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Serum Zinc in the Progression of Alzheimer's Disease

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Abstract

Previous studies show significantly decreased levels of zinc transporter 1 (ZnT-1) in the brain of subjects with mild cognitive impairment (MCI) but significantly increased ZnT-1 in late stage AD (LAD). However, the reason for the apparent dichotomy is unclear. Based on *in vivo* studies that show animals provided a zinc (Zn) deficient diet demonstrate decreased brain ZnT-1, we used inductively coupled plasma-mass spectrometry (ICP-MS) to quantify serum Zn levels from 18 living mild to moderate AD patients (9 men, 9 women), 19 MCI patients (9 men, 10 women) and 16 age-matched normal control (NC) subjects (9 men, 7 women). Zinc levels for all subjects were not significantly different between any of the three subject groups. However, there was a statistically significant decrease of serum Zn (11.7 ± 0.5 μM) in men with MCI compared to women with MCI (13.7 ± 0.6 μM) and NC men (13.9 ± 0.6 μM). Serum Zn levels in probable AD patients were comparable to those in NC subjects. Overall, these data suggest a significant decrease of serum Zn in men with MCI may explain the loss of ZnT-1 observed in previous studies and suggest there may be more pronounced sex differences in MCI than were previously recognized.

Keywords

Alzheimer's disease; zinc; mild cognitive impairment; zinc transporters; serum

1. Introduction

Alzheimer's disease (AD) is the fourth leading cause of death in the United States and, in 2000, affected 4.5 million Americans [34]. Estimates indicate that ~3% of Americans between ages 65 and 74, 19% ages 75 – 84, and 47% over age 85 are victims of the disease [25] and that ~ 60% of nursing home patients over age 65 suffer from AD. Clinically AD is characterized by a progressive decline in multiple cognitive functions and is thought to begin with mild cognitive impairment (MCI), widely considered to be a transition between normal aging and dementia. Current data suggest that conversion from MCI to dementia occurs at a rate of 10 to 15% per year [57] with ~80% conversion by the sixth year of followup; although ~5% of MCI subjects remain stable or convert to normal [5,19].

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Over the past 25 to 30 years the potential role of trace elements, particularly iron (Fe), copper (Cu), and zinc (Zn) in the pathogenesis of AD has been the focus of a variety of studies [62]. Multiple lines of evidence show alterations of redox active Fe and Cu in vulnerable regions of the late stage AD (LAD) brain and suggest a potential role for these metals in the increased oxidative stress associated with LAD (reviewed in [62]). Additionally, Zn, a redox inactive, essential trace metal is elevated in vulnerable regions of the LAD brain. Zn serves structural, catalytic and regulatory roles [6,30,70] and functions as a crucial component in over 300 enzymes and transcription factors where it serves as an essential cofactor for catalytic activity [27] or by conferring structural stability to Zn finger domains of DNA binding proteins [12] including stimulating protein-1 (sp-1), a transcription factor responsible for ~30% of amyloid precursor protein transcription [7,15,16].

In the brain, Zn concentrations are highest in the hippocampus, amygdala, and neocortex and are relatively low in cerebellum [18,28,38], a pattern that mirrors the distribution of pathologic features in AD. Overall, brain Zn concentrations are ~10X serum Zn levels [67] and range between 150 and 200 μM [22,58]. Transport of Zn into the brain occurs via the blood/brain and blood/cerebrospinal fluid barriers [68], where brain capillary endothelial cells respond to changes in systemic Zn status by increasing or decreasing Zn uptake [41].

Maintenance of Zn balance in brain is maintained by 3 families of proteins including metallothioneins (MTs), Zrt-Irt like (ZIP) proteins, and Zn transporter (ZnT) proteins. Despite the critical functions of these proteins, there has been relatively little study of ZnTs in the progression of AD. Our previous studies of ZnT proteins show alterations of ZnT-1, ZnT-4, and ZnT-6 in the progression of AD [44,64]. Of particular interest is our observation of a significant decrease of ZnT-1, the protein responsible for maintaining low intracellular Zn in the hippocampus/parahippocampal gyrus (HPG) of subjects with MCI that rebounds to a significant increase in LAD HPG [43]. The mechanism for ZnT-1 to maintain low intracellular Zn is not well understood. Palmiter [51,53] proposed that ZnT-1 transports zinc out of the cell to protect Zn toxicity. Recent studies [49,50,61] demonstrated that ZnT-1 reduces Zn influx through the L-type calcium channels (LTCC) without increasing Zn efflux.

To determine if alterations of serum Zn occur in the progression of AD and contribute to alterations of ZnT-1, we analyzed serum Zn concentrations in thoroughly evaluated, well-characterized, living MCI, and probable AD patients and age-matched normal control (NC) subjects using ICP-MS.

2. Materials and Methods

2.1 Subjects

Serum samples were collected from 18 mild to moderate probable AD patients (9 men, 9 women), 19 MCI patients (9 men, 10 women) and 16 age-matched normal control subjects (9 men, 7 women) through the University of Kentucky Alzheimer's Disease Center (UK-ADC) using University of Kentucky Institutional Review Board approved protocols. All AD patients were followed longitudinally in the Clinical Core of the UK-ADC and had annual mental status testing, and physical and neurological examinations. Probable AD patients demonstrated progressive intellectual decline and met NINCDS-ADRDA Workgroup criteria for the clinical diagnosis of probable AD [45]. Control subjects were from a population of 446 normal participants followed longitudinally in the healthy brain aging clinic of the UK-ADC. Control subjects had neuropsychologic testing, and physical, and neurological examinations annually. All control subjects had neuropsychologic scores in the normal range and showed no evidence of memory decline. Patients with MCI were derived from the control group and were followed longitudinally in the UK-ADC clinic. MCI patients were normal on enrollment and developed MCI during follow-up. The clinical criteria for diagnosis of amnestic MCI are those of Petersen

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et al. [56] and include: a) memory complaints, b) objective memory impairment for age and education, c) intact general cognitive function, d) intact activities of daily living (ADLs), and e) the subject is not demented. Objective memory test impairment was based on a score of ≤ 1.5 standard deviations from the mean of controls on the CERAD Word List Learning Task [48] and corroborated in some cases with the Free and Cued Selective Reminding Test [9].

2.2 Serum sample collection and processing

Blood samples were collected into vacutainer tubes and centrifuged to isolate serum. Serum samples were aliquoted into 2 mL samples and stored at -80° C until used for analysis. For blank corrections, identical vacutainer tubes were filled with an equal volume of distilled/deionized water. Buffy coat specimens isolated during centrifugation were collected for APOE-4 genotyping and were stored at -80 ° C until used for analysis.

For analysis, serum samples were thawed to room temperature in a clean air hood for approximately 2 hours. The samples were vortexed 10 seconds and 100 μL serum transferred to pre-weighed and pre-cleaned trace-metal-free polypropylene tubes (Stockwell, Scottsdale, AZ). The tubes were weighed and centrifuged for 2 minutes and 300 μL of OPTIMA HNO_3 and 100 μL of Fluka Trace SELECT grade H_2O_2 (Sigma-Aldrich, St. Louis, MO) were added to each sample. The sample tubes were tightly capped and placed in an ultrasonic water bath at 60°C for one hour. The capped tubes dried overnight in a clean air hood. The samples were then gravimetrically diluted with 10 mL 18 MΩ-cm deionized water and 100 μL 50 μg/g cadmium (Cd) added as an internal standard. The tubes were mixed by inversion and centrifuged 10 minutes immediately before ICP-MS analysis.

2.3 ICP Analysis

High resolution inductively coupled plasma mass spectrometry (HR-ICP-MS) analysis was performed at the Research Reactor of the University of Missouri-Columbia using a VG Axiom ICP-MS operated with an radio frequency power of 1400W and an argon flow rate of 14 L/min. The instrument resolution was set at 6000 for ^{66}Zn and ^{111}Cd with a dwell time of 10 ms. Data were quantified using a 7-point (0, 2, 5, 10, 20, 50, 100 ng/g) calibration curve. To determine Zn recovery, 150 μL of a 1 μg/g Zn single element standard solution was used to spike duplicate samples. Zero point standard (0 ng/g Zn) and quality control Zn standard (10 ng/g Zn) samples were analyzed every 8 samples during the analyses to measure background and to monitor instrument response. To prevent protein deposition and minimize nebulizer clogging, the sample probe was flushed with concentrated HNO_3 for 15 seconds followed by 45 seconds normal washing after each sample.

2.4 Statistical analysis

Subject demographic data were compared using analysis of variance (ANOVA) and the commercially available ABSTAT software (AndersonBell, Avada, CO). A 1-sample Kolmogorov-Smirnov test demonstrated serum Zn concentrations were normally distributed and Levene's Test for Equality of Variances demonstrated that the populations had equal variances. Therefore, a general linear model, Univariate: Two Facto ANOVA, was used for comparison.

3. Results

3.1 Method validation

The calibration standard solutions yielded a linear response for Zn over the concentration range of 0 ~ 100 ng/g ($r^2 \geq 0.999$). Zn concentrations in the blank solutions were less than 0.4 ng/g. Zn concentrations in the sample tubes ranged from 1.86 to 22.04 ng/g with an average of 8.38

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ng/g and a standard deviation of 2.55 ng/g. When blank subtraction was performed, the blank signal was less than 10% of the total sample signal in all cases except one. The relative standard deviation of the measured Zn concentrations was less than 5% in 6 measurements of a 10 ng/g Zn standard solution over the course of a day. The detection limit of the instrument was 0.2 ng/g and was calculated as 3 times the standard deviation of the analytical signal from the blank solution. The amounts of the spiked Zn were approximately 200% of the Zn in the spiked samples and the recovery ranged from 89% to 109%. The concentrations of Zn in the original human serum samples ranged from 0.58 to 1.20 μg/g after recovery adjustment. These numbers were then converted to molar concentrations (μM) assuming a sample density of 1 g/mL.

3.1 Comparison of all subjects

Comparison of data for all subjects combined showed no significant differences in age or the number of APOE-4 alleles between the three groups (Table 1A&B). Mini-Mental State Examination (MMSE) and Clinical Dementia Rating scale (CDR) scores showed a disease progression related change with significantly higher CDR scores in MCI (0.5 ± 0.03) and probable AD patients (1.4 ± 0.2) compared to NC subjects (0 ± 0). MMSE scores showed a significant disease progression related decrease with significantly lower scores in AD patients (18.0 ± 2.1) compared to NC subjects (28.8 ± 0.4). Patients with MCI also showed lower MMSE scores (27.7 ± 0.4), although the difference was not statistically significant. Serum Zn concentrations for all subjects showed no significant differences between the 3 groups. Correlation analyses showed a positive relationship between Zn concentrations and the number of APOE-4 alleles for control subjects but a negative relationship for MCI and AD patients although the correlation coefficients were not statistically significant.

3.2 Comparison of data segregated by sex

Demographic data for the subject populations separated by sex are shown in Table 2. There were no significant differences in age or the number of APOE-4 alleles for men compared to women for any of the 3 subject groups. Similarly, there were no differences in age or the number of APOE-4 alleles for men or women between the subject groups.

CDR scores showed a significant disease progression-related increase for both men and women with significant elevations for men (0.4 ± 0.06) and women (0.5 ± 0.0) with MCI and men (1.4 ± 0.35) and women (1.35 ± 0.2) with AD compared to NC men (0.0 ± 0.0) and NC women (0 ± 0). MMSE scores for women showed a significant decrease for MCI patients (26.7 ± 0.5) and mild to moderate AD patients (18.5 ± 3.1) compared to NC subjects (29.6 ± 0.2). MMSE scores in men with MCI (28.9 ± 0.4) were not significantly different than NC men (28.2 ± 0.6). Mild to moderate AD patients showed a significant decrease in MMSE (17.2 ± 2.7) compared to MCI and NC subjects. MMSE scores between sexes showed a significant decrease in women with MCI compared to men with MCI. Serum Zn concentrations by sex showed a statistically significant decrease ($p < 0.05$) of Zn in men with MCI (11.7 ± 0.5 μM) compared to NC men (13.9 ± 0.6 μM) that rebounded to NC levels in subjects with AD (12.7 ± 0.4 μM). In contrast, serum Zn levels between the 3 groups for women were not significantly different. Serum Zn was significantly lower ($p < 0.05$) in men with MCI (11.7 ± 0.5 μM) compared to women with MCI (13.7 ± 0.6 μM). Correlation coefficients between serum Zn concentrations and MMSE or CDR scores were not significant. Correlation analyses showed serum Zn concentrations were positively related to the number of APOE-4 alleles in control men but negatively related to the number of APOE-4 alleles in men with MCI and AD and all women including controls. However, the correlation coefficients were not statistically significant.

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4. Discussion

This is the first study of serum Zn concentrations in well characterized subjects in the progression of AD from MCI to mid stage disease. Our data show no significant differences in Zn when all subjects were combined. However, when the subjects were analyzed by sex, we observed a statistically significant decrease in Zn concentration in men with MCI compared to women with MCI and men who are neurologically normal. In addition, Zn concentrations in men increased to control levels in probable AD subjects. The reason for decreased serum Zn in men with MCI is unclear. However, our previous study of ZnT-1 in the brain of a small number of MCI subjects suggested a more pronounced decrease of ZnT-1 in men compared to women with MCI supporting a relationship between extraparenchymal Zn and ZnT-1 levels in brain [43]. Our current data are consistent with previous studies [32,47,63] that showed no significant differences between control and AD serum Zn levels but are in contrast to our previous study of postmortem serum from a small number of LAD and control subjects [59] when subjects of both sexes were combined.

There has been considerable interest in the potential role of Zn in the pathogenesis of AD since 1981 when Burnet [8] first proposed Zn deficiencies led to dementia. Since that time multiple studies of Zn in LAD and control brain have been contradictory. Initial studies showed significantly decreased Zn in the hippocampus, inferior parietal lobule, and occipital cortex of LAD subjects [1,2,14,21], although later studies using short postmortem interval tissue specimens from well characterized LAD and control subjects showed significant elevations of Zn in LAD hippocampus, amygdala, and multiple neocortical areas [13,18,20,23,60,71]. The differences observed between the studies may lie in the fact that formalin-fixed tissues were used in some of the earlier studies that also included control subjects that were not prospectively evaluated. Although multiple studies show alterations of Zn at the bulk level, the cellular localization of Zn alterations in AD is unclear. Studies of Zn distribution in AD have primarily focused on the association of Zn with senile plaques. Using micro-particle induced x-ray emission (micro-PIXE) we initially showed increased Zn in senile plaques compared to adjacent neuropil and an elevation of Zn in LAD neuropil compared to age-matched control subjects [42]. Several subsequent studies have confirmed those findings in AD [10,46,65,66] and in amyloid plaques in Tg2576 transgenic mice expressing mutant APP [29,40].

The reasons for elevated Zn in the brain in AD are unclear. Although several studies have attempted to relate changes in peripheral Zn to elevated brain levels, these studies have been contradictory. Haines et al. [32], Molina et al. [47], and Shore et al. [63] showed no significant differences between AD and control serum Zn, whereas Jeandel et al. [37] showed a significant decrease in Zn and other nutrients and antioxidant properties in AD serum, although the AD group may have contained malnourished subjects. The study of Haines et al. [32] is also questionable because it included control subjects whose MMSE scores were considered cognitively impaired. In contrast, Rulon et al. [59] and Gonzales et al. [31] showed significant elevations of Zn in AD serum. Additionally, Gonzales et al. [31] showed that serum Zn correlated with the presence of APOE4 alleles and concluded that, of the parameters analyzed in their study, only serum Zn appeared to be an independent risk factor associated with the development of AD.

Although our current serum data appear at odds with observations in brain, they are consistent with previous *in vivo* animal studies that show systemic Zn deficiencies lead to diminished brain ZnT-1 levels and increased brain Zn [11,69], suggesting brain capillary epithelial cells increase Zn uptake in the presence of diminished extra-parenchymal Zn to maintain Zn stores.

In the brain, Zn is distributed in 3 pools including: a) a membrane-bound metalloprotein or protein-metal complex pool involved in metabolic reactions and nonmetabolic functions such

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as biomembrane structure and support; b) a vesicular pool localized in nerve terminal synaptic vesicles; and c) an ionic pool of free or loosely bound ions in the cytoplasm [27]. Of these sources, the vesicular pool, which is easily chelated, is thought to be the most important [17, 26,33,54,55] because it is released during neurotransmission and may reach concentrations of 300 μM in the synapse. Unless these Zn gradients are immediately sequestered, they could potentially induce neurodegeneration. Overall, mean brain Zn concentrations are ~ 10 times serum Zn levels [67] and range between 150 and 200 μM [22,58]. Peak extracellular Zn concentrations my reach 300 μM during seizure activity or during neurotransmission [4,35]. Therefore, it is imperative that cells regulate Zn levels through control of influx and efflux and through chelation to Zn sequestering proteins.

Although the transport of Zn from brain extracellular environments to intracellular compartments in neurons and glia is not completely understood, it is thought to involve members of the ZIP family of proteins [39]. ZIP proteins are predicted to have 8 transmembrane domains with a histidine-rich intracellular loop between domains 3 and 4 [36] and are part of the plasma membrane or membranes of intracellular organelles. To date, 14 mammalian ZIP proteins that function to increase intracellular Zn by increasing Zn uptake (ZIP 1- 5; 7 – 15) or by releasing Zn from intracellular stores when Zn is deficient (ZIP 6 and 7) have been identified using mouse and human sequence analysis.

Export and sequestration of Zn are carried out by the ZnT family of proteins that are predicted to have 6 transmembrane domains with a histidine-rich loop between transmembrane domains 4 and 5. To date, 8 ZnT proteins have been described (reviewed [24]). ZnT-1 is located at the plasma membrane and is expressed in the brain and other organs [52], whereas the other ZnT proteins are expressed at the membrane of intracellular organelles. ZnT-1 expression is induced in the presence of elevated cytoplasmic Zn through direct binding of Zn to the Zn-finger domain of metal response element-binding transcription factor-1 (reviewed [3]). In the only study of ZnT-1 in AD, our Western blot analyses show significantly decreased ZnT-1 in the HPG of MCI, but significant elevations in EAD and LAD [43]. Recent studies of Chowanadisai et al. [11] showed rats provided a Zn-deficient diet demonstrated decreased brain ZnT-1, suggesting low systemic Zn could decrease ZnT-1 to maintain brain Zn stores. These studies are consistent with those of Takeda et al. [69] who showed rats on a Zn-deficient diet showed increased brain Zn.

To determine if low extra-parenchymal Zn may contribute to alterations of ZnT-1 observed in MCI, we quantified serum Zn concentrations in MCI, AD, and NC subjects. The data obtained in this study suggest decreased serum Zn levels, particularly in men, may lead to decreased ZnT-1 expression in brain and subsequent alterations of cellular Zn distributions that may contribute to the progression of AD through increased Aβ processing and deposition. Because low serum Zn could easily be treated, it presents a potential therapeutic target.

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Table 1

Table 1A

	Mean ± SEM Age (years)	**Sex**	**Number of APOE-4 alleles**	**Mean ± SEM CDR**	**Mean ± SEM MMSE**
All NC	77.9 ± 1.7	9 men/7 women	7 subjects with 1-E4 allele	0 ± 0	28.8 ± 0.1
All MCI	78.4 ± 1.6	9 men, 10 women	7 patients with1-E4 allele	0.5 ± 0.03	27.7 ± 0.4
All AD	80.3 ± 1.7	9 men/9 women	6 patients with 1-E4 allele; 3 patients with 2-E4 alleles	1.4 ± 0.2*	18.0 ± 2.1*

Table 1B

	Mean ± SEM Age (years)	Sex	Number of APOE-4 alleles	Mean ± SEM CDR	Mean ± SEM MMSE
NC Men	77.9 ± 1.6	9	4 subjects with 1-E4 allele	0 ± 0	28.2 ± 0.6
NC Women	77.9 ± 3.6	7	3 subjects with 1-E4 allele	0 ± 0	29.6 ± 0.2
MCI Men	77.8 ± 1.5	9	4 patients with 1-E4 allele	0.4 ± 0.06	28.9 ± 0.4
MCI Women	79.2 ±2.9	10	3 patients with 1-E4 allele	0.5 ± 0.0	26.7 ± 0.5*
AD Men	79.6 ± 2.3	9	4 patients with 1-E4 allele; 2 patients with 2-E4 alleles	1.4 ± 0.4*	17.2 ± 2.7*
AD Women	81.0 ± 2.6	9	2 patients with 1-E4 allele; 1 patient with 2-E4 alleles	1.4 ± 0.2*	18.5 ± 3.1*

NC = normal control; MCI = mild cognitive impairment; AD = mild to moderate Alzheimer's disease; CDR = Clinical Dementia Rating scale; MMSE = Mini-Mental State Examination.

* $p < 0.05$

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Table 2

Zinc concentrations (µM) in the original serum samples by groups

Group	Male		Female		Overall
	Mean ± S.E.M. µM	**Range µM**	**Mean ± S.E.M. µM**	**Range µM**	**Mean ± S.E.M. µM**
NC (9M, 7F)	13.9 ± 0.6	10.9 ~ 17.3	12.5 ± 1.0	9.5 ~ 16.6	13.3 ± 0.6
MCI (9M, 10F)	11.7 ± 0.5	9.6 ~ 14.2	13.7 ± 0.6	11.4 ~ 16.6	12.8 ± 0.5
AD (9M, 9F)	12.7 ± 0.4	11.1 ~ 14.9	12.1 ± 0.8	8.9 ~ 15.9	12.4 ± 0.4

NC = normal control; MCI = mild cognitive impairment; AD = mild to moderate Alzheimer's disease

Ages in the NC, MCI, and AD groups were not significantly different, $F(2, 50)=0.249$, $p=0.781$. There was a significant interaction between group and sex in the Zn level, $F(2, 47)=3.433$, $p=0.041$. For males, the NC group had significantly higher Zn level than the MCI group, $p=0.028$. For females, the MCI group had marginally significantly higher Zn level than the AD group, $p=0.088$. For the MCI group, females had significantly higher Zn level than males, $p=0.035$. There was no significant difference among the three groups (NC, MCI and AD) in Zn level if sex was not taken into account, $F(2, 47)=0.649$, $p=0.527$. There was no significant difference between males and females in Zn level without considering group, $F(1, 47)=0.002$, $p=0.965$.