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Oral zinc reduces amyloid burden in Tg2576 mice

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Abstract

The aggregation of amyloid beta in Alzheimer's disease can be affected by free transition metals such as copper and zinc in the brain. Addition of copper and zinc with amyloid acts to increase aggregation and copper additionally promotes the formation of reactive oxygen species. We propose that reduction of brain copper by blocking uptake of copper from the diet is a viable strategy to regulate the formation of insoluble amyloid beta in the brain of Tg2576 mice. Mice were treated with regimens of zinc acetate, which acts with metallothionein to block copper uptake in the gut, at various times along their lifespan to model prevention and treatment paradigms. We found that the mice tolerated zinc acetate well over the six month course of study. While we did not observe significant changes in cognition and behavior, there was a reduction in insoluble amyloid beta in the brain. This observation coincided with a reduction in brain copper and interestingly no change in brain zinc. Our findings show that blocking copper uptake from the diet can redistribute copper from the brain and reduce amyloid beta aggregation.

Keywords

Copper; Alzheimer's Disease; Amyloid beta-protein; Transgenic Mice

INTRODUCTION

Alzheimer's disease (AD) is a chronic degenerative disease characterized by the presence of extracellular amyloid plaques and intracellular neurofibrillary tangles (NFTs). The plaques are formed through accumulation of β amyloid (A β) protein [1]. A β is formed by β and γ -secretase cleavage of the amyloid β precursor protein (A β PP) [2]. NFTs are composed of accumulated hyperphosphorylated tau protein [3]. Together the A β plaques and NFTs cause loss of cholinergic neurons, deterioration of cognitive function, and memory loss [4–6]. A β plaques and NFTs form primarily in the cortex and hippocampal regions of the brain resulting in cellular dysfunction and death in these regions and leading to memory loss [7].

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Currently, treatments for AD focus on reducing the symptoms associated with the disease after onset; however future research is focused on treating or preventing the pathological aspects of the disease.

Environmental factors are thought to play a large role in increasing the risk of developing AD. An emerging field of study focuses on how transition metals influence the progression of amyloid formation in the brain [8–16]. Several zinc and copper binding sites have been identified in the A β PP protein [8, 9]. These sites have been linked with many important functions involving zinc binding affinity and inhibition of α -secretase [10]. As well, there is increasing evidence that copper affects the pathology of AD in a number of ways. For instance, brain copper has been shown to increase with age [11], copper up-regulates the expression of the A β PP protein [12], and the presence of copper in vitro and in vivo affects the aggregation and neurotoxicity of A β [13–16]. Therefore, treatments focused on lowering copper levels in aged brain could mitigate A β pathology and the resulting symptoms.

The Tg2576 mouse line is a transgenic AD model containing the A β PP_{SWE} mutation (K670N/M671L) [17]. This animal displays increased A β PP production leading to increased A β accumulation in the cortex and hippocampus. A β PP begins to accumulate from birth, and a measurable increase in A β is observed around 6–8 months of age. By 12–15 months of age, behavioral deficits occur with an increase in amyloid plaque accumulation [1]. Previous work in the Tg2576 model has examined the usage of a copper-lowering agent, Clioquinol (CQ), in the prevention of plaque formation [14]. CQ is a bioavailable antibiotic Cu⁺⁺/Zn⁺⁺ chelator. CQ was found to cross the blood brain barrier (BBB) and bind copper, resulting in lowered A β pathology without lowering brain copper. CQ went onto a small phase II clinical study in patients with moderately severe AD [15]. In the study, subjects on CQ had a minimal deterioration of cognitive subscale scores on the Alzheimer's Disease Assessment Scale, as compared to the placebo group, which had a substantial worsening of scores. However, further clinical testing has not been conducted due to complications in good manufacturing practices and concerns about CQ's safety [16]. Alternative copper-complexing agents, such as CQ analogs, are in development [18, 19]. One of these analogs, PBT2, has been investigated in a phase IIa clinical trial. Patients taking PBT2 showed a decrease in CSF A β 1-42, and decreased decline of function in the neuropsychological test battery as compared to placebo. Further analysis of the original clinical study revealed improvement in Composite z-scores, Executive Factor z-scores and ADAS-cog [20].

Mechanistically, CQ and PBT2 are thought to work by crossing the BBB and binding extracellular copper and zinc, without altering plasma or brain copper levels. This results in decreased hydroxyl radical formation and activation of intracellular protective mechanisms [18, 21–26]. There is modest evidence that copper-chelating agents must cross the BBB to effectively modulate A β deposition. For example, trientine, another copper chelating agent, does not cross the blood brain barrier and did not lower brain A β in the Tg2576 model [14]. Interestingly, AD pathology can be influenced by excess systemic copper levels [27]. We have previously reported that the use of Tetrathiomolybdate (TM) lowers brain copper levels by lowering systemic copper [28]. The proposed mechanism of TM, which does not cross the BBB, creates a negative systemic copper balance, and brain copper and A β levels are reduced [28]. However, TM, which was an investigational agent in human subjects with the

genetic copper accumulation disorder known as Wilson's disease, [29–31], has shown safety concerns in human subjects and FDA approval was abandoned.

We sought to develop alternative copper lowering strategies that have similar mechanisms as TM, but pose less safety concerns. Zinc acetate, when taken orally, is used as a mild copper lowering agent for maintenance therapy in subjects with Wilson's disease. Oral zinc increases the concentration of metallothionein in the intestine resulting in decreased copper absorption from the diet and gastric juices. We hypothesized that by using zinc acetate we could lower brain copper levels and consequently plaque load and neurotoxicity. We tested the ability of oral zinc to attenuate A β pathology and memory impairment in the Tg2576 mouse model (Fig 1). Mice were aged 7, 8, or 9 months, and then administered a 6 month regimen of oral zinc acetate in their drinking water. The levels of plasma ceruloplasmin (Cp), a copper dependent enzyme, were used as a surrogate marker for circulating free copper. We also confirmed metal changes in the brain and plasma by inductively coupled plasma mass spectroscopy. We found zinc acetate reduced copper levels in the brain and caused a reduction in insoluble A β if the treatments were begun at 7 months. However, we did not observe any statistical behavioral changes. The copper-lowering aspects of this strategy are distinct from the approach with clioquinol and its derivatives, and may point to a viable alternative copper-modulating strategy for clinical trials in AD.

MATERIALS AND METHODS

Animals and diet

Tg2576 are transgenic mice containing a mutant transgene of human A β PP₆₉₅ (K670N/M671L) on a hamster prion promoter. Mice were generated from a breeding pair generously provided by Dr. Karen Hsiao-Ashe (Mayo Clinic, MN). The transgene was carried on a genetic background of C57BL/6J X SJL/J. Male mice were harem bred with either C57BL/6J or SJL/J females alternating between strains for each generation. C57BL/6J or SJL/J females were obtained from Jackson Laboratory (Bar Harbor, ME) at 8 weeks of age. Following weaning, litters were genotyped and group housed (4–5 per cage) until the commencement of experiments. All of the mice in this study were female because male Tg2576 mice are prone to marked aggression towards cage mates. Mice were maintained in a climate-controlled environment with a 12-hr light/12-hr dark cycle, and fed AIN-93M Purified Rodent Diet (Dyets Inc., Bethlehem, PA). AIN-93M Purified Rodent Diet was used as a maintenance diet with a controlled amount of cupric carbonate (6 mg/kg) and zinc carbonate (30 mg/kg) among other vitamins and minerals [32]. This diet allowed us to control and ensure baseline copper and zinc ingestion was the same throughout and within all cohorts. AIN-93M contains 70 g/kg of soybean oil, 7% of which is linolenic acid, an omega-3 fatty acid. Diet and water were supplied ad libitum. All procedures were conducted in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and were approved by the institutional Animal Care and Use Committee of the Portland VA Medical Center.

Treatments

Tg2576 and wild type mice were randomized to normal drinking water or drinking water with zinc acetate (Mallinckrodt Baker, NJ). Animal weights and Cp levels were monitored

to ensure zinc levels were not detrimental to the animal's well-being. In all of the treatment cohorts, oral zinc acetate was administered at a dose of 2 g/L vehicle in their drinking water (vehicle = DiH_2O). In the 7 month start cohort (7msc), 6.9–7.2 month-old mice were treated for six months as follows: 25 wild-type littermate controls (vehicle n=12, zinc acetate n=13) and 18 Tg2576 mice (vehicle n=9, zinc acetate n=9). In the 8 month start cohort (8msc), 8.4–8.5 month-old mice were treated for six months as follows: 21 wild-type littermate controls (vehicle n=11, zinc acetate n=10) and 19 Tg2576 mice (vehicle n=9, zinc acetate n=10). In the 9 month start cohort (9msc), 8.9–9.7 month old mice were treated for six months as follows: 19 wild-type littermate controls (vehicle n=10, zinc acetate n=9) and 13 Tg2576 mice (vehicle n=6, zinc acetate n=7).

Ceruloplasmin assay

For the 7msc, blood was collected at baseline for every animal and then every 4–6 weeks from 6 animals per treatment group, distributed so that all animals had blood collected a total of 3 times. For the 8 and 9msc blood was collected at the beginning and end of treatments. Up to 200 μL was obtained from the saphenous vein. Cp activity was measured from the beginning and end of treatment using a modified version of the oxidase method that we had used in our previous paper [28]. Data was expressed as the mean $\pm$ standard error of the mean (SEM).

Behavioral analysis

To assess possible neurological deficits caused by copper reduction, the rotarod and open field tests were conducted. Rotarod testing was performed using the same apparatus and methods in our previous studies [28]. A customized Rotarod (Med Associates, Georgia, VT) was used to test mice for 10 trials each day for two days. An electronic beam measured the latency from test start until a mouse fell. The intertrial interval (ITI) was 1 minute. Performance was evaluated for each mouse and expressed as the mean $\pm$ SEM of the time spent on the rod (in seconds) for each treatment group.

Low open field performance can be indicative of locomotor defects. Increased open-field activity has been reported in the Tg2576 model [33]. We evaluated whether copper deficiency would affect this behavior. Open-field testing was performed in an apparatus as described in our previous experiment [28]. Mice were tested two times for 5 minutes on two consecutive days. Movement was tracked using ANY-maze video tracking system (Stoelting Co, Wood Dale, IL). Performance was expressed as the mean $\pm$ SEM of the total distance moved (in meters), for each treatment group.

To assess hippocampal-dependent spatial learning and memory, mice were trained in a standard Morris water maze (MWM) task using the same apparatus and protocol used in our previous studies [28, 34, 35]. Mice were placed into the MWM tank for 6 trials each day for 7 days. The first two days were visual platform testing in which the platform was slightly above the water with a graduated cylinder on top. Platform location was rotated equally between each quadrant to prevent bias towards any given quadrant. Mice were given 60 seconds to find the platform before being placed back in their cage. Forty-eight hours after visible platform training, hidden platform training was conducted for five days. The target

quadrant became the upper right quadrant and the platform was not moved during hidden platform training. The platform was slightly submerged in opaque water and mice were given 60 seconds to find the platform. On the last day of hidden platform training, the platform was removed from the tank and mice were given a probe trial 2 hours after the final training, in which they were allowed to swim freely in the pool for 60 seconds. Seventy-two hours following the first probe trial a second probe trial was conducted. The ITI for each trial was 10 minutes, except for between trials 3 and 4 which were 2 hours and correspond to the beginning of session one and session two for each day. All MWM tasks were evaluated using an ANY-maze video tracking system. Performance on visible and hidden platform training was expressed as the mean $\pm$ SEM of the latency to platform (in seconds). Performance on probe trials was expressed as the mean $\pm$ SEM of the percent time in target quadrant ((seconds in quadrant/60)*100) for all mice in each treatment group.

Analysis of body metals

Zinc and copper from plasma and brain tissue and levels were measured using inductively coupled plasma mass spectroscopy (ICP-MS). For the analysis, brain tissue (approximately 40–80 mg wet weight) were digested with 1 ml concentrated HNO₃ (trace metal grade, Fisher Scientific) solution. Following overnight, room temperature digestion of the samples, 5 ml with distilled water was added to yield solutions containing 19.5% nitric acid. For the measurement the samples were spun at 4,700 $\times$ g for 10 min to remove insoluble residues, and the supernatant transferred into clean, acid treated 15 ml conical tubes and diluted with 1% HNO₃ (trace metal grade, Fisher Scientific). For the analysis of plasma, samples were directly diluted into 1% HNO₃ (trace metal grade, Fisher Scientific). Inductively coupled plasma mass spectrometry (ICPMS) analysis was performed using an Agilent 7700x equipped with an ASX 250 autosampler. The system was operated at a radio frequency power of 1550 W, an argon plasma gas flow rate of 15 L/min, and argon carrier gas flow rate of 1.04 L/min. Elements were measured in kinetic energy discrimination (KED) mode using He gas (4.3 ml/min). Data were quantified using a 9-point calibration curve (0, 0.5, 1, 2, 5, 10, 50, 100, 1000 ppb (ng/g)) with external standards for Fe, Cu, and Zn (Common Elements Mix 2, Multi-Element Aqueous Standard, VHG Labs) in 1% HNO₃ (trace metal grade, Fisher Scientific). For each sample data were acquired in triplicates and averaged. An internal standard (Internal Standard Multi-Element Mix 3, VHG Labs) introduced with the sample was used to correct for plasma instabilities, frequent measurements of a 10 ppb Cu solution as well as a blank (containing 1% HNO₃ only) were used as quality control and to determine the coefficient of variance. To access recovery rates of elements and probe background contamination from buffers and containers the following controls were treated, prepared, and analyzed by the same method as the samples: certified NIST standard reference material (serum; 1598a), homogenization buffers, acid control (containing 50% HNO₃ only), and certified elemental standard for ICPMS (Common Elements Mix 2, Multi-Element Aqueous Standard, VHG Labs).

Tissue homogenates and ELISA

After behavioral testing, animals were euthanized by CO₂ inhalation, followed by cervical dislocation. Blood samples were collected on ice, spun down, and the plasma was removed and frozen. One lobe of the liver was removed and snap frozen for future analysis of metals.

The anterior 2 mm of bilateral frontal cortex (“frontal cortex slice”) was dissected and snap frozen. A 2mm section behind the frontal cortex (“frontal slice”) was dissected and snap frozen for inductively coupled plasma mass spectroscopy (ICPMS). The cerebellums were also dissected and snap frozen. The right hemisphere was fixed in 4% formaldehyde in phosphate buffered saline (PBS) for 24 hours, immersed in PBS for 24 hours, 15% sucrose for 24 hours, and then 30% sucrose for 24 hours. The right hemisphere was then sliced into 40 micron coronal sections for use in histochemical experiments. The contralateral hemisphere was frozen and separated into anterior and posterior. The anterior left hemisphere (“ALH”) was homogenized for enzyme-linked immunosorbent assays (ELISAs) and western blots while the posterior left hemisphere, frontal cortex slice, cerebellum, and liver were kept for other studies to be determined.

The anterior left hemisphere tissue was homogenized using a glass mortar and pestle in TRIS-buffered saline (TBS) containing protease and phosphatase inhibitor cocktail (Thermo Scientific, Rockford, IL) and 1% Triton X-100 (Roche, Indianapolis, IL), then centrifuged at $100,000 \times g$ for 30 min at 4°C. The resulting supernatant was labeled “soluble fraction”. The resulting pellet was collected, washed in TBS with protease and phosphatase inhibitors and re-centrifuged. The final pellet was sonicated for 30 seconds at 4°C in 70% formic acid (Sigma, St. Louis, Mo), incubated for 1 hour at room temperature, and centrifuged at $100,000 \times g$ for 30 min at 4°C. The final supernatant was collected and labeled “insoluble fraction”. Commercial ELISA kits (Life Technologies, Camarillo, CA) were used to measure A β ₄₀ and A β ₄₂ insoluble fractions. The amount of A β was determined by absorbance at 450nm and represented as the mean $\pm$ SEM of all mice in each treatment group.

Immunohistochemistry

Immunohistochemistry was performed according to the procedures laid out in our previous paper [28]. A freezing microtome was used to slice forty micron coronal sections. Floating sections were briefly quenched in a 10% methanol solution, then incubated for 3 hours in blocking buffer (100mM TBS, pH 8.0, 2 mg/ml bovine serum albumin (Fisher Scientific)). Brains were incubated overnight in primary antibody against A β ₄₀ (rabbit polyclonal antibody 1:100 in blocking buffer (Life Technologies)). Following primary antibody incubation, sections were washed and subsequently incubated for two hours with biotin labeled rabbit secondary antibody (1:200, Vector Labs, Burlingame, CA). After another set of washes, sections were incubated in a solution containing an avidin-linked peroxidase complex (ABC, Vector Labs), and developed in diaminobenzidine (DAB, Sigma). Mounted sections were washed, dehydrated, and finally cover-slipped using Permount (Fisher Scientific). Protein expression was analyzed using Image J software (Image J, Wayne Rasband, NIH, USA). Three coronal sections per mouse were used representing anterior, middle and posterior hippocampus. The degree of staining for A β was expressed as particles per area (in inch²) analyzed in the cortex or hippocampus. Represented values were averaged for the three coronal sections for each animal. The mean $\pm$ SEM of each animal’s values were then averaged for each treatment group.

Statistics

Data sets were analyzed and plotted with GraphPad Prism 5 software (GraphPad software Inc.) and R 3.0 (R Foundation for Statistical Computing). Analysis was carried out using a combination of linear mixed-effects regression and one-tailed Student's t-tests with a 95% confidence interval with multiple comparisons corrected using Tukey HSD for the t-tests and Holm-Bonferroni adjustments for the linear models. Mixed-effects models were specifically utilized for longitudinal assessment of the hidden platform training over the repeated days of training and session within days. Differences between the end of one training day and the following day were also considered. Mixed-effects models were used to account for the serial correlation of repeated measures within individual mice.

Model integrity was evaluated using standard diagnostics techniques to determine normality of outcomes and homogeneity of error variance. Outliers for the t-tests were determined using the Grubbs' outlier test < <http://www.graphpad.com/quickcalcs/Grubbs1.cfm> > at an alpha = 0.05 while regression model outliers were assessed using Cook's distance and inspection of the residual plots. We removed one outlier from the following open field data sets: 7msc Tg2576 vehicle, 8msc wild type zinc, and 9msc Tg2576 zinc. We removed one outlier from the following plasma zinc data sets: 7msc Tg2576 zinc. We removed one outlier from the following brain zinc data sets: 7msc Tg2576 zinc, 8msc wild type vehicle, 8msc wild type zinc. We removed one outlier from the following plasma copper data sets: 8msc Tg2576 vehicle, 9msc Tg2576 vehicle. We removed one outlier from the following brain copper data sets: Tg2576 vehicle.

Results

Treatment with oral zinc acetate was well tolerated

The copper lowering paradigm was examined by the addition of zinc acetate to the drinking water (Fig 1). Consumption of zinc acetate was monitored by measuring the starting and ending volumes of water in each bottle and adjusting for the number of mice per cage. We found that the average dose for the 7 month start cohort (7msc) was 314.24 mg Zn/kg, while the 8 month start cohort (8msc) consumed less per day at 202.50 mg Zn/kg, and the 9 month start cohort (9msc) was intermediate at 273.05 mg Zn/kg.

Oral zinc acetate has been shown to be well tolerated in humans as a copper lowering therapy for Wilson's disease [36]. However, these patients have increased copper levels at baseline compared to normal controls. We wanted to determine if our copper lowering therapy in mice that have normal copper levels was toxic. The first measure of health effects of copper lowering was changes in body weight. We considered weight loss of 10% or more to be a potential indicator of toxicity of treatment. Mice were analyzed for body weight approximately every 3–4 days for the six month course of treatment and averaged. We found that the average body weight over the treatment course of six months did not vary significantly between vehicle and zinc treated controls in either the Tg2576 or wild-type genotypes during the 7, 8 or 9mscs (Fig 2).

Copper deficiency in humans is a rare condition, and recently was found associated with myelopathy where patients usually present with gait difficulties [37]. Therefore, we wanted

to test whether treatment with zinc caused any balance dysfunction or locomotor changes. Balance dysfunction was assessed using the rotarod performance test (Fig 3A). We found that in general the Tg2576 mice stayed on the rotarod for shorter durations than the wild type mice, however this was not significant. We also observed that there was no significant difference between the treatment groups for either genotype. To address the possibility of zinc treatment affecting locomotor performance, we assessed the mice using the open field test which measured the total distance moved by the mice. We found that in the 7msc, there was no significant difference between the genotypes or the treatment groups (Fig 3B). We also found that with the 8msc and 9msc there was also no significant difference in movement activity between genotypes or treatment groups (Fig 3C, D). Taken together the data indicates no significant health or neurological defects with the administration of oral zinc acetate.

Levels of ceruloplasmin are mildly affected in response to zinc acetate administration

The protein ceruloplasmin is the major copper carrying enzyme in the blood. As copper levels are lowered, less copper is bound to ceruloplasmin and the apoenzyme becomes unstable and is degraded. We utilized the plasma ceruloplasmin levels to determine if the copper lowering strategy was effective, thus resulting in lower levels of ceruloplasmin. We found that in the 7msc both the Tg2576 and wild type mice treated with zinc had lower ceruloplasmin as compared to the respective genotype vehicle controls, however only the wild type zinc-treated mice showed significantly lower ceruloplasmin levels (Figure 4A). In the 8msc we also found that the ceruloplasmin levels were less in the zinc treated animals, however not statistically so (Figure 4B). Finally, in the 9msc the levels of ceruloplasmin were only slightly reduced as compared to the vehicle mice (Figure 4C).

Levels of plasma and brain metals are altered by oral zinc acetate

In order to assess the efficacy of treatment, we examined the plasma and brain levels of copper and zinc using IC-PMS (Fig 5). We hypothesized that with increased intake of zinc acetate as a copper lowering therapy; there would be an increase in the plasma and brain levels of zinc, with a corresponding decrease of copper in the brain, but levels of copper in the plasma would remain unchanged. We found that there was a significant increase in the levels of zinc in the plasma of all zinc treated mice, regardless of genotype or cohort start time (Fig 5A). Interestingly, the levels of plasma zinc in the 9msc treated with zinc were lower than in the 7 and 8msc despite the levels of plasma zinc in the vehicle treated mice being the same between all groups. Contrary to our hypothesis, we found that the levels of zinc in the brain were unchanged with treatment across all cohorts despite the approximately doubled levels in the plasma (Fig 5B). Surprisingly, only the 7msc wild type zinc treated mice had reduced copper levels in the plasma as compared to vehicle treated mice (Fig 5C). This result corresponds with a significant decrease in ceruloplasmin levels found in the same mice (Fig 4A). Finally, we observed a significant decrease in the amount of brain copper with zinc treatment in all cohorts and all genotypes as compared to the matched vehicle controls (Fig 5D).

Zinc acetate treatment did not affect the Morris Water Maze

After treatment, the spatial memory function of each cohort was evaluated by the Morris Water Maze task. During the visible platform task, all genotypes and treatments successfully learned the task as determined by a decrease in the duration to find the hidden platform from day 1 to day 2 (Fig 6A). Interestingly in the 9msc, the zinc treated Tg2576 mice found the visible platform faster than the vehicle treated Tg2576 and the zinc treated wild type mice took longer to find the visible platform than the vehicle treated wild type mice. However, all mice in the 9msc found the visible platform faster in the second day as compared to the first day. In the 7msc (Fig 6B), 8msc (Fig 6C) and 9msc (Fig 6D) hidden platform training all mice had minor improvement in finding the hidden platform from training day one to training day five. There was a non-significant trend for the wild type mice (open and closed triangles) to reach the platform in a shorter amount of time at the end of the five days of training compared to the Tg2576 mice (open and closed squares) for all cohorts. In an effort to determine subtle learning changes, we analyzed the retention between training days, defined as the difference between the duration to find the platform of the first hidden platform trial of the day and the duration to find the platform of the last hidden platform trial of the previous day. We found a significant reduction for time to locate the hidden platform in zinc treated Tg2576 mice when compared to vehicle treated Tg2576 mice. However, this difference was only observed during the earliest hidden platform training (days 1 and 2) and only in the 7msc cohort. No other differences in training between zinc and vehicle treated wild type or Tg2576 mice were found during mixed model analysis. In the 2 hour probe trial, the wild type mice in all cohorts showed memory of the platform as indicated by spending more than 25% of the time in the target quadrant (Fig 6E). Interestingly the 8msc wild type zinc treated mice showed decreased memory of the platform as compared to the vehicle treated counterpart. The Tg2576 vehicle treated mice on average showed no learning and memory of the task as indicated by spending 25% or less time in the target quadrant. The Tg2576 zinc treated 8msc and 9msc showed higher than 25% dwell time, but this was not statistically significant from the vehicle treated Tg2576 mice. In the 72 hour probe trial the 7, 8 and 9msc showed no learning behaviors as indicated by 25% or less dwell times for all treatment groups and genotypes (Fig 6F). Taken together, treatment of both wild type and Tg2576 mice showed no real improvement in the learning and memory tasks utilizing the MWM.

Insoluble amyloid is reduced in response to copper lowering

The Swedish mutation in A β PP is known to promote the formation of insoluble A β species [38]. We hypothesized that our copper lowering treatment would reduce the burden of insoluble A β in the brain. We measured the A β burden in the brain by both ELISA, for the quantity of change overall, and IHC for changes by location. The anterior left hemisphere of each mouse was homogenized and subjected to an ultracentrifugation protocol to isolate insoluble proteins from soluble species. We then analyzed the amount of soluble and insoluble A β_{40} and A β_{42} in each homogenate by commercially available ELISA kits which have antibodies specific for each form. The amount of insoluble A β_{40} was significantly reduced in the zinc treated Tg2576 mice, as compared to the vehicle treated mice, in the 7msc, but not the 8 or 9msc (Fig 7A). The amount of insoluble A β_{42} was also significantly reduced in the zinc treated Tg2576 7 and 8msc, but not the 9msc, as compared to vehicle

treated mice (Fig 7C). We observed a non-significant trend of increased insoluble A β ₄₀ and A β ₄₂ in the 9msc treated with zinc, as compared to the vehicle treated mice (Fig 7A, C). The amount of soluble A β ₄₀ and A β ₄₂ was not significantly affected in any of the treatment groups (Fig 7B, D). It is interesting to note that the amount of soluble A β ₄₀ and A β ₄₂ is orders of magnitude lower than the amount of insoluble A β ₄₀ and A β ₄₂, indicating that A β species in the Tg2576 mice at these ages is mostly insoluble. The right hemisphere of each mouse was sectioned and probed with an anti-A β ₄₀ antibody (both soluble and insoluble forms) (Fig 7E). We found that with age the amount of A β ₄₀ in the cortex is increased, but not in the hippocampus, similar to previously reported results. We also observed no significant reduction in the number of A β particles per in.² measured in any of the cohorts in both the cortex and hippocampus when treated with zinc as compared to vehicle. Taken together, this data suggests that copper lowering therapy can reduce insoluble A β , while having no effect on total soluble A β , and has little effect on overall plaque formation.

Discussion

These data show that systemic copper-lowering with oral zinc acetate can lower cerebral levels of copper and insoluble A β . This treatment is distinct from prior studies of clioquinol and PBT2 because the study drug does not cross the blood brain barrier, but acts to lower copper systemically. Remarkably, oral zinc treatment does not alter brain levels of zinc, which is important because increased brain zinc might be expected to exacerbate A β pathology. The reason for stable brain zinc levels in these mice with elevated plasma zinc is unknown, but the finding implies that brain zinc is tightly regulated.

These findings are also consistent with a recent study showing that lower copper disrupts A β homeostasis [39]. The findings also support previous studies showing that zinc deficiency exacerbates A β pathology in A β PP mice [40], and complement our previous report of attenuated A β pathology in A β PP mice with systemic copper-lowering achieved by the investigational drug Tetrathiomolybdate [28]. In contrast to Tetrathiomolybdate, however, oral zinc is associated with very little toxicity, with no evidence of any adverse effects of oral zinc upon body weight, rotarod, or open field behavior. The lack of toxicity makes oral zinc a candidate for use in human subjects.

However, the lack of an effect of zinc therapy upon hippocampal memory function in the MWM is discouraging with respect to the potential clinical value of this approach. The MWM results are reminiscent of our observations with Tetrathiomolybdate, which also failed to improve memory despite reduced brain levels of A β . This may reflect a need for a greater degree of A β lowering in order to see an effect on human brain tissue, or could be explained by several other mechanisms. One alternative explanation holds that the pathologic correlate of the cognitive change in AD may be the neurofibrillary tangle, rather than the A β plaque, and since Tg2576 mice lack significant NFT pathology, this set of experiments does not address this possibility.

In support of NFT pathology playing a role in the hippocampal memory changes, a previous study showed that a low dose of zinc sulfate given from 1 month of age in 3xTg mice not only lowered A β plaque and tau tangle load, but improved memory [41]. A follow up study

showed that even a low dose of zinc for a long time was sufficient to lower brain copper levels [42]. This is important as the 3xTg strain is genetically different from our Tg2576, as well as the wild-type, indicating the mechanism of copper lowering is applicable to various genotypes.

Interestingly, a previous study examining zinc treatment in the Tg2576 model showed a worsening of cognition, but a reduction in plaque size and number with zinc carbonate treatment [43]. A key point to emphasize about the study design is that mice were administered zinc in their water prenatally and if the treatment acts in a similar manner to our study by lowering systemic copper, the mice born to zinc treated mothers would have a copper deficiency from birth, most likely resulting in developmental issues. Unfortunately, the study by Linkous et al. did not examine the copper and zinc levels in the plasma and brain of the mice. The data however, supports the idea that zinc supplementation can reduce A β plaque burden, as has been seen in both the Corona et al. and our current study. Taken together, the idea of enhancing dietary zinc to lower systemic copper in an effort to reduce plaque burden has been replicated in multiple studies. The interesting effects of how zinc supplementation may affect learning and memory requires additional testing with mice that contain human tau, as the 3xTg model indicates that tau is important in this process. A similar set of experiments to our current study utilizing murine models specific to NFT pathology is under way, with the expectation that those models will help us clarify whether systemic copper lowering strategies are feasible to be translated to human subjects.

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Figure 1.

Timeline of pathology and treatments. The progression of pathology in Tg2576 mice is illustrated over time. Mice begin showing A β accumulation around 6–7 months of age with increasing plaque accumulation at 7–8 months of age and rapidly developing until 18 months of age. After this point the amount of accumulation remains similar as the mice age further. Behavioral changes begin around 9–10 months of age and worsen with aging. The treatment cohorts were designed to begin corresponding to the human dementia condition. The 7msc is meant to represent a mild cognitive impairment case with some plaque formation, but few cognitive changes. The 8msc is meant to represent mild AD with learning and memory changes and more plaque formation. The 9msc is meant to represent moderate AD with robust plaque formation and more robust behavioral changes.

Figure 2.

Body weight changes upon treatment with zinc acetate. The mean body weight over the treatment course (6 months) for each mouse is displayed as closed circles (●). The mean $\pm$ SEM for the entire treatment group is plotted for the 7msc (A), 8msc (B), and 9msc (C). The weights did not vary significantly over the course of treatment for either the Tg2576 or wild type mice, when analyzed by a student's t-test.

Figure 3.

The assessment of balance and locomotor function with zinc acetate treatment. (A) Tg2576 and wild type mice were assessed for balance changes after treatment with zinc acetate (6 months after start) using the rotarod test. Data is displayed as the mean $\pm$ SEM duration on the rotarod at the beginning of trials [B] and the end of trials [E]. No significant changes were observed between vehicle and zinc acetate treated groups. The 7msec (B), 8msec (C), and 9msec (D) were analyzed for locomotor function by the open field movement test. Data points (●) are represented as the total distance traveled in 5 minutes for each mouse. The

mean $\pm$ SEM is plotted for all mice in each treatment group. No significant differences were detected between vehicle and zinc acetate treated groups for either the Tg2576 or wild type mice, when analyzed by a student's t-test.

Figure 4.

Ceruloplasmin activity after six months of treatment. The activity of ceruloplasmin was measured as a marker for copper reduction in the plasma of 7msc (A), 8msc (B), and 9msc (C) at the conclusion of the treatment (6 months after start). Two replicates were analyzed for each mouse. The mean $\pm$ SEM for all mice in the zinc acetate treated group of the reported genotype/cohort is plotted as a percentage of the corresponding vehicle group for each genotype. *** = $p < 0.001$.

Figure 5.

Analysis of plasma and brain zinc and copper. Samples of plasma and brain tissue were collected for each mouse and analyzed by ICPMS for copper and zinc levels. (A) Zinc or (B) copper levels in the plasma represented in parts per billion (ppb). (C) Zinc or (D) copper levels in the brain tissue represented as μg of zinc per gram of brain tissue homogenized. Data is represented as the mean $\pm$ SEM of all mice in the treatment group. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Figure 6.

Spatial learning and memory assessment after treatment with zinc acetate for six months.

(A) The visual platform was used to train mice to find a platform raised above the water level. Mice were trained for two days. Data is represented as the mean $\pm$ SEM of the time to reach the platform in seconds, for all mice in the treatment group. (B–D) the hidden platform was used to examine the learning ability of mice to find a platform hidden beneath the surface of the water using visual cues around the room for the 7msc (B), 8msc (C), and 9msc (D). Each training day is the average of 6 trials per day for each mouse in the treatment group. Data is represented as the mean $\pm$ SEM for the time to find the hidden platform in

seconds. The probe test is used to measure how well the mice remember where the hidden platform was located using the visual cues provided. Mice were tested at 2 hours (E) and 72 hours (F) after the final hidden platform training trial. Data represents the mean $\pm$ SEM of the percentage of time the mouse spent in the target quadrant for all mice in the treatment group. * = $p < 0.05$, ** = $p < 0.01$.

Figure 7.

Assessment of Aβ levels in response to zinc acetate treatment. The amount of total insoluble Aβ₄₀ (A) or Aβ₄₂ (C) and soluble Aβ₄₀ (B) or Aβ₄₂ (D) from brain tissue was analyzed by ELISA for the Tg2576 mice treated with vehicle or zinc acetate. The data is represented as the mean ± SEM absorbance of all mice in the treatment group. (E) Immunohistochemistry was performed on three sections of the right hemisphere of each mouse for total Aβ. Sections were analyzed for the total staining present in the cortex [C] or hippocampus [H]. Data is represented as the mean ± SEM number of particles measured perin². * = p < 0.05.