Research Article

Decreased Copper in Alzheimer's Disease Brain Is Predominantly in the Soluble Extractable Fraction

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Alzheimer's disease (AD) is the leading cause of dementia and represents a significant burden on the global economy and society. The role of transition metals, in particular copper (Cu), in AD has become of significant interest due to the dyshomeostasis of these essential elements, which can impart profound effects on cell viability and neuronal function. We tested the hypothesis that there is a systemic perturbation in Cu compartmentalization in AD, within the brain as well as in the periphery, specifically within erythrocytes. Our results showed that the previously reported decrease in Cu within the human frontal cortex was confined to the soluble ($P < 0.05$) and total homogenate ($P < 0.05$) fractions. No differences were observed in Cu concentration in erythrocytes. Our data indicate that there is a brain specific alteration in Cu levels in AD localized to the soluble extracted material, which is not reflected in erythrocytes. Further studies using metalloproteomics approaches will be able to elucidate the metabolic mechanism(s) that results in the decreased brain Cu levels during the progression of AD.

1. Introduction

Alzheimer's disease (AD) is the predominant cause of dementia in the aging population and represents a mounting health epidemic [1]. Despite advances in understanding the events leading to the onset of cognitive decline, the principal cause of AD is still undetermined. The role of copper (Cu), iron (Fe), and zinc (Zn) in AD has become of significant interest because the dyshomeostasis of essential trace elements has been observed to have profound effects on cell viability and neuronal function [2, 3], which have been previously reviewed [4].

Cu, an essential element in the central nervous system (CNS), is crucial for life, but its unique redox propensity renders it toxic in circumstances of an increase pool of labile species [5–8]. Specific lesions in the Cu pathway can lead to a severe but treatable neurological impairment,

including Menkes and Wilson's disease [9–11]. Cu displays a distinctly compartmentalized distribution throughout the brain, reflecting its diverse function in various neurological processes [12, 13].

Within the CNS, Cu is known to decrease in the frontal, occipital, and parietal lobes [14] amygdala and hippocampus in AD [15]. The process for this decline is not well understood, though extracellular plaques of aggregated amyloid-β (Aβ) are reported to be enriched with trace elements including Fe, Zn, and Cu [16]. Recently, it was also reported that frontal cortex from AD subjects had an increased propensity to bind exchangeable Cu, which correlated with oxidative damage observed in the tissue [17].

In cerebral spinal fluid (CSF), Cu levels are not observed at significantly different concentrations between AD and healthy controls (HC) [18–20]. However, within peripheral fluids, Cu dyshomeostasis has been more intensely studied.

Reports of increased [19, 21], decreased [22, 23], or unchanged [24–26] serum or plasma Cu in AD have rendered total Cu levels too variable to be of diagnostic utility, for review see [27]. Yet, many studies have concluded that there is a subtle but consistent excess of nonbound or diffusible Cu in serum [21, 22, 28–34]. Despite this, a consensus has been thwarted by a lack of standardization and limitations driven by covariate influences on peripheral "high throughout" screening of Cu concentrations [35]. In other peripheral tissues, such as erythrocytes, superoxide dismutase 1 (SOD1) activity has been found to be diminished in AD [23]. This is thought to be due to a Cu deficiency in the enzyme, as reported previously [36].

In this study, we tested the hypothesis that there is a systemic perturbation in Cu compartmentalization in AD, within the frontal cortex as well as in the periphery, within erythrocytes.

2. Methods

2.1. Subjects. The AIBL study incorporates longitudinal neuroimaging, biomarker, neuropsychometric, and lifestyle data, see [37] for a detailed description of methods. Briefly, participants over the age of 65 years and fluent in English were divided into three groups; cognitively healthy individuals (HC), participants with mild cognitive impairment (MCI) based on the established criteria [38, 39], and participants diagnosed with *possible* or *probable* AD as defined by NINCDS-ADRDA criteria [40]. Written informed consent was obtained from all participants, and the study was approved by the appropriate institutional ethics committees.

2.2. Erythrocyte Preparation. Whole blood was collected from overnight fasted participants with a 27 g needle, into Sarstedt S-Monovette Lithium-Heparin 7.5 mL tubes (01.1608.100). The tubes were spun at 3,200 ×g for 30 min at room temperature, and the plasma was carefully taken off the hematocrit. The buffy coat was prepared by ficoll gradient centrifugation to extract the white blood cells. The erythrocytefraction was washed 3 times by adding 0.9% normal saline to an end volume of approximately 14 mL. Erythrocytes were dispersed by gently inverting the tubes 10 times and then centrifuged at 650 ×g for 10 minutes at 20°C with braking on. The final centrifugation was 1,500 ×g for 10 minutes at 20°C with braking on. The final saline wash was discarded, and the erythrocytes resuspended to an end volume of 6 mL in phosphate buffered saline (PBS) (pH 7.4), then aliquoted into polypropylene (Nunc cryobank, Denmark) tubes and snap-frozen in liquid nitrogen.

2.3. Fractionation of Brain Tissue for Biochemical Analysis. Brain tissues were obtained from the Victorian brain bank network, and all experiments were approved by the University of Melbourne health sciences, human ethics sub-committee (ID1136882). Hemisected frozen brains at −80°C

were thawed to −20°C and sectioned in 1 cm slices. The meninges were removed from approximately 5 g of frontal cortex (Brodmann area 9), and the grey matter was dissected in to 0.2–0.5 g aliquots and stored at −80°C. The grey matter was allowed to thaw on ice and then homogenized using a BioMasher (Omni International). Tissue was placed in the BioMasher, the plunger was inserted, and then the apparatus was centrifuged at 100,000 ×g with a desktop centrifuge. After centrifugation, Tris buffered saline (TBS, 50 mM Tris pH 8.0, 150 mM NaCl) containing EDTA free protease inhibitors (Roche, 05056489001) was added at a ratio of 1 : 4 (tissue : buffer, w/v). The sample was then transferred to ultracentrifuge tubes and centrifuged at 100,000 ×g for 30 minutes at 4°C. The TBS supernatant, or "soluble" material, was collected and stored at −80°C before Western blot analysis. The pellet was resuspended in 100 mM $NaCO_3$ pH 11.0 (1 : 4, tissue:buffer) and further centrifuged at 100,000 ×g for 30 minutes at 4°C. The supernatant, "peripheral membrane/vesicular" material was recovered, and the pellet was resuspended with 7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 30 mM Bicine pH 8.5, and centrifuged at 100,000 ×g for 30 minutes at 4°C. The supernatant, "membrane" material, was recovered, and the resulting pellet was then incubated at room temperature with 70% formic acid for 16–18 hours before being centrifuged at 100,000 ×g for 30 minutes. After the sequential extraction, little to no observable material remained.

2.4. Induction Coupled Plasma-Mass Spectrometry (ICP-MS). Frozen aliquots of erythrocytes or brain tissue homogenate fractions were thawed at room temperature. For brain homogenates, 50 μL was diluted (1 : 20) with 950 μL of 1% HNO_3 (v/v). 50 μL of washed erythrocytes were digested in equivalent volumes of concentrated (65%) HNO_3 and H_2O_2 (Merck Millipore) at 80°C for 5 minutes, then diluted 1 : 20 with 1% HNO_3. Cu concentration was determined using an Agilent Technologies 7700x ICP-MS system. The sample introduction system used a Teflon MiraMist parallel path nebulizer (Burgener Research Inc.) and standard Scott-type double-pass spray chamber (Glass Expansion). Helium was used as a collision gas. ICP-MS conditions were replicated from previously reported studies from our laboratory [35]. The instrument was calibrated using multielement standards (Accustandard, ICP-MS-2-1, ICP-MS-3, and ICP-MS-4; total of 44 elements) containing copper at 0, 5, 10, 50, and 100 ppb with ^{89}Y as the internal standard for all isotopes of Cu. Interday relative standard deviations were determined using a quality control serum (Seronorm) with a certified copper level (84.55 μg/L 95% CI 80.35–88.75 μg/L) and were consistently between 2.0–5.0%.

2.5. Statistical Analysis. Statistical analyses were performed with Prism version 6.0 (Graphpad Inc). To compare differences between the groups, a one-way ANOVA Bonferroni's multiple comparison test was used. Significant P values were <0.05.

TABLE 1: Distribution of Cu in different cellular fractions and total levels from human brain.

Brain fraction	Cu (μg/g of wet weight)		P value
	HC	AD	
Soluble	1.93 ± 0.6 (1.0–3.2)	1.46 ± 0.6 (0.6–3.3)	**<0.05**
Peripheral/vesicular	0.57 ± 0.2 (0.3–0.9)	0.38 ± 0.3 (0.7–1.1)	>0.05
Membrane	0.52 ± 0.2 (0.2–1.2)	0.36 ± 0.2 (0.05–0.8)	>0.05
Formic acid[¶]	0.52 ± 0.2 (0.3–1.0)	0.38 ± 0.2 (0.05–0.7)	>0.05
Total homogenate[§]	3.33 ± 2.2 (1.5–13)	2.29 ± 1.0 (0.9–4.7)	**<0.0001**

Concentration based on wet weight of tissue, mean ± standard deviation (range).
[¶](HC) $N = 20$, (AD) $N = 22$, [§](HC) $N = 24$, and (AD) $N = 23$. Numbers in brackets are the 95% confidence intervals. P values were calculated using one-way ANOVA Bonferroni multiple comparison post hoc test. (NS: nonsignificant).
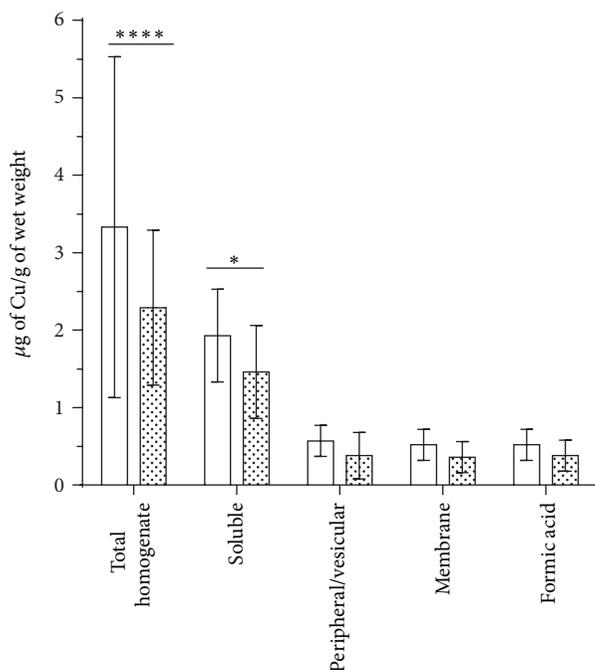

FIGURE 1: Cu content in human brain. Illustrates a significant decrease in total Cu in the soluble fraction of the extracted tissue. [*]$P < 0.05$, [****]$P < 0.0001$; one-way ANOVA with Bonferroni's multiple comparison post hoc test of log transformed data. Cu is decreased in AD frontal cortex. Copper is significantly decreased in the total homogenate and soluble extracted material ($P < 0.05$). The only fraction that had a significant decrease was the soluble fraction indicating that the decrease in Cu observed in the total homogenate is localized to changes in the soluble fraction. HC *Healthy Control* (clear boxes), AD *Alzheimer's disease* (filled boxes).

3. Results

Table 1 and Figure 1 show that changes in Cu within the human frontal cortex were localized to the soluble fraction. We observed a significant decrease in total Cu levels consistent with previous studies [14, 15]. To investigate if the change in Cu was global or localized to a specific cellular compartment, we fractionated the brain tissue into four groups: soluble, peripheral membrane, and vesicular material, integral membrane and formic acid extractable material that contains predominantly insoluble plaques [41].
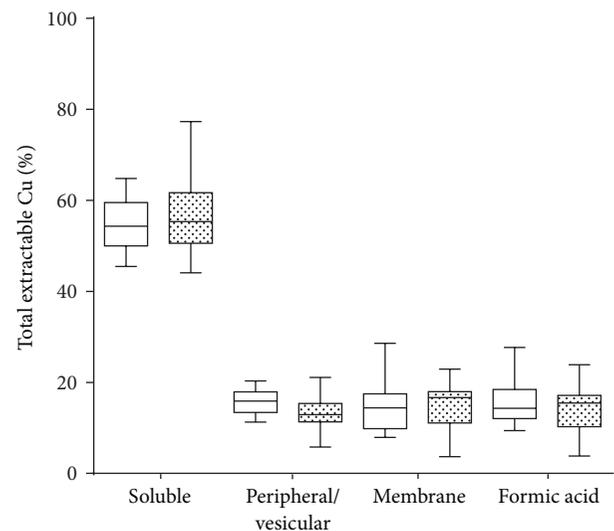

FIGURE 2: Cu content in human brain expressed as percentage distribution. The percent distribution of Cu extracted from human frontal cortex in the brain is conserved in AD and HC (total pooled). Box and whisker plots show the range, interquartile range, and median values. No significant difference was observed in the percentage of Cu in each of the corresponding fractions. Between 50–60% of the total Cu in human brain tissue is extractable in the soluble phase. No significant difference was observed between HC (clear boxes) and AD (filled boxes).

We only observed a significant decrease in Cu in the soluble fraction between AD and HC (Figure 1, $P < 0.05$, one-way ANOVA Bonferroni's multiple comparison test). Table 2 shows the demography for the postmortem brain samples. Although we did observe a decreasing trend for Cu in each, we found that when Cu is expressed as a percentage of total Cu for each individual (Figure 2), no significant differences were observed, suggesting a conservation of Cu equilibrium that may be homoeostatically regulated. We observed that 50–60% of total tissue Cu content was localized to the soluble extractable material (Figure 2).

Previous studies have associated AD specific changes in erythrocytes [42, 43], including changes in the Cu dependent enzyme Cu, Zn-superoxide dismutase 1 (SOD1) [23]. We used well-characterized samples from the AIBL study to investigate the level of Cu in erythrocytes. Table 3 shows
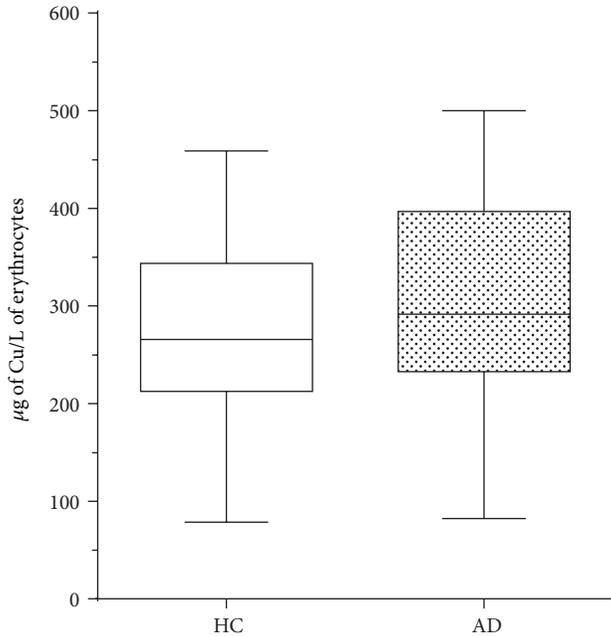
FIGURE 3: Cu concentration in human erythrocytes. The total Cu content of erythrocytes was determined using ICP-MS. No significant change ($P = 0.53$) in the level of Cu in red blood cells was observed between HC ($N = 40$) (clear boxes) and AD ($N = 40$) (filled boxes).

TABLE 2: Post-mortem subject demography.

	AD (N = 30)	HC (N = 27)	P value
Age (years)	78.0 (9.2)	77.0 (7.6)	>0.05
Gender females (%)	27	33	>0.05
ApoEε4 carriers (%)	76	15	**<0.0001**
PMI (hours)	33.9 (22)	38.4 (14.3)	>0.05

Values are means (SD) unless noted above as otherwise. *P* values were calculated using *t*-test (two tailed). HC: healthy control, AD: Alzheimer's disease, *ApoEε4*: Apolipoprotein E epsilon 4, PMI: postmortem interval.

the AIBL cohort demographics for individuals analysed for erythrocyte Cu concentrations. Figure 3 shows that there was no significant difference in erythrocytic Cu concentration observed between AD and control samples.

4. Discussion

The aim of this study was to investigate the distribution and concentration of Cu in the frontal cortex and periphery of AD subjects when compared to age-matched healthy control samples. A number of studies have indicated that there is a significant perturbation in Cu coordination in AD within the frontal cortex [14, 17, 44] and periphery [3, 27]. Using well-characterized subjects, we were unable to demonstrate a significant difference in Cu levels within erythrocytes, a finding which is consistent with our investigations of serum Cu levels [35]. However, we did observe a significant difference within the frontal cortex, where AD tissue had

TABLE 3: AIBL cohort demographics for individuals analysed for erythrocyte Cu levels.

	HC (N = 40)	AD (N = 40)	P value
Age (years)	76.8 (8.0)	77.3 (8.0)	>0.05
Gender females (%)	47.5	55	>0.05
ApoEε4 carriers (%)	37.5	60	**<0.05**
MMSE	28.4 (1.4)	18.1 (6.0)	**<0.0001**
CDR	0.075 (0.2)	6.175 (3.2)	**<0.0001**

Values are means (SD) unless noted above as otherwise. *P* values were calculated using one-way ANOVA Bonferroni multiple comparison post hoc test. HC: healthy control, AD: Alzheimer's disease, and MMSE: Mini-Mental State Examination. CDR: clinical dementia rating scale, *ApoEε4*: Apolipoprotein E epsilon 4.

significantly less Cu than controls ($P = <0.0001$). This observation is consistent with the frontal cortex having a unique susceptibility for Cu deficiency, compared to the periphery. Previous studies have used fractionation to investigate trace elements in AD brain [45], but data on changes to Cu concentrations is lacking. By fractionating brain tissue into several biochemical distinct subunits we observed that the decrease in Cu is mainly confined to the soluble fraction. The change in the soluble fraction is consistent with the reported deficiency of metallothioneins in the AD brain [46], though further metalloproteomic investigations are required to determine the extent that the Cu proteome is altered in AD neuropathology [47]. Surprisingly, no significant difference was observed in the formic acid fraction (Figure 1), contrary to our expectation that the AD plaques would demonstrate increased Cu in line with previous reports [48, 49]. The absence of an increase in Cu in the formic acid fraction highlights the importance of using spatially resolved techniques to measure tissue distribution of trace elements, including X-ray microfluorescence microscopy [50] and laser ablation ICP-MS [51, 52].

We have shown that over 50% of the Cu in human brain tissue can be extracted in the soluble or cytosolic portion of the homogenate. Further, the changes observed in the AD brain are due to specific changes in this soluble phase. As Cu has a strong propensity to participate in free radical chemistry, the distribution and delivery of Cu are carefully controlled by a set of Cu specific protein machinery [13, 53, 54]. This Cu handling system maintains less than one free Cu ion per cell [55]. Therefore, essentially all of the Cu is bound to biological ligands or is chaperoned by Cu regulatory proteins. Future investigations using a metalloproteomic approach [56–58] will be able to determine if the changes in the soluble Cu levels are specific to changes in binding partners, such as the reported decrease in metallothionein observed in AD [46]. As Cu is tightly regulated and exists as a ligated entity in the cell, it will be interesting to investigate if the change in soluble Cu are global, suggesting all Cu-proteins are decreased in AD, or are the changes restricted to selective proteins. In particular, detailed investigations of the stoichiometry of Cu proteins, like ceruloplasmin and the three reported metallothionein isoforms, will also be

informative concerning Cu perturbations that may lead to deficiency in the CNS.

In conclusion, this study has examined two pools of Cu in AD compared to age matched HCs. We showed that there is a specific change in the frontal cortex, indicating there is a perturbation in Cu homeostasis leading to a local diminution in concentration. We did not detect a significant difference in erythrocytes, suggesting that Cu disturbance may be confined to the brain in AD, precluding peripheral Cu levels as a useful biomarker for AD. The timing, systemic covariates, and mechanism(s) of this alteration still need to be systemically investigated, and advanced analytical metalloproteomic techniques will go a long way to answer these questions in the future.

Acknowledgments

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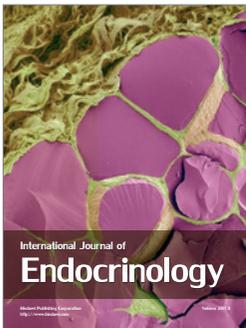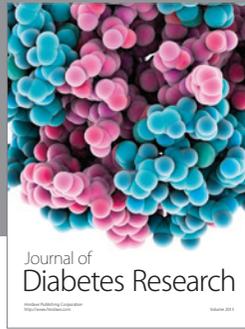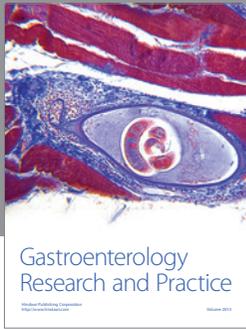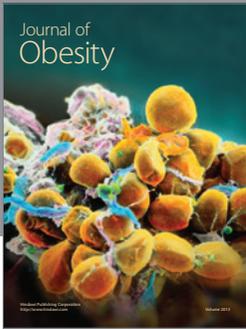
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