated by phase alterations induced by local field inhomogeneities (R₂' or γB_0 where R₂^{*} = R₂ + R₂' and 1/T₂^{*} = 1/T₂ + γB_0). In white matter, the R₂^{*} decrease is directly related to the reduction in field inhomogeneity upon Fe⁺³ chelation. Iron chelation of A β plaques also causes a reduction in R₂^{*} relaxation rate; however much of the relaxation remains, indicating another factor is influencing the multifaceted relaxation of A β plaques.

Alzheimer's A β plaque morphology provides clues for the transverse relaxation mechanism. The compositional A β fibrils that makeup plaques contain numerous hydrophobic and hydrophilic amino-acid residues which relate to the amyloidogenic property of plaques. In such case, water molecules are cycled between being repelled from the hydrophobic moieties and bound to hydrophilic regions of the plaques. The rapid hydrogen bond cycling of water molecules would result in a first order cross relaxation via proton-proton magnetization exchange, leading to rapid transverse relaxation. Fast chemical exchange of protons between bound and bulk phases accounts for the macromolecular interaction and is a significant contributor to the hypo-intense contrast in the plaques (45). In addition, the magnetic susceptibility differences between the highly compact A β protein mass and surrounding tissue are hypothesized to induce static magnetic field inhomogeneities in addition to iron. Plaques contain numerous infiltrated gaps or channels (7), which allow

water molecules to readily diffuse in and out the plaques. Structured A β plaque hydration due to water molecule infiltration into the interior of plaques along these channels advances the aforementioned interactions and collectively increases the rate of proton transverse relaxation. The relative decrease in R_2^* rate with iron chelation compared to the remaining relaxation suggests that plaque related relaxation appears to be a dominant factor over iron loading in the shortening transverse relaxation in the A β plaques.

Histological visualization of iron supports the R_2^* relaxation rate measurements. R_2^* relaxation rates of DFO chelated tissue samples indicate they have a decreased iron concentration compared to unchelated tissue. Qualitative histological results visually show less iron staining in chelated amyloid-beta plaques, white, and gray matter. Also of interest is the finding that the transverse relaxation rate in iron chelated DFO treated and untreated AD white matter is higher than control samples. The relaxation data indicate that AD white matter contains more iron than control tissue in both chelated and unchelated states. While there is a similar percent decrease in white matter R_2^* rate upon chelation in AD and control tissue, indicating a similar decrease in iron content, the R_2^* rate of AD tissue remains greater than control tissue. Considering that GRE R_2^* relaxation rate is a factor of both iron content and tissue structure, this pattern could be indicative of white matter alterations in Alzheimer's disease. This theory is congruent with the hypothesis that AD has an integral white matter component (46,47).

The present study frames the synergistic role of iron in magnetic resonance imaging of A β plaques. The interpretation of the data should be considered in the wider context of the literature base. Tissue fixation is known to cause alterations in proton transverse relaxation (48) and care must be taken when comparing *ex vivo* to *in vivo* MRI datasets. The present study compared only *ex vivo* paraformalin fixed AD and control tissue. As the tissue was handled in a similar fashion, the results from each cohort are comparable to one another and are meant to approximate an *in vivo* condition only. The literature contains some heterogeneity in regards to effect of prolonged formalin fixation and the leaching of iron from tissue samples. With this said, the tissue samples in the current study demonstrate increased iron content based on histology and MRI measures especially with regards to co-localization of focal iron deposition and A β plaques in Alzheimer's tissue samples. While it appears that the majority of the GRE hypo-intensities in the AD tissue are A β plaque, iron, and vessel related, a comprehensive characterization of all hypo-intensities was not possible in the current study. The limited number of specimens utilized putatively is a factor in the study design and statistical outcome; as such the interpretation of the data should be considered with this context in mind. The histological staining of iron with Perl's and DAB enhancement does not allow quantification of iron within neuronal tissue. Future study quantifying iron in MR imaged A β plaques is required to establish the definitive degree to which iron and amyloid are responsible for the synergistic proton relaxation observed.

This work aids in understating the role of iron and amyloid-beta plaque induced transverse relaxation within *ex vivo* Alzheimer's disease tissue. Further *in vivo* characterization of the relationship between MRI metrics and Alzheimer's etiology remains to be determined. Ascertaining the pathological factors responsible for transverse relaxation in AD tissue at the microscopic level is directly translatable towards the determination of *in vivo* amyloid

and iron load. The present data represents a step forward in understanding the relaxation mechanisms associated with A β plaques for future utilization in clinical and translational research.

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Abbreviations

Aβ	Amyloid Beta
AD	Alzheimer's disease
APP	Amyloid Precursor Protein
CT	Control
DAB	Diaminobenzidine
dH₂O	Deionized Water
DFO	Deferoxamine
FA	Flip Angle
Fe⁺²	Iron (II)
Fe⁺³	Iron (III)
GRE	Gradient-Echo
H₂O₂	Hydrogen Peroxide
MRI	Magnetic Resonance Imaging
PBS	Phosphate Buffered Saline
PET	Positron Emission Tomography
PS1	Presenilin 1
ROI	Region of Interest
T₂	Transverse Proton Relaxation Decay Time (s, 1/R ₂)
T₂[*]	Transverse Proton Relaxation Decay Time with field inhomogeneities (s, 1/R ₂ [*])
TE	Echo Time
TR	Repetition Time
R₂	Transverse Proton Relaxation Decay Rate (1/s, 1/T ₂)
R₂[*]	Transverse Proton Relaxation Decay Rate with field inhomogeneities (1/s, 1/T ₂ [*])

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Figure 1.

T₂*-weighted MR images (a, b) and low magnification microscopy (c, d) images of sequentially cut 60 μ m sections from the same AD entorhinal cortex tissue sample which have been treated with saline (a, c) and deferoxamine b, d). The MR images of DFO unchelated saline treated tissue show MR hypo-intensities characteristic in of high iron in white matter tracks verified microscopically in (c). In addition, punctate hypo-intensities are found throughout cortical gray matter in saline treated tissue. The DFO treated tissue exhibits less contrast loss within in white matter tracks on the MR images (b). The histological data (d) corroborate this as less iron is stained within these white matter tracks. Of particular interest are the focal MR hypo-intensities within cortical regions which remain following iron chelation. The scale bar is set to 1mm in length.

Figure 2.

T_2^* -weighted MR images (a, d), microscopy images of Perl's stain for iron (b, e), and fluorescent thioflavin-S for A β plaques (c, f) of sequentially cut AD tissue samples treated with saline (a, b, and c) and deferoxamine (d, e, and f). The gross anatomical scale MR images show punctate hypo-intensities in both the saline and DFO treated samples. The enlarged 40 $\times$ regions of the saline treated tissue shows hypo-intensities in the MR images that are associated with high iron content and A β plaques. The DFO treated tissue shows a lack of positive iron staining associated with A β plaques but still exhibit hypo-intensities on the MR images associated with plaque deposition. The scale bars are set at 1mm and 250 μ m in length for the gross anatomical and enlarged levels images, respectively.

Figure 3.

Amyloid-beta staining (a,e), iron deposition (b,f), T_2^* -weighted MR images (c,g), and susceptibility weighted images (d,h) of saline (left) and DFO treated (right) AD tissue samples. Thioflavin-S stained A β plaques in saline treated (red arrows in a - d) positive for iron deposition (b) are viewable as punctate hypo-intensities on gradient-echo MR images (c). The ability to view plaques is magnified with incorporation of phase data in the susceptibility weighted datasets (d). A β plaques treated with deferoxamine (red arrows in e - h) show a reduced amount of associated iron (f) and are viewable on both T_2^* -weighted (g) and susceptibility weighted imaging datasets (h). Blue arrows indicate hypo-intense regions on the GRE dataset that are not associated with A β plaques. Histological evaluation demonstrates that they are associated with blood products within vasculature. The B_0 field

orientation is indicated and images are presented at 40× magnification with calibrated 100 μm scale bars.

Figure 4. Transverse R_2^* rates for saline and deferoxamine treated AD tissue samples within A β plaque, gray, and white matter and control tissue samples within gray and white matter regions of interest. Comparison and significance of these measures can be found in tables 1 and 2. Error bars represent the standard error of the mean.

Table 1Transverse R_2^* Rate Measures in Alzheimer's Disease and Control Tissue Unchelated verses Chelated.

Alzheimer's Tissue ROI	Alzheimer's Unchelated (1/s)	Alzheimer's Chelated Fe (1/s)	Percent Change	<i>P</i> -value
A β Plaques	33.5 $\pm$ 0.76	28.6 $\pm$ 0.66	-14.6 %	<i>p</i> < 0.001
Gray Matter	19.7 $\pm$ 0.29	18.7 $\pm$ 0.38	-5.0 %	<i>p</i> = 0.038
White Matter	35.1 $\pm$ 0.79	30.3 $\pm$ 0.55	-13.6 %	<i>p</i> < 0.001

Control Tissue ROI	Control Unchelated (1/s)	Control Unchelated (1/s)	Percent Change	<i>P</i> -value
Gray Matter	21.7 $\pm$ 0.74	20.1 $\pm$ 0.70	-7.2 %	<i>p</i> = 0.147
White Matter	25.0 $\pm$ 1.01	22.0 $\pm$ 0.72	-11.8 %	<i>p</i> = 0.021

Italicized *P* values convey significance greater than $\alpha = 0.05$

Table 2Statistical Comparison of Transverse R_2^* Rate Measures

AD vs. Control	Unchelated (DFO -)	Chelated (DFO +)
Gray Matter	<i>p</i> = 0.018	p = 0.088
White Matter	<i>p</i> < 0.001	<i>p</i> < 0.001

Unchelated Tissue (DFO -)	Alzheimer's Disease	Control
A β Plaques vs. Gray Matter	<i>p</i> < 0.001	-----
A β Plaques vs. White Matter	<i>p</i> = 0.155	-----
Gray vs. White Matter	<i>p</i> < 0.001	<i>p</i> = 0.009

Chelated Tissue (DFO +)	Alzheimer's Disease	Control
A β Plaques vs. Gray Matter	<i>p</i> < 0.001	-----
A β Plaques vs. White Matter	p = 0.054	-----
Gray vs. White Matter	<i>p</i> < 0.001	p = 0.061

Italicized *P* values convey significance greater than $\alpha = 0.05$

A dashed line indicates that this comparison is not applicable.