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MICEST: a Potential Tool for Non-invasive Detection of Molecular Changes in Alzheimer's Disease

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Abstract

Myo-Inositol (mIns) is a marker of glial cells proliferation and has been shown to increase in early Alzheimer's disease (AD) pathology. mIns exhibits a concentration dependent chemical-exchange-saturation-transfer (CEST) effect (MICEST) between its hydroxyl groups and bulk water protons. Using the endogenous MICEST technique brain mIns concentration and glial cells proliferation can be mapped at high spatial resolution. The high resolution mapping of mIns was performed using MICEST technique on ~20 months old APP-PS1 transgenic mouse model of AD as well as on age matched wild type (WT) control (n=5). The APP-PS1 mice show ~50% higher MICEST contrast than WT control with concomitant increase in mIns concentration as measured through proton spectroscopy. Immunostaining against glial-fibrillary-acidic protein also depicts proliferative glial cells in larger extent in APP-PS1 than WT mice, which correspond to the higher mIns concentration. Potential significance of MICEST in early detection of AD pathology is discussed in detail.

Keywords

Alzheimer's disease; myo-inositol; magnetic resonance spectroscopy; chemical exchange saturation transfer imaging; glial cells proliferation

Introduction

Alzheimer's disease (AD) is the most common cause of dementia in elderly, affecting approximately 2–4 million individuals in the United States and more than 30 millions worldwide (Petrella et al., 2003). The pathological hallmarks of AD include extracellular deposition of amyloid beta (A β), neurofibrillary tangles, neuronal and synaptic loss, and characterized by reactive gliosis and inflammation (Lee et al., 2001; Nagy et al., 1998). The neurodegeneration in AD is associated with the activation of glial cells in brain parenchyma (von Bernhardi, 2007) and the most common glial cell type activated in AD is astrocytes (Nagele et al., 2004). Although the nature of the trigger that causes activation of astrocytes in AD brain *in vivo* is not clear, *in vitro* studies have shown that both aggregated A β protein and the intact cores of amyloid plaques isolated from human AD brain tissue can stimulate

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activation of astrocytes (Nagele et al., 2004). Once activated these cells secrete cytokines and chemokines which in turn damage the neuronal cells (Aschner et al., 1999; Chen et al., 2010; Streit et al., 2004; von Bernhardi, 2007). Currently, the elevated expression of activated glial cells in AD brain pathology is evaluated through immunostaining against glial fibrillary acidic protein (GFAP) (Chen et al., 2010; Nagele et al., 2004). This method is invasive and requires extensive *in vitro* tissue processing with antibodies treatment and doesn't allow for longitudinal studies of measuring glial cell proliferation *in vivo*.

Positron Emission Tomography (PET) exploits radioactive ligands that bind to the receptors of the glial cells (Cagnin et al., 2001; Hammoud et al., 2005). The radioactive ligand used to study the glial cells proliferation in AD is PK11195 (1-[2-chlorophenyl]-N-methyl-N-[1-methylpropyl]-3-isoquinoline carboxamide), which showed increased regional binding for the benzodiazepine receptor in AD compared to control (Cagnin et al., 2001). The major shortcomings of PET are radiation exposure, low resolution, imaging logistics due to short half-lives of radio ligands. While magnetic resonance imaging (MRI) is noninvasive and provides high resolution and exquisite structural details, existing MRI methods are not capable of imaging the distribution of glial cells in brain. Proton magnetic resonance spectroscopy (¹H MRS) provides a non-invasive way to monitor the biochemical changes in the brain during disease progression. ¹H MRS has been widely used to detect the changes in various metabolites in AD both in transgenic animal model as well as human brain (Chen et al., 2009; Huang et al., 2001; Kantarci et al., 2003; Marjanska et al., 2005; Siger et al., 2009). Each metabolite provides unique information about the disease pathology as they are sensitive to pathological changes at cellular or molecular level. For example- N-acetyl aspartate (NAA), a marker of neuronal density and glutamate excitatory neurotransmitters has been shown to decrease in AD pathology (Chen et al., 2009; Huang et al., 2001; Kantarci et al., 2003; Marjanska et al., 2005; Siger et al., 2009). On the other hand myoinositol (mIns), which is a marker of glial cells proliferation, has been shown to increase in AD pathology (Chen et al., 2009; Huang et al., 2001; Kantarci et al., 2003; Marjanska et al., 2005; Siger et al., 2009). ¹H MRS studies have shown progressively increased mIns concentration with advancing age in APP-PS1 transgenic AD mouse brain compared to wild type (WT) control (Marjanska et al., 2005). Although, ¹H MRS has been widely used to monitor mIns changes in AD it suffers from poor resolution and also does not provide the map of brain region with active glial cells proliferation/activation at high resolution. As, it has been already reported that brain region with increased concentration of mIns is associated with the glial cells proliferation/activation, the high resolution mapping of mIns may serve as the surrogate map of the glial cells distribution in the brain.

Recently, high-resolution mapping of mIns in human brain has been introduced by exploiting exchangeable –OH protons present on mIns using a technique commonly known as chemical exchange saturation transfer imaging (MICEST) (Haris et al., 2011). In CEST, the exchangeable protons of a solute molecule can be selectively irradiated by application of the radio-frequency (RF) pulse and their saturated magnetization transfer with bulk water leads to decrease in bulk water signal (Sherry and Woods, 2008; Ward et al., 2000) in a solute concentration dependent manner. The CEST technique has been earlier used to map the pH (Zhou et al., 2003) as well as protein concentration changes (Jones et al., 2006) in the brain by exploiting the amide proton (-NH). The exchangeable –OH proton has been used to monitor the glycogen change in liver (van Zijl et al., 2007) and proteoglycan in the knee cartilage (Ling et al., 2008).

In the current study, we demonstrated high resolution mapping of mIns using MICEST technique in transgenic APP-PS1 AD mice and shown elevated MICEST contrast compared to WT mice. The increase in MICEST was further compared to changes in mIns signal amplitude on ¹H MRS as well as GFAP immunohistochemistry results in both AD and WT

mice. Finally, the potential implication of the MICEST in detecting early AD pathology is discussed in detail.

Materials and Methods

Animal Preparation

Five APP-PS1 mice and 5 WT mice age spanning from 18 to 20 months were used in this study. Mice were transferred to a 9.4T horizontal bore small animal MR scanner (Varian, Palo Alto, CA) and placed in a 20-mm diameter commercial quadrature proton coil (m2m Imaging Corp., Cleveland, OH). Animals were kept under anesthesia (1.5% isoflurane in 1 liters/min oxygen) and their body temperature was maintained with the air generated and blowing through a heater (SA Instruments, Inc., Stony Brook, NY). Respiration and body temperature were continuously monitored using a MRI compatible small animal monitor system (SA Instruments, Inc., Stony Brook, NY). Experimental protocols were approved by the Institutional Animal Care and Use Committees.

Imaging Protocols

CEST imaging of the mouse brain was performed using a custom-programmed segmented radiofrequency spoiled gradient echo (GRE) readout pulse sequence with a frequency selective continuous wave saturation preparation pulse. The sequence parameters were: field of view =20×20 mm², slice thickness = 2 mm, flip angle=15°, GRE readout TR=6.2 ms (64 segments), TE =2.9 ms, matrix size=128×128, number of averages=1. For every 8s one saturation pulse and 64 acquisition segments were applied. CEST images were collected using a 5 second saturation pulse at peak B₁ of 75 Hz with varying frequencies from 0 – 2 ppm in step size of 0.1ppm. B₁ and B₀ maps were also acquired and used to correct the MICEST contrast as described previously (Haris et al., 2011). MT imaging was performed at 20 ppm using 250 Hz saturation power and 1s saturation duration. The total imaging time including B₀ and B₁ mapping was around 20 min.

Image Processing

All image processing and data analysis were performed using software written in MATLAB (version 7.5, R2007b). MICEST contrast was calculated at ±0.6ppm by normalizing with 20ppm signal using the Equation [1] (Haris et al., 2011):

$$MICEST = 100 * \left| \frac{S_{-ve} - S_{+ve}}{S_0} \right| \quad \text{Eq. [1]}$$

where S_{-ve}, S_{+ve} and S₀ are the B₀ corrected MR signals at -0.6ppm, +0.6ppm and 20ppm respectively. MICEST contrast was further corrected for B₁ inhomogeneity. z-spectra were generated by plotting CEST image intensity as a function of resonance offset of saturation pulse. For CEST asymmetry curves the relative water signal difference were plotted at the frequency offsets from 0 to 1.2ppm.

MTR maps were computed using equation [2]-

$$MTR = \frac{M_0 - M_{sat}}{M_0} \times 100\% \quad \text{Eq. [2]}$$

Where M₀ is the magnetization off and M_{sat} is the magnetization with a saturation pulse applied at 20 ppm.

Spectroscopy

Single voxel spectra (SVS) were performed with stimulated echo acquisition mode using a vendor (Varian) provided pulse sequence with the following parameters: voxel size=4mm×4mm×2mm (Voxel volume 32 μ L), spectral width=4 kHz, number of points=4006, averages=264, TE=8 ms, Tm=7 ms, and TR=5s. Water suppression was achieved using the variable pulse power and optimized relaxation delays method. Localized shimming was performed to obtain localized water line width values of 0.075ppm or less. SVS spectra were obtained from the raw free induction decay data by exponential apodization (20Hz), Fourier transformation, phase correction and baseline removal. Peak integrals of mIns peak and unsuppressed water peak were calculated using a non-linear least squares fitting (MATLAB “nlinfit” routine) program. Peak integrals were calculated and normalized with creatine peak integral.

Histopathology

All the excised mice brains were fixed in 10% buffered formalin and embedded in paraffin. The whole specimen was blocked and 6 μ m sections were taken for the immunohistochemistry analysis.

Immunohistochemistry

The slides were deparaffinized in Xylene (Surgipath, 03672) followed by rehydration with 100%, 95%, 70% ethanol. Antigen retrieval was performed in the digital decloaking chamber (Biocare medical, DC2002) by using citric acid based antigen unmasking solution (Vector laboratories, H-3300). The sections were blocked with 4% BSA (Sigma, A-7906) and 2% goat or horse serum in TT buffer (10mM of Tris-HCl, 500mM NaCl, and 0.05% Tween-20) at room temperature for 30 minutes. Sections were then incubated with primary antibody diluted in blocking buffer at 4 °C overnight. The primary antibody concentration for GFAP was 1:20. Following washing in Tris buffer, secondary antibody diluted at 1:200 (Vector laboratories, BA-1000 or BA-9200) in blocking buffer was added and incubated at room temperature for 45 minutes. After three washes, the sections were incubated with Vectastain ABC reagent (Vector laboratories, PK-6100) for 40 minutes at room temperature. The sections were again washed thrice in Tris buffer and incubated with the chromogendiaminobenzidine-tetrahydrochloride (DAB) ((Sigma, D4293) substrate followed by counterstaining in hematoxylin. The sections were mounted in DPX mountant. Parallel control slides were run with all batches including positive and negative controls. For evaluation of GFAP immunostain cells, each GFAP immunostained slide was digitized with a 1.25 $\times$ and 20 $\times$ objective using Olympus camera.

Results

Figure 1 illustrates the sensitivity advantage of MICEST at physiological concentration and temperature. It shows over two orders of magnitude higher sensitivity for MICEST over 1 H MRS of mIns.

The brain anatomical images, static magnetic field (B_0), radiofrequency field (B_1) maps, MICEST maps and MRS spectra from WT and APP-PS1 mice are shown in figures 2 and 3. Shimming of the main magnetic field provided a fairly uniform B_0 field (heterogeneity <0.2 ppm) across the brain image while B_1 map was quite homogeneous. To further validate the change in MICEST contrast is due to alter mIns concentration we performed 1 HMRS spectra for the voxel shown on the anatomical images (Figs. 2A & 3A). This voxel was placed in an area devoid of cerebral spinal fluid. 1 HMRS spectra clearly show elevated mIns peak signal in APP-PS1 compared to WT mice. Comparative analysis showed ~50% higher MICEST

($p < 0.05$) contrast and ~51% increase in mIns/Cr ($p < 0.05$) in APP-PS1 mice brain than in that of WT mice.

Z-spectra and z-spectra asymmetry curves are shown in figure 4. The z-spectra reflects the water signal intensity at difference frequency offset after application of saturation pulse. The z-spectra asymmetry curve typically reflects the relative water signal change at different frequency offset due to the exchange between saturated labile protons with water protons. The 0.6ppm line on the z-spectra asymmetry curves corresponds to MICEST contrast. The z-spectra asymmetry curves clearly depict the higher MICEST contrast in APP-PS1 mice brain compared to WT mice.

The bar graphs shown in figure 5 represent the percentage change in the mIns/Cr and MICEST in APP-PS1 compared to WT mice. The ~50% increased MICEST in APP-PS1 mice is comparable to the ~51% increase in mIns/Cr ratio.

The other factor that may significantly contribute to MICEST is magnetization transfer effect from bound water pool. To evaluate the bound water pool in APP-PS1 and WT mice, MT imaging was performed. No significant difference in MTR contrast was observed between APP-PS1 (48.3 ± 1.5) and WT mice (48.0 ± 1.1). However, any decrease in MTR contrast in APP-PS1 mouse will lead to increase in MICEST contrast.

Immunohistochemistry

To observe the glial cells proliferation, immunostaining against GFAP was performed both on APP-PS1 and WT mice brain slices and the results are shown in figure 7. Higher intensity of GFAP immunoreactivity was observed in APP-PS1 mice brain compared to WT which signifying glial cells proliferation/activation in APP-PS1 mice. The GFAP-positive cells were seen interlocking in to meshes in APP-PS1 mice. In the selected voxel region, where MICEST and MRS showed ~50% increase of mIns/Cr, a corresponding higher intensity of GFAP immunoreactivity in APP-PS1 mice was observed.

Discussion

In the current study, for the first time we have performed high resolution mapping of mIns in APP-PS1 transgenic mouse model of AD using MICEST technique. Higher MICEST contrast in APP-PS1 mice was observed compared to WT mice and the results were further validated through comparable change of mIns/Cr on ^1H MRS. Immunohistochemistry analysis showed elevated proliferation of glial cells in APP-PS1 than WT mice. The higher MICEST contrast in APP-PS1 mice correlates with higher glial cells proliferation and potentially can be used to monitor glial cells proliferation non-invasively.

In this work, we used APP-PS1 mouse model as this model matches most closely with the neurochemical and neurological profile in human AD (Marjanska et al., 2005). In an earlier MRS study, most profound increase in mIns in APP-PS1 mouse was observed at the age of 20 months (Marjanska et al., 2005). All mice used in the current study were also ~20 months old and higher mIns signals were observed in APP-PS1 mice brain. Compared to WT mice, reduced signals from NAA and glutamate were found in APP-PS1 mice, which is consistent with the previously reported studies, where a reduction in the levels of NAA and glutamate with advancing age was found in APP-PS1 mice (Chen et al., 2009; Marjanska et al., 2005). A gradually increased mIns during AD disease progression in APP-PS1 mice model starting from 3 months to 23 months has been reported using ^1H MRS (Marjanska et al., 2005). They attributed this increased mIns concentration to the active glial cells proliferation during these periods. Chen et al. have shown significantly increased mIns in 3 months old APP-PS1 transgenic mice along with higher GFAP expression compared to age matched WT mice

(Chen et al., 2009). The above discussion shows that it is indeed possible to track the glial cell proliferation in transgenic animal models of AD using ^1H MRS mIns. However, using ^1H MRS, mIns cannot be imaged at high resolution and thus brain regions with different amount of glial cells proliferation cannot be easily monitored.

It is worth comparing the potential advantage of detecting sensitivity of CEST compared to traditional MRS measurement based on aliphatic protons (-CH). The experimental data demonstrates, with experimental parameters used, the actual sensitivity of CEST realizable experimentally is ~ 700 times higher than ^1H MRS. This sensitivity amplification should make it feasible to detect relatively small changes in mIns levels at high resolution (voxel size of 0.02 cc).

The z-spectra showed the water signal amplitude at different frequency offset and has contribution both from direct water saturation and CEST effect. The direct water saturation can underestimate the MICEST contrast by decreasing the saturation transfer efficiency.

The z-spectra asymmetry curves are rather broad and showed MICEST peak at ~0.6ppm which is consistent with the previous study on human brain (Haris et al., 2011). MICEST map not only reflects the spatial distribution of mIns in brain, but also highlights the regions with active glial cells proliferation. Higher MICEST contrast in APP-PS1 mice reflects higher [mIns] and active glial cells proliferation which is supported by the ^1H MRS as well as GFAP immunohistochemistry analysis. However, direct correlation between MICEST and GFAP expression cannot be performed due to the difference in their spatial resolution as well as slice positions. Although in the current study, we examined the ~20 months old mice, based on the published MRS results on a range of ages of AD mice and potential higher sensitivity of MICEST, we expect that the MICEST can map glial cell changes in young (>3 month) AD mice as well. In the current study, we observed heterogeneous distribution of MICEST contrast in brain of APP-PS1 mice which might be due to the varied nature of AD related pathological changes. Using ^1H MRS the heterogeneity in AD pathology cannot be evaluated as it suffer from poor spatial resolution.

No apparent change in the MTR contrast has been observed in APP-PS1 compared to WT mice suggests that the CEST contrast at 0.6 ppm is due to the change in the mIns concentration. The other metabolites with functional -OH groups present in the brain are glucose and glycogen. Given the concentration of these metabolites in the brain and their peak CEST position i.e. 1 ppm and saturation parameters used in the current study we do not expect any appreciable contribution from these metabolites to MICEST.

Most patients with middle cognitive impairment (MCI) ultimately develop dementia, it is reasonable to regard neuroimaging findings that are sensitive to the changes in the brains of people with MCI as markers of early or “prodromal” AD pathologic changes. In human, gradual increase in mIns concentration has been shown from control to MCI to full onset of AD (Huang et al., 2001; Kantarci et al., 2003; Siger et al., 2009). It has been suggested that ^1H MRS quantified mIns can be used as a biomarker to detect the early changes in AD pathology at the stage of MCI and may serve as a tool to monitor the therapeutic response. However, due to large voxel size (8cm^3) it is difficult to monitor the regional changes in [mIns] as well as glial cells proliferation. However, *in vivo* mapping of the regional glial cells proliferation in AD patients was performed earlier by exploiting the PET ligands (Cagnin et al., 2001; Hammoud et al., 2005). The wide clinical utility of PET technique is hampered by the use of radioactive ligand and low spatial resolution. Mapping of mIns through MICEST technique will allow monitoring the regional changes in mIns concentration at high resolution (~200 micron) during the disease course and in response to

treatment. Also, the MICEST technique do not require any exogenous contrast administration and quite easy to setup in the routine clinical MR imaging.

It is worth of discussing some technical details of the MICEST method. Precise saturation of exchangeable protons requires perfect B_0 homogeneity. B_0 inhomogeneity could interfere with the selective saturation frequency and makes the two selective frequencies asymmetric to bulk water. In that case, the CEST effect would reflect differences between direct saturation and magnetization transfer effects. The CEST effect is also highly dependent on saturation B_1 amplitude (Ward and Balaban, 2000). With fixed saturation duration, a higher B_1 can induce a higher CEST effect (Winter et al., 2006), and B_1 homogeneity is a critical requirement for quantitative CEST imaging. Artifacts induced by B_0 and B_1 field inhomogeneity and the corresponding correction algorithms have been previously discussed in detail (Sun et al., 2007). In the current study, we used methods described previously to correct the B_1 and B_0 induced artifacts due to small B_0 and B_1 variations. For proof of principle purposes, in the current study only a single slice with a two dimensional imaging sequence was imaged. However, it would be relatively straightforward to implement a three dimensional acquisition of MICEST map.

The findings of this preliminary study suggest that the *in vivo* detection of altered regional mIns concentration in AD is feasible at high resolution using the MICEST technique, and provides a novel non-invasive contrast method to detect the glial cells proliferation. Further, MICEST mapping could serve as *in vivo* surrogate marker of therapeutic efficacy in trials of agents designed to reduce neurotoxicity mediated by the activated glial cells during early disease onset in AD. Because of its noninvasive and repeatable nature, MICEST mapping could aid drug discovery efforts in transgenic models of AD.

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- We image the changes in myo-inositol at high spatial resolution in mouse model of Alzheimer's disease.
- This method provides over two orders of magnitude higher sensitivity compared to ¹HMRS.
- Glial Cells proliferation/activation in Alzheimer's disease can be monitored in-vivo noninvasively.
- It has potential for investigating the changes in myo-inositol level at early stage of AD.
- It can be used as a new imaging biomarker in early diagnosis of AD at the stage of mild cognitive impairments.

Figure 1.

A. Image showing the single voxel localization from a phantom of 10 mM mIns (pH=7.0, 37°C) solution prepared in phosphate buffer saline. **B.** ¹H MRS PRESS water suppressed spectrum (TR=20s, TE=16ms, 4averages) from the single voxel shown in the image A. The mIns peak at ~3.56 ppm is used in quantifying mIns from brain. The measured integration of mIns peak at ~3.56 ppm is 146.16 units. **C.** Water ¹H resonance spectra obtained while saturating at ± 0.6 ppm as well as their difference spectrum. **D.** The difference spectrum of water resonance is the absolute difference of two spectra obtained while saturating at ± 0.6 ppm using peak B₁ of 75Hz and duration of 5s. The difference spectrum represents the +0.6 ppm point on the CEST asymmetry spectrum and its integration value is 106546.6 units.

Figure 2.

A. Anatomical image from 20 months old wild type (WT) mouse, **B.** MICEST map for the corresponding slice shows the distribution of mIns in the brain. The MICEST map was corrected for any B_0 and B_1 fields inhomogeneity (figure **C & D**). B_0 and B_{1ref} maps show the field inhomogeneity on the brain. B_0 map shows less than 0.2 ppm variation on the brain, while B_1 map is quite homogenous. **E.** ^1H MRS spectrum for the voxel as shown in **A**, demonstrate the metabolites present in the mouse brain.

(Lip = lipid, NAA = N-acetyl aspartate, Glu = glutamate, tCr = total creatine, Cho = choline, Tau = taurine, Mi = Myo-inositol,)

Figure 3.

A. Anatomical image from 20 months old APP-PS1 transgenic mouse model of AD, **B.** MICEST maps for the corresponding slice shows the distribution of mIns in the brain. Higher MICEST contrast was depicted in the brain compared to WT mouse. The MICEST map was also corrected for any B_0 and B_1 fields inhomogeneity (figures **C & D**). **E.** 1H MRS spectrum for the voxel placed in A shows increase in myo-inositol peak amplitude.

Figure 4.

Z-spectra and z-spectra asymmetry curve for the voxels shown in figures 2A and 3A. The dotted line at 0.6ppm shows the MICEST contrast. Z-spectra clearly depict higher MICEST contrast in APP-PS1 than WT mice.

Figure 5. The bar graphs show the percentage change in the mIns/Cr and MICEST in APP-PS1 compared to WT mice. The ~50% increased MICEST in APP-PS1 mice is comparable to the ~51% increase in mIns/Cr ratio.

Figure 6. Shows the GFAP immunostain brain slices from WT and APP-PS1 mouse. The high resolution (20×) images show higher expression of GFAP in APP-PS1 mouse than WT mouse. The astrocytes proliferation in APP-PS1 mouse is apparent in much larger area. The GFAP-positive cells are interlocking in to meshes in APP-PS1 mouse.