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Brain oxidative stress: Detection and mapping of anti-oxidant marker 'Glutathione' in different brain regions of healthy male/female, MCI and Alzheimer patients using non-invasive magnetic resonance spectroscopy

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ABSTRACT

Glutathione (GSH) serves as an important anti-oxidant in the brain by scavenging harmful reactive oxygen species that are generated during different molecular processes. The GSH level in the brain provides indirect information on oxidative stress of the brain. We report *in vivo* detection of GSH non-invasively from various brain regions (frontal cortex, parietal cortex, hippocampus and cerebellum) in bilateral hemispheres of healthy male and female subjects and from bi-lateral frontal cortices in patients with mild cognitive impairment (MCI) and Alzheimer's disease (AD). All AD patients who participated in this study were on medication with cholinesterase inhibitors. Healthy young male (age 26.4 ± 3.0) and healthy young female (age 23.6 ± 2.1) subjects have higher amount of GSH in the parietal cortical region and a specific GSH distribution pattern (parietal cortex > frontal cortex > hippocampus ~ cerebellum) has been found. Overall mean GSH content is higher in healthy young female compared to healthy young male subjects and GSH is distributed differently in two hemispheres among male and female subjects. In both young female and male subjects, statistically significant ($p = 0.02$ for young female and $p = 0.001$ for young male) difference in mean GSH content is found when compared between left frontal cortex (LFC) and right frontal cortex (RFC). In healthy young female subjects, we report statistically significant positive correlation of GSH content between RFC and LFC ($r = 0.641$, $p = 0.004$) as well as right parietal cortex (RPC) and left parietal cortex (LPC) ($r = 0.797$, $p = 0.000$) regions. In healthy young male subjects, statistically significant positive correlation of GSH content was observed between LFC and LPC ($r = 0.481$, $p = 0.032$) regions. This statistical analysis implicates that in case of a high GSH content in LPC of a young male, his LFC region would also contain high GSH and vice versa. The difference in mean of GSH content between healthy young female control and female AD patients in RFC region ($p = 0.003$) and difference in mean of GSH content between healthy young male control and male AD patients ($p = 0.05$) in LFC region is found to be statistically significant. It is the first scientific report correlating alteration (in selective brain regions) of GSH level with clinical status of male and female subjects using non-invasive imaging technique.

1. Introduction

Glutathione (γ-L-glutamyl-L-cysteinylglycine; GSH) is a major antioxidant in the human body particularly in major organs like brain, liver and kidney. GSH is a highly efficient scavenger for reactive oxygen species (ROS) like superoxides, hydroxyl radicals ($HO^{\cdot}$) and oxidant peroxynitrites ($ONOO^{-}$) [1]. Importantly, GSH is the only compound capable of scavenging hydroxyl radicals as there is no known enzymatic defense system against these radicals [2]. In addition, GSH acts as a storage form or carrier for cysteine, as higher concentrations of free cysteine are potentially neurotoxic [3]. Apart from these intracellular effects, GSH also functions as a neuromodulator/neurotransmitter mediating neuronal response via NMDA (N-Methyl-D-Aspartate) receptors [3] and plays a role in the regulation of apoptosis, cell proliferation and neuronal differentiation [4]. Convincingly, GSH is an important molecule, with multi-tasking performance capabilities in the brain. In this context, the measurement of GSH levels in healthy and neurodegenerative conditions (MCI and AD in this study), is a pursuit of neuroscientific importance.

GSH is synthesized de novo in the brain and the supply of GSH from outside the brain is restricted for various reasons, primarily

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due to difficulty of the molecule crossing the blood–brain-barrier [5,6]. Orally administered GSH supplement is hydrolyzed by dipeptidase enzyme in the gastrointestinal tract [1] and intravenously administered GSH is also rapidly eliminated, with a half-life span of 7 min due to the interaction with γ-glutamyltransferase [7]. Therefore, the intracellular content of GSH is regulated only by the synthesis of GSH within the neurons and by the regeneration of GSH via reduction of oxidized glutathione (GSSG).

Oxidative stress plays an important role in different neurodegenerative disorders, in particular to AD. Recently, analysis on postmortem brain (frontal cortex of MCI ($n = 8$), AD ($n = 4$), and late-stage AD ($n = 9$)) implicated an involvement of oxidative stress in AD related synaptic loss [8]. This being the case, detection of GSH in healthy and diseased conditions should provide critical information pertaining to "overall neuronal health".

Magnetic resonance spectroscopy (MRS) is a state-of-the-art non-invasive technique for the detection of neurochemicals [9–11]. The smaller sized anti-oxidant, GSH, can be detected by *in vivo* proton MRS studies. Due to the overlap of ^{1}H resonance peaks of GSH with other brain metabolites, unambiguous detection of GSH cannot be accomplished by PRESS [12] or STEAM [13] pulse sequences. Hence, MEGA-PRESS [14] pulse sequence was developed by incorporating additional "selective 180° editing pulses" in the original PRESS pulse sequence to detect GSH. The detection of GSH in singular brain region using MEGA-PRESS pulse has been applied by various researcher groups [14–19]; however, the distribution of GSH in various brain regions of healthy young male and female as well as *in vivo* detection of GSH in AD or MCI patients has never been investigated. This motivated us to be on a path forward in AD research and other neurodegenerative disorders, using a non-invasive and state-of-the-art imaging technique which provides GSH level quantitatively in the specific brain region.

The objectives of this study are: (1) non-invasive detection and quantitation of GSH level from four brain regions (bilateral frontal cortices, parietal cortices, hippocampus and cerebellum) and to investigate any variation in the GSH content between the two hemispheres; (2) to investigate variations of GSH content between healthy young male and female subjects; (3) to assess GSH levels in healthy young (male and female) subjects and in patients (MCI and AD).

2. Materials and methods

2.1. Participants of this human study

MRS data from total eighty-five participants [healthy young male, $n = 25$; healthy young female, $n = 20$; healthy older male $n = 9$; healthy older female, $n = 6$; MCI male, $n = 5$; MCI female, $n = 6$; AD male, $n = 7$, AD female, $n = 7$] were taken in this study. The experimental protocol for *in vivo* GSH detection studies was approved by the institutional review board (NBRC) and all patients were recruited from the Department of Neurology, All India Institute of Medical Sciences (AIIMS), New Delhi. MCI and AD patients were diagnosed as per guidelines and through clinical evaluation [20,21] by a team of clinicians at Department of Neurology, AIIMS, New Delhi. Recruitment of healthy male and female subjects was performed at the National Brain Research Center. Inclusion criteria for control subjects were no report of any neurological disorders. For MCI subjects age was 50Y and above and for AD subjects age was 55Y and above. Progressive cognitive decline of the AD patients was attributed only due to AD, and they did not present any other comorbid conditions (e.g., cerebrovascular disease, PD). Subjects and patients with metallic device, MRI incompatibility and claustrophobia were excluded. Patients who participated in this study were on medication with cholinesterase inhibitors and were not taking any psychoactive medication. All control subjects and patients and/or family member signed informed consent forms before the imaging study.

2.2. Experimental design

All subjects ($N = 85$) underwent the same experimental protocol. Briefly, MRI images (coronal, sagittal, and axial) were initially obtained by placing the voxel in the preferred brain regions for spectroscopic study. For the detection of GSH using MEGA-PRESS pulse sequence [14], the α-H of cysteine amino acid residue of GSH was inverted using selective 180° (on-resonance) pulse in the first set of experiments. In the second set of experiments, the selective 180° was not applied on α-H of cysteine amino acid resonance position. These two sets of experiments were executed in an interleaved fashion. The subtraction of the two spectra (from the two sets of experiments) yielded β-CH_2 of cysteine appearing at ~2.90 ppm (parts per million) as a characteristic of the GSH molecule. The characteristic β-CH_2 of cysteine (~2.90 ppm) is clearly detectable; thereby enabling an unambiguous assignment of GSH molecule from the brain region under investigation.

All *in vivo* data were acquired using a Philips Achieva 3T MRI scanner equipped with state-of-the-art dual tuned transmit-receive head coil (Rapid Corporation, Germany) and same experimental parameters (TR = 2500 ms, TE = 120 ms, SW = 2500 Hz, number of data points, 2048, voxel size $2.5 \times 2.5 \times 2.5$ cm^3 = 15.6 cc) were used for phantom as well as for all human subjects. The refocusing 180° pulse was set at 4.40 ppm and water suppression was accomplished with Chemical Shift Selective Suppression (CHESS) pulse sequence [22]. Total experimental time using MEGA-PRESS for each voxel was 13 min 20 s. We also performed MEGA-PRESS experiments by setting the selective refocusing 180° pulse for α-H of cysteine at different resonance positions (i.e. 4.40, 4.45, 4.50 and 4.56 ppm respectively). All MRS data were apodized, processed using Philips software package and further analyzed for quantitation of GSH content. Statistical analysis was carried out using SPSS (version 14) software package.

3. Results

In order to confirm the spectral pattern of GSH in human brain using PRESS pulse sequence, first the MEGA-PRESS pulse sequence was used on a phantom containing pure GSH (Sigma–Aldrich) solution prepared in a physiologic pH phosphate buffer condition. Same pulse sequence and experimental parameters (TR = 2.5 s, TE = 120 ms, NSA = 16) including voxel size ($2.5 \times 2.5 \times 2.5$ cm^3) were used to generate data for human studies as well as in phantoms. Fig. 1 shows side-by-side presentation of GSH spectra from the phantom containing pure GSH in a physiologic phosphate buffer medium as well as from the parietal cortical region of a young healthy female. The spectral patterns from the two cases (Fig. 1) are the same and confirmed the identity (β-CH_2) of cysteine residue of GSH in the human brain. Brain MRS spectra generated using MEGA-PRESS sequence contained two resonance peaks (Fig. 1), one corresponding to β-CH_2 of cysteine from GSH and an additional peak corresponding to the NAA (N-acetyl aspartate), in conformity with previous report [16].

To demonstrate the importance of setting the selective refocusing 180° pulse on α-H of cysteine, we have performed four MEGA-PRESS experiments on the parietal region of the healthy male subject, where the selective 180° pulse on α-H of cysteine was set at four different frequency regions (i.e. 4.40, 4.45, 4.50 and 4.56 ppm) and all other experimental parameters remained the same (Fig. 2). It is important to note that when the selective 180° pulse on α-H of cysteine is set at 4.40 ppm, best quality GSH

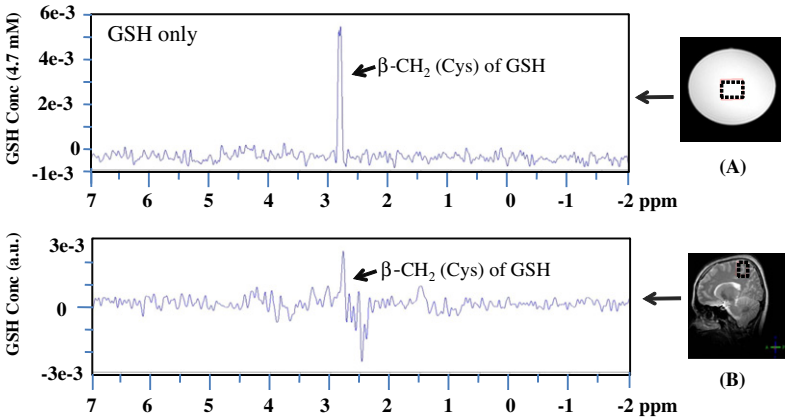

Fig. 1. Detection of GSH from a single voxel ($2.5 \times 2.5 \times 2.5$ cm^3) from a phantom as well as from parietal cortex of a young normal female brain using MEGA-PRESS pulse sequence at 3T scanner. All experiments to detect GSH were performed in same experimental conditions and voxel size. The MRS data were then processed using same protocol. The amplitude of original GSH, which is proportional to the GSH concentration, was calculated.

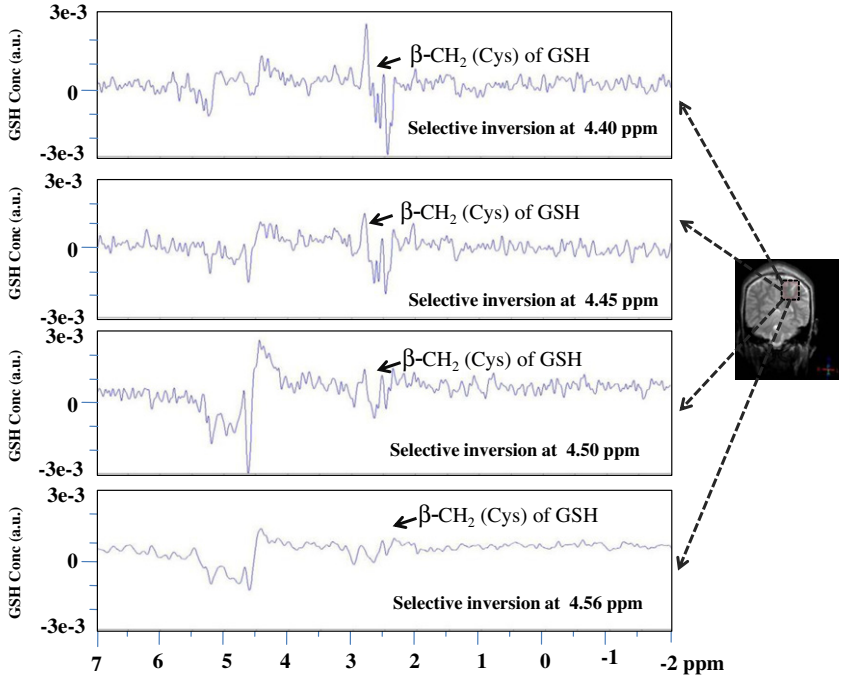

Fig. 2. Detection of GSH at various excitation frequency position (4.40, 4.45, 4.50 and 4.56 ppm) using MEGA-PRESS sequence on a young male while keeping other experimental parameters the same. It is clearly evident that when the selective 180° excitation pulse is set at 4.40 ppm, the best quality GSH spectra are obtained.

spectra from β-CH_2 peaks were obtained. This demonstrates that the setting of selective 180° pulse on α-H of cysteine is crucial.

Quantitative presentation of mean GSH amount from various brain regions (right frontal cortex; left frontal cortex; right parietal cortex; left parietal cortex; right hippocampus; left hippocampus; right cerebellum and left cerebellum) from each hemisphere of healthy male and healthy female subjects are presented in Fig. 3A. The mean of GSH content is higher in healthy female subjects compared to healthy male subjects. In seven regions out of eight (exception being left hippocampus), the mean value, indicated by a bar, is higher in female than in male subjects. However, we could find statistically significant difference in mean in only three regions namely; LFC ($p = 0.006$), RPC ($p = 0.01$) and LPC ($p = 0.04$). MRS data indicate that the parietal cortex region (in both healthy male and female) has more GSH content than the frontal cortex, hippocampus and cerebellum (figure not shown). The GSH content is more disperse in young male subjects. On the contrary, in healthy young females, the GSH levels were not dispersed. It is important to note that six healthy male subjects among the 25 subjects have no detectable GSH level (subject-1, LFC, RPC, LPC, RC, subject-2: LFC, RPC; subject-3: LFC; subject-4: LFC; subject-5: LFC and subject6: RPC). Comparatively, all healthy young female subjects ($n = 20$) had higher GSH levels in LFC, RPC and LPC regions. Comparative analysis of GSH content in two regions (RFC and LFC) in healthy young (female and male) and patients (male and female) is presented in Fig. 3B. We have found statistically significant depletion of GSH levels in RFC region of AD female patients and in LFC region of AD male patients. In case of MCI patients, GSH level in both RFC and LFC regions is depleted as compared to healthy young (male and female) subjects but it did not reach statistical significance.

Fig. 4 represents GSH level in the RFC region of female subjects in four cases (healthy young, healthy aged, MCI and AD). We have shown that GSH level is depleted in the aged normal female

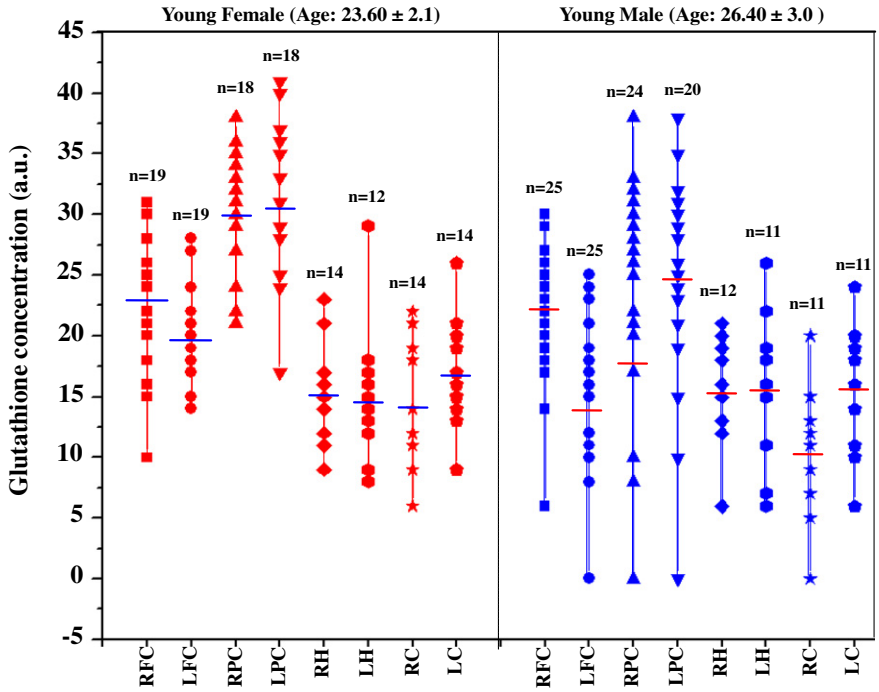

Fig. 3A. Quantitative representation of GSH concentration (in a.u) in healthy young male and female subjects in right frontal cortex (RFC), left frontal cortex (LFC), right parietal cortex (RPC), left parietal cortex (LPC), right hippocampus (RH), left hippocampus (LH), right cerebellum (RC) , left cerebellum (LC) brain regions. Sample size for each region is indicated as *n*. In some cases, multiple subjects had same GSH concentration leading to less number of points in graph than as indicated by the sample size. GSH was not detectable in six young males in four brain regions. Mean of the GSH concentration for each region is indicated as a bar. The mean of GSH content has been found to have higher values for female subjects compared to male in measured seven brain regions. Mean GSH content is higher for male in left hippocampus.

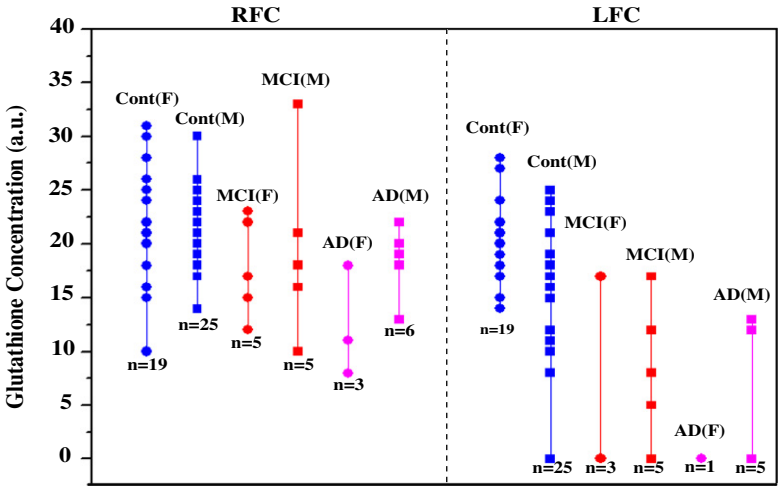

Fig. 3B. Quantitative representation of GSH concentration (in a.u) in RFC and LFC regions of healthy young male, healthy young female, MCI (male and female) and AD male and female patients. M and F symbols refer to male and female subject while sample size is indicated as *n*. It is clearly evident that GSH level is depleted in male and female AD patients as compared to healthy male and female subjects. Depletion of GSH level was statistically significant in RFC region among AD female patients compared to healthy female subjects and LFC region in male AD patients when compared to healthy male subjects. Although amount of GSH was found to be less in MCI cases than in healthy subjects, it did not reach significant value.

compared to young female subject due to aging. Interestingly, the GSH level is much depleted in MCI subject compared to the aged normal female. In turn, in the case of AD female subject, the GSH level is much lower than in the MCI subject. This trend can also be noted in Fig. 3B. In all the four cases, experimental conditions were the same for collecting MRS data. The lowered GSH level in the aged subjects, with further lowering of GSH levels in the patients with MCI and AD would suggest depletion of GSH with aging and in the patient with MCI and AD possibly due to neuronal degeneration.

4. Discussion

Oxidative stress may be a common mechanism underlying various forms of cell death including necrosis, apoptosis, and toxicity [2]. The deficiency of anti-oxidant marker GSH also leads to mitochondrial damage in brain [6]. Oxidative stress is involved in different brain disorders, and detection of GSH and correlating it with clinical status is a major thrust area in different laboratories. GSH depletion has been implicated in oxidative stress, resulting in neuronal degeneration. Involvement of oxidative stress in relation

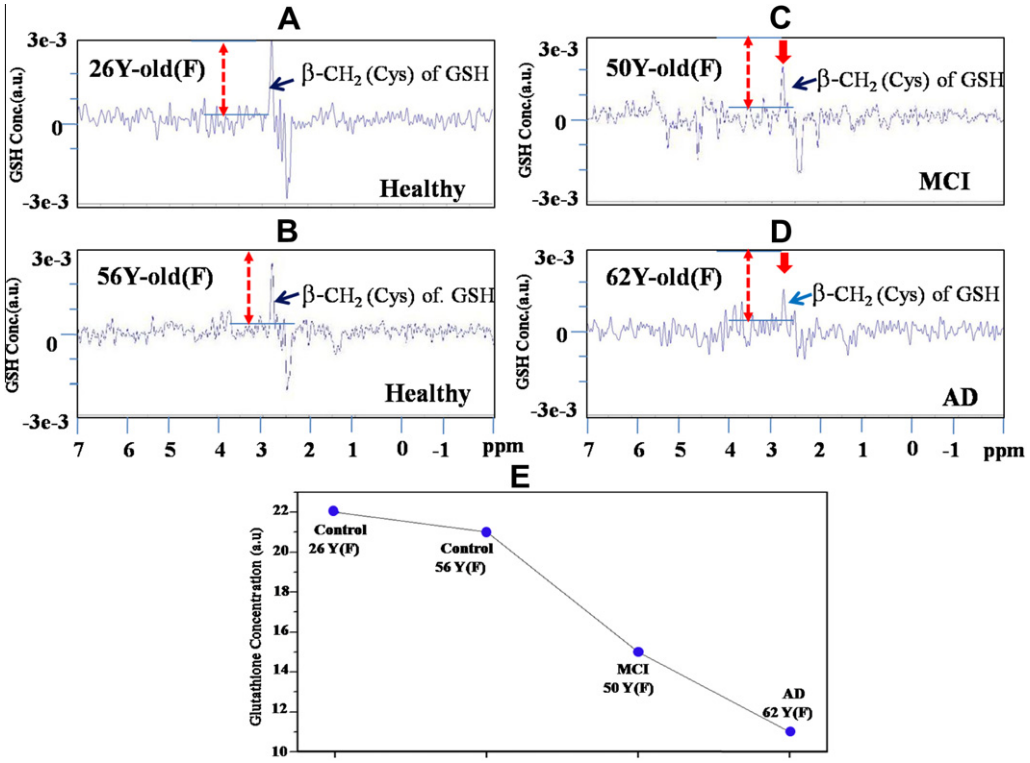

Fig. 4. GSH level in right frontal cortex in various cases: (A) healthy female (26Y old); (B) healthy female (56Y old); (C) MCI female (50Y old) and (D) probable AD female patient (62Y old) using MEGA-PRESS pulse sequence in a 3T MRI scanner. The decrease of GSH content is indicated by red arrow. (E) Quantitative presentation of GSH concentration in four different cases (A–D) as mentioned above. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

to impaired metabolism has been reported in schizophrenia patients [16,23,24]. Oxidative stress is considered a major pathogenic factor in AD and Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) and other neurodegenerative conditions. Varying levels of GSH have been reported in autopsy brains of AD and PD patients [25]. Hence, *in vivo* detection of GSH content in various brain regions of neurodegenerative patients will have a profound clinical significance.

In this context, it is critical to know GSH distribution level in normal young male and female population and this will serve as baseline for other studies. Fig. 3A indicates the quantitative distribution of GSH in healthy young female and male subjects. Our *in vivo* study clearly shows that there is a different distribution of GSH in brain regions in the two hemispheres and distribution of GSH is much higher in the parietal cortex region compared to other brain regions. The quantitative distribution of GSH level follows the same pattern in various regions (parietal cortex > frontal cortex > hippocampus ~ cerebellum) in healthy young male and female subjects.

4.1. Statistical analysis of this study

The statistical analysis was performed using SPSS (version 14) software. For statistical analysis, GSH content from various subjects (healthy young female (age 23.6 ± 2.1), and healthy young male (age 26.4 ± 3.0) and patients (male and female) were considered for analysis. Both in healthy young male and female subjects, differences in mean GSH content in LFC and RFC are statistically significant ($p = 0.02$ in young females, $p = 0.001$ in young males) as determined by paired *t*-test. In healthy young female subjects, we report statistically significant positive correlation of GSH content between regions RFC and LFC ($r = 0.641$, $p = 0.004$) and between RPC and LPC ($r = 0.794$, $p = 0.0001$) regions. In healthy young male subjects, statistically significant positive correlation of GSH content was observed between LFC and LPC ($r = 0.481$, $p = 0.032$). The difference in mean GSH content between healthy young female control and female AD patients in RFC region ($p = 0.003$) and the difference in mean GSH content in young male and male AD patients in LFC region ($p = 0.05$) are found to be statistically significant.

Leven's test for equality of variance shows significant difference between males and females for LFC ($p = 0.008$), RPC ($p = 0.01$) clearly indicating the dispersion of GSH levels in healthy male subjects.

The mean GSH content in healthy young female is high compared to healthy young male. Significant difference in mean GSH are found in three brain regions (LFC ($p = 0.006$), RPC ($p = 0.01$) and LPC ($p = 0.04$)) between young female and young male subjects. It is clearly evident that GSH level is depleted in male and female AD patients as compared to healthy male and female subjects (Fig. 3B). Depletion of GSH level was statistically significant in RFC region among AD female patients compared to healthy female subjects ($p = 0.003$) and LFC region in male AD patients when compared to healthy male subjects ($p = 0.05$). Although amount of GSH was found to be decreased in MCI subjects compared to healthy subjects, but did not reach statistically significant value.

4.2. Relevance of this study

Oxidative stress is an important factor in aging as well as in neurodegenerative disorders. It has been shown from the blood samples of AD patients (male and female), that the GSH concentration was decreased in red blood cells of AD patients when compared with age- and gender-matched controls [26]. Identification

of any neurochemical which shows alterations with healthy aging and neurodegeneration is of immense importance. In this respect, GSH can serve as an important molecule which needs to be monitored in the disease progression. Fig. 4 indicates a comparative analysis of GSH levels in the same experimental condition from female subjects of various ages as well as from MCI and AD female subjects. It is very important to note that there is a gradual decrease of GSH levels from healthy 26 Y old female to healthy 56 Y old female. The GSH level is also reduced in MCI patient compared to aged female control subject (56Y old). The GSH level is further depleted significantly in female AD patients. The gradual depletion of GSH level is presented quantitatively in AD and MCI patients (both male and female) compared to control subjects (male and female).

This is the first *in vivo* study for quantitative analysis of GSH level distribution in young male and female subjects in normal healthy condition as well as MCI and AD subjects in various brain regions. Gender specific difference of GSH is clearly evident and some of these differences are statistically significant. Maximum amount of GSH is found to be in the parietal cortex region compared to other brain region in both healthy male and female subjects. This novel study will add new valued information in AD research and similar studies are extended to other neurodegenerative disorders.

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