CONCENTRATION OF METAL'S IN ALZHEIMER'S DISEASE PLAQUES

by

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ABSTRACT

CONCENTRATION OF METALS IN ALZHEIMER'S DISEASE PLAQUES

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Alzheimer's disease (AD) is the most common form of dementia in the world. This study focused on the effects of how different metals added to drinking water affected the metal concentrations in the plaques. There is disagreement in the field about some of the underlying mechanisms in relation to trace metals where concern is if these trace metals are harmful or beneficial. It was hypothesized that adding trace metals to drinking water would have a significant effect on the metal concentration of the amyloid plaque. This study examined the metal concentrations in the plaques in the Tg2576 mouse brain raised on 4 different types of waters, specifically with different metals added to the water, in addition to a lab water group. Mice were raised on water treatments from 3-14 months postnatal. They were broken up into four groups reflective of the metal added to the water: 10ppm $ZnCO_3$ (Zinc carbonate), 10ppm of $ZnCO_3$ & 0.25ppm $CuCO_3$ (Zinc & Copper carbonate), 10ppm of $Fe(NO_3)_3$ (Iron nitrate), and unspiked (tap) lab water. Data were collected from spectroscopes at National Synchrotron Light Source at Brookhaven

National Labs using the electron beam at X27a, while analysis was conducted at George Mason University. This study found water treated with various trace metals was significantly different among zinc and iron metal levels in the amyloid plaque, but came back non-significant for copper metal. The results of this study support that the trace metals added to the drinking to Tg2576 mice does have an effect on amyloid metal concentration. There is a limited amount of research focused solely on the amyloid plaque and the effects of trace metals in drinking water. This study adds to the complex dynamic of Alzheimer's disease by adding further the behavior of the amyloid plaque when exposed to different types of water.

1. INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia in the world. Currently, there are promising directions towards curing this disease or, at the very least, methods of slowing down its progression (Hebert el al., 2003). Alzheimer's disease is a degenerative disease that currently affects more than 5 million Americans (Hebert el al., 2003). Notable symptoms of AD include memory loss, aggression, and cognitive impairment each with increasing severity over time. Alzheimer's disease progresses to serious memory loss, depression, and confusion, which can also include sleeplessness and loss of appetite. The importance of understanding this disease is paramount, because as our population continues to age (e.g. baby boomers), the diagnosis of Alzheimer's disease will continue to rise. It is predicted by 2050, that the Alzheimer's disease rate will quadruple, with an estimated 43% prevalence of the cases requiring a high level of care (Brookmeyer, et al., 2007). It was predicted that a delay in both onset and progression of the disease by a year would reduce nearly 9.2 million cases in 2050 (Brookmeyer, et al., 2007). This is particularly important to late onset care. Alzheimer's disease has been found to have a genetic causes (early onset), and genetic risk factors (late onset) (Lambert et al., 2009). When detected early, measures can be taken to improve the quality of life for both caregivers and patients.

The impairment in memory is one of the hallmark features of Alzheimer's disease. It is characterized by the loss of episodic memory, which leads to progressive impairment in other domains of cognition. This leads to difficulties in activities of everyday living for both the person afflicted with the disease and the family/people involved. Examples include losing memories of past and significant events, people's names and faces, and a sense of self. It equates to the individual with Alzheimer's disease losing themselves as the disease progresses. The disease eventually progresses to the point of such severe cognitive impairment that the people afflicted need constant assistance for daily living. Caring for a person with Alzheimer's disease can be challenging because impairment in communications and cognition makes it problematic.

There has been research involved to detect Alzheimer's disease as early as possible. Fernandez et al. (2010) found that the behavioral protocol, Behavioral and Psychological Symptoms of Dementia (BPSD), is a useful evaluation tool in detecting a possibility that the person has Alzheimer's disease. Another useful measure for detecting Alzheimer's disease is the use of Pittsburgh Compound-B (PIB) developed to detect amyloid in the brain, (Klunk et al., 2004). The goal was to detect Alzheimer's disease through the use of in vivo imaging techniques. Klunk et al. (2004) were able to develop a dye that bound to the amyloid and was able to cross the blood brain barrier. In their research, AD patients showed the most retention of PIB compared to controls. The amyloid was detected with the use of a positron emission tomography (PET).

Molecularly, Alzheimer's disease is characterized by the extracellular accumulation of the protein amyloid beta and the intracellular accumulation of tau

protein, which cause plaques and tangles, respectively (Braak & Braak, 1991). This is followed by a decrease in neuronal density and atrophy of the brain. Magnetic resonance imaging (MRI) shows shrinkage in the gyri and widening of sulci (Whitwell, 2010). Alzheimer's disease patients show atrophy involving the medial temporal lobe, specifically affecting the hippocampus, enthorhinal cortex, and the temporoparietal association neocortex (as cited in Whitwell, 2010).

In a basic overview, amyloid-beta is formed when APP (amyloid precursor protein) is cut abnormally. A number of enzymes are involved with the cutting of APP: alpha (α), beta (β), and gamma (γ) secretase. When APP is cut by alpha secretase, it prevents the formation of amyloid fragment which the body can clear out. This is known as the non-amyloidogenic pathway and produces cleavage.

When APP is cut by ß and γ in different locations, this produces the harmful amyloid fragment, which consequently forms amyloid plaques, a common feature in Alzheimer's disease. Gamma and beta secretase produce amyloid-beta in various lengths, generally 39 to 43 peptides long (Lorenzo et al. 2000). The most common strains are $A\beta_{42}$ and $A\beta_{40}$, in which the subscript denotes the length of the peptide strain. $A\beta_{40}$ is the most commonly produced, but research shows $A\beta_{42}$ is more dangerous as it aggregates more readily (Selkoe, 2001). Colletier et al. (2011) found evidence that suggested the polymorphic structure of $A\beta_{42}$ contributes to the toxicity by exhibiting an elevated aggregation with the stacking of β pleated sheets by both parallel and anti-parallel terminals versus the other various forms of Aβ found in plaques.

This study focused on the effects of how different metals added to drinking water affected the metal concentrations in the plaques of each group. As mentioned previously, the plaques are thought to have a role in AD.

Specifically, we looked at the effects of adding zinc, copper & zinc, and iron to drinking water, and examined the effects of the metal concentration of the plaques in the Tg2576 mice. There has been a lot of positive progression towards slowing down Alzheimer's disease, but still no cure. There is disagreement in the field about some of the underlying mechanisms in relation to trace metals. It mostly concerns whether these trace metals are harmful or beneficial, or if they have any impact at all as these three metals are found in amyloid plaques. It is suggested that high concentration of metals in amyloid plaques contribute to the higher amounts of oxidative stress in Alzheimer's pathology (Huang et al., 2004).

The present study uses an animal model to study Alzheimer's disease, as the methods involved in this study would be unethical if used on humans. The Tg2576 mice contain the Swedish mutation of human APP; they were genetically engineered to develop AD plaques by the addition of human APP with two mutations in order to speed up plaque production (Hsiao, 1998). The Tg2576 mouse acts as an animal model of Alzheimer's disease, mimicking the amyloid plaques and memory loss found in human patients with Alzheimer's disease. This paper analyzed metals in plaques of Tg2576 mice using X-ray spectroscopy. X-ray spectroscopy examines the metal concentrations in a given sample. (George & McIntyre, 1994; Jenkins & DeVries, 1968).

Zinc

Research studies involving zinc are numerous, and opinions on the role of zinc in Alzheimer's disease are divided. As early as 1980, Burnet proposed that zinc deficiency led to the causation of dementia (Burnet, 1981). Subsequent studies seem to point towards an imbalance of zinc levels that could be contributing to accelerating Alzheimer's disease. Research finds zinc promotes the aggregation of endogenous amyloid beta in cerebrospinal fluid in low concentrations (Brown et al., 1997). This suggests the possibility that zinc is a key factor in AD plaque formation and/or stabilization. High or low levels of zinc can be neurotoxic, leading to neuronal death. As people age, the homeostatic mechanisms that keep zinc within normal levels can lead to neuronal death (Cuajungco et al., 2000).

In related research, it was found that Aβ is rapidly aggregated by zinc at pH 7.4 (Bush et al. 1994). A review by Sensi et al. (2009) reported studies that found the modulation of zinc binding to amyloid is regulated by the metallothionein 3 (MT3), a protein released by astrocytes in the central nervous system (CNS). It was found that in AD, these proteins' activity is reduced, possibly explaining the high concentration of metals in the Alzheimer's plaques. Sensi also stated that the AD brain is under oxidative stress. This has the potential to increase zinc release, leading to increased beta aggregation in the synapse. Copper absorption through the intestinal wall can be impeded by high amounts of zinc in the system (Maret & Sandstead, 2006).

Zinc has been found to inhibit the α secreatase enzyme, responsible for cutting APP into $A\beta_{40}$., leading to higher levels of $A\beta_{42}$, with increased likelihood of production of insoluble plaques (Cuajungco & Faget, 2003). Evidence was found that zinc changes

the confirmation of Aβ, interfering with copper binding, preventing the formation of free radicals in vitro (Huang et al., 1999a). This suggests that zinc acts a protectorate against oxidative interactions from copper and iron (Cuajungco et al., 2000).

In the brain, zinc is found in high concentrations in the hippocampus, amygdala, and neocortex (as cited Dong et al., 2008). As stated earlier, zinc has been found to be a key player in the role of amyloid formation. Experiments involving ZnT3 knockout mice have further revealed the role of zinc. ZnT3 is an enzyme that is responsible for Zinc transport from the pre-synaptic neuron into the vesicles. In ZnT3 knockout mice, loss of zinc release reduced Aβ formation in mutant APP transgenic mice. This was further supported by a study conducted by Deshpande et al. (2009) who hypothesized that the increase in zinc concentration attracted and promoted additional amyloid beta accumulation at the synapse. A study conducted by Cole et al. (1999), using the ZnT3 knock out mouse, resulted in the complete disappearance of zinc in synaptic vesicles throughout the brain without affecting other nonvesicular pools of zinc. This led to the conclusion that zinc release in the synapse is critical for amyloid beta synaptic accumulation.

Linkous et al. (2009) studied the effects of zinc added to the drinking water, comparing the Tg2576 (Tg) mice versus wild type mice (Wt), forming of four groups.

Linkous et al. (2009) used the Morris water maze to test the spatial memory of the mice and found transgenic mice raised on zinc-treated water had the most impairment. Overall, they found that zinc impaired spatial memory for both wild type and transgenic mice, but the impairment to spatial memory was more profound in the transgenic mice.

Linkous et al. (2009) also found the Tg2576 mice with the zinc-treated water had smaller and fewer plaques than the Tg2576 mice raised on lab water. Interesting to note that these are the same mice that had the poorest performance with the Morris water maze. In contrast, the Tg2576 mice raised on lab water showed the best performance. This indicated a complex relationship of plaques and behavior that is not well understood.

Copper

Copper, when oxidized leads to free radicals in the system, which consequently leads to further damage to brain tissue (as cited Shcherbatykh & Carpenter, 2007). Huang et al. (1999b) found APP has a high affinity copper binding site in the extracelluar domain, reducing Cu^{2+} to Cu^{+}, which is then used as a catalyst for the production of H_2O_2, an enzyme which is known to be harmful to neural tissue. This further leads to free radical production (oxidative stress), consequently leading to furthering the pathogenesis of Alzheimer's disease.

Copper is believed to induce aggregation of Aβ peptides into plaques. There is an association of increased levels of copper in the brain with higher levels of Aβ deposition. In a review of their work, Bush and Tanzi (2008) stated that with decreased activity from the MT3 protein, there is an increase in copper (as well as zinc) left in the synapse. Another factor in aging is the decreased release of the MT3 protein, a protein that helps regulate the amount of copper in the synapse. With the lessened activity, it results in the excess copper and zinc metal ions free to aggregate to amyloid beta oligomers.

The view of the role of copper in the field is divided. There are studies that show copper to be a contributing factor in Alzheimer's disease, while other studies show it can

restore behavioral impairments. Copper is used everywhere throughout the body, and is essential in every day enzyme activity. Copper has been looked at as another factor in Alzheimer's disease pathogenesis because it has been found in amyloid plaques (Bush, 2000). Researchers have found that there is a dysfunction in the balance of copper in the brain, whether there is an excess or a deficiency (Zucooni, 2007). It is well known that dietary zinc inhibits the absorption of copper intestinally, leading to a copper deficiency (Sandstead, 1995). This was subsequently supported in that rats raised on zinc enhanced water showed high levels of zinc in the brain, specifically in the cortex, while copper was found to be deficient (Flinn et al., 2005). When copper was added to zinc treated water, it restored spatial memory impairments as well as fear extinction, suggesting that reduction in copper levels plays an important factor in memory (Micheli et al. 2007).

In postmortem AD neural tissue, Bayer and Multhaup (2005) found significantly lower levels of copper in transgenic mice compared to controls, supporting an inverse relationship between copper levels and Aβ accumulation. In support of Linkous et al. (2009), the administration of copper lowered Aβ in the brain (Bayer & Multhaup, 2005).

Iron

Iron has also been found to be factor in Alzheimer's disease, as it contributes to oxidative damage to the brain (Morgan et al., 2004). Just like copper, iron forms reactive oxygen species that damages neural tissue. Iron is found in both neurofibrillary tangles and plaques. Over the progression of Alzheimer's disease, iron levels in the body increase, while the levels of ferritin, a protein that regulates levels of iron, do not increase

(Zecca et al., 2004). This in turn leads to great availability of iron to produce higher oxidative damage to the brain.

In a study examining the effects of iron, it was found that iron redox activity was significantly high at the earlier preclinical stages of AD than previously thought (Smith et al., 2010). Mattson & Butterfield (1995) originally predicted that oxidative stress was the end stage manifestation of Alzheimer's disease. Smith et al. (2010) reported dysfunction in the homeostatic mechanisms and found numerous cortical regions were affected. Areas that were considered unaffected in Alzheimer's disease & iron redox activity (i.e. the cerebellum) were found to contain oxidative stress. It is known that Aβ adds further oxidative stress, and in combination with the decreased function in iron regulation, leaves neural tissue to further oxidative damage.

Mitochondria and cellular metabolism are compromised both structurally and chemically in Alzheimer's disease, with an overall reduction in size, shape and intact cristae (Blass et al., 1997). Few mitochondria exhibited normal morphology in AD neurons (Hirai et al., 2001). In combination, this cycle of Reactive Oxygen Species increasing along with Alzheimer's disease, would lead to continuing damage to neural tissue, further exacerbating AD pathology.

Metal Chelators

By examining zinc (as well as copper), researchers have been able to narrow down the mechanisms that cause amyloid accumulation at the synapse. It has been demonstrated by previous studies that zinc release leads to amyloid beta accumulation in the synapse (Bush et al., 1994). Factors include aging, which has lead to the disruption in

the homeostatic mechanisms that keep metal levels in the brain within normal parameters (Bush and Tanzi, 2008). With the disruption of the homeostatic mechanisms, this has led to increased levels of zinc in the synapse. In Alzheimer's disease, amyloid beta is being formed and released into the synapse. This, combined with the increasing amounts of zinc, leads to amyloid beta oligomers, as amyloid beta has a high affinity for zinc. Research has shown that zinc amyloid beta oligomers favor binding to the NMDA receptor, leading to the disruption of the NMDA receptor (as reviewed by Sensi et al. 2009), which is critical in long term potentiation (LTP), the mechanism involved in learning (Kodirov et al., 2006). At the same time, copper is being released from the post synaptic neuron as a protectorate to the NMDA receptor. This leads to a increase in both zinc and copper in the synapse. MT3, a protein released by astrocytes, mediates the levels of copper and zinc in the synapse. With aging as a factor, there is a marked decrease in the release of the MT3 protein, which results in the excess copper and zinc metal ions free to aggregate to amyloid beta oligomers (Sensi et al, 2009).

Disruption of the LTP can explain the disruption in memory in aging adults, as metals bind to the NMDA receptor, involved in LTP. Factors in aging lead to dysfunction in the mitochondria in the hippocampus. Loss of ATP because of mitochondria failure leads to further disruption in the homeostatic mechanism, as zinc reuptake is energy dependent (Bush & Tanzi, 2008). With impairment to the reuptake of zinc, this leads to more zinc being left in the synapse to further aggregate with amyloid beta. A study conducted by Li et al. (2011) found Aß oligomers binding and inhibiting NMDA receptors in the hippocampus. Overall, they found at low nanomolar levels, Aß oligomers

present increase the activation of extrasynaptic NR2B-containing receptors which impair synaptic plasticity. Li et al. (2011) suggest a development of an effective extrasynaptic NR2B antagonsists could have the potential to alleviate impairment in the early stages of Alzheimer's disease.

It appears research is approaching a remedy for the accumulation of amyloid beta in the synapse. One goal was to use metal chelators to rid the excess metal ions that cause amyloid beta build up. Early metal chelators were not successful as they were chelating other metal ions too, leading to an impairment in daily functioning of Alzheimer's diseased patients during clinical trials (Bush and Tanzi, 2009). Compounds such as clioquinol and PBT2 have led to more promising results, as they target only zinc and copper specifically, and have not led to impairment in daily functioning. Clioquinol has been found to restore the levels of zinc and copper in the neurons, as previous studies have shown that rats, prior to trials, experienced zinc and copper neuronal deficiencies. It has shown promise, as not only did clioquinol reduce amyloid beta accumulation, but most importantly restored cognitive functioning. PBT2 produced the same results, but achieved this at a faster rate. PBT2 has better blood brain barrier penetration and reversed cognitive dysfunction faster than clioquinol (Bush and Tanzi, 2009).

X-Ray Spectroscopy

This current study used X-ray spectroscopy to analyze Tg mouse brain tissue. Specifically, we analyzed the metal concentration of the mouse plaques using X-ray fluorescence emission. X-ray emission spectroscopy allows researchers to see the relative concentration of metals and where these metals are located.

X-ray spectroscopy operates by using high energy X-ray focused on the sample. This causes the atoms of the sample to be excited, and subsequently, moves the electrons from lower energy orbitals to higher energy orbitals. These high energy orbitals are not stable, and when the electrons fall back down to their original low energy orbital, they emit photons with wavelengths in the X-ray regions. Detectors then can determine the wavelength of the photon emitted. Metals (as all atoms) have unique patterns of filled orbitals, so it is possible to determine which metals are present by the wavelengths that are emitted by the photon from the sample (Jenkins & DeVries, 1967).

The X-ray spectrometry was performed at the National Synchrotron Light Source at Brookhaven National labs in New York. The advantages of using spectroscopy from the Brookhaven labs were higher resolution and higher accuracy from the data. Synchrotrons power the Brookhaven labs which release x-rays as they travel, they are cyclical particle accelerators creating high energy beams of electrons using powerful magnetic fields. They offer considerable advantage when analyzing small samples over smaller x-ray spectrometers, such as the sample from the Tg2576 mice neural tissue from this study (Miller et al., 2006).

Hypothesis

In order to further understand the role of the different metals, this study examined the metal concentrations in the plaques in the Tg2576 mouse brain raised on different types of waters, specifically with different metals added to the water, in addition to a lab water group. Ashley Bush, an authority in metals research in Alzheimer's disease, believes the metals cannot pass through the blood brain barrier (Bush & Tanzi, 2008).

Despite the inconclusiveness of the evidence of the effects of metals added to drinking water, it was predicted that a difference would be detected among the groups. With the lack of unity on the subject, the precise direction on where these changes lie cannot be specifically predicted. The objective of this study was to further understand if metals present in drinking water would have an effect on the metal concentration of amyloid beta plaques present in an AD animal model.

2. METHODS

Mouse Rearing and care

This experiment used transgenic 2576 mice that possessed a genetic APP mutation. The Tg2576 mouse produces high levels of Aβ in the cortex, specifically in the areas of the hippocampus, entorhinal cortex, frontal cortex, and temporal cortex. The Tg2576 mouse was designed to emulate Alzheimer's disease in humans, and has also been found to develop long term and spatial memory impairments (Hsiao, 1998).

The mice in this experiment were part of a cohort, specifically cohort 4. The mice of cohort 4 mice were raised in our colony, at George Mason University, on three different types of metals added to the water. Mice were raised on water treatments from 3-14 months postnatal. They were broken up into four groups reflective of the metal added to the water: 10ppm $ZnCO_3$ (Zinc carbonate), 10ppm of $ZnCO_3$ & 0.25ppm $CuCO_3$ (Zinc & Copper carbonate), 10ppm of $Fe(NO_3)_3$ (Iron nitrate), and unspiked (tap) lab water. These values were verified by using a mass spectrometer at the US Geological Survey. All mice were sacrificed at 18 months of age. Brains were extracted at the time of sacrifice and flash frozen in dry ice.

Prior to the start of the analysis, there were a total of forty-six Tg2576 mice. For reasons unknown, some of the data from the Tg2576 mice went missing and/or became unusable. This data that were corrupted were tossed out of the study. The groups were

originally broken down as such: 31 mice were given water treated with 10ppm of Zinc carbonate, 21 mice were given water treated with 10ppm of Zn with 0.25ppm of Cu (Zinc and Copper carbonate), 32 mice were given water treated with 10ppm of Fe nitrate, and 25 mice were given unspiked lab water. Total number of subjects used in this study came out to 109 Tg2576 mice.

Brains were sliced using a cryostat at -20 degrees Celsius. Each brain was sliced at 20 microns and affixed to zinc free slides for analysis. Congo red protocol was used to dye brain slides to confirm the location of AD plaques within the mouse brain.

Data was collected from spectroscopes at National Synchrotron Light Source at Brookhaven National Labs using the electron beam at X27a, while analysis was conducted at George Mason University. The data was measured using counts per second, where the counts is a measure of the number of photons released from the sample. The advantage of using the counts per second measurement allows for observation of metal concentration. However there is a disadvantage using the counts per second measurement, since it only gives us a relative concentration of the metal in a sample and not an exact amount. The conversion from counts per second to parts per million does not stay constant between metal groups. Calibration standards are used to determine more accurate amounts of metal in a given sample. The standards that were used are of sheep brain tissue spiked with known amounts of metals. A standard curve is created from which actual ppm values can be calculated. Standards were created for each trip to Brookhaven National Labs, as standards degrade over time and can only be used once.

Once X-ray spectroscope has finished analyzing and collecting the wavelengths from the photons from a sample, they are converted into peaks. These peaks indicate the number of counts per second recorded for each metal in the sample. It is required to preprocess these peaks before further analysis, because of an anomaly of the driver program. The driver program collects and formats data from the X27a beam. The program used for data collection, using the X27a beam, requires users to indicate peaks of metals that pertain to the study. Unfortunately the program does not allow the user to manually enter values for the peaks, it conversely estimates these values. Computer programs X27a plotter and Multi-Channel Analysis (MCA) plotter were used to conduct peak fitting. After samples have been peak fitted, X27a Plotter was used to obtain the amounts of individual metals in a given sample.

3. RESULTS

The focus of this study was the effect of exposure to metals in drinking water on metal concentrations in plaques of the Tg 2576 animal model for Alzheimer's disease. Descriptive statistics comparing the four water groups (lab water, iron, zinc water, and zinc & copper water) for each metal (zinc, iron, and copper metal) found that zinc levels were highest in the lab water group with a mean of 42.70 parts per million. Iron levels were found to be the highest in the lab water group with a mean of 49.76 parts per million compared to the other water groups. Overall, lab water and iron water groups had higher amounts of zinc and iron found in the plaques that were analyzed in this study (see Table 1).

Table 1

Descriptive Statistics of Metals in Plaque by Water Type

	Water Type	Ppm	Sample Size
Zinc	Lab Water	42.70	25
	Iron Water	31.29	32
	Zinc & Copper Water	14.96	21
	Zinc Water	11.42	31
Copper	Zinc & Copper Water	7.50	21
	Zinc Water	6.98	31
	Iron Water	6.10	32
	Lab Water	3.11	25
Iron	Lab Water	49.76	25
	Iron Water	38.75	32
	Zinc & Copper	13.93	21
	Zinc Water	12.35	31

Due to assumption violations of normality, homogeneity of variance (see Appendix 1) related to parametric testing, nonparametric Kruskal-Wallis tests were used to compare water types for each metal studied to determine if any differences were statistically significant. Since the Kruskal-Wallis tests use ordinal data, the data were changed to ranks (see Table 2).

Table 2

Ranked Means of Metals in the Plaques by Water Type

	Water Type	Mean Rank	Sample Size
Zinc	Lab Water	77.80	25
	Iron Water	64.16	32
	Zinc & Copper Water	41.76	21
	Zinc Water	36.13	31
Copper	Iron Water	54.75	32
	Zinc Water	60.32	31
	Zinc & Copper Water	60.10	21
	Lab Water	44.44	25
Iron	Lab Water	77.36	25
	Iron Water	64.41	32
	Zinc & Copper	36.62	21
	Zinc Water	39.71	31

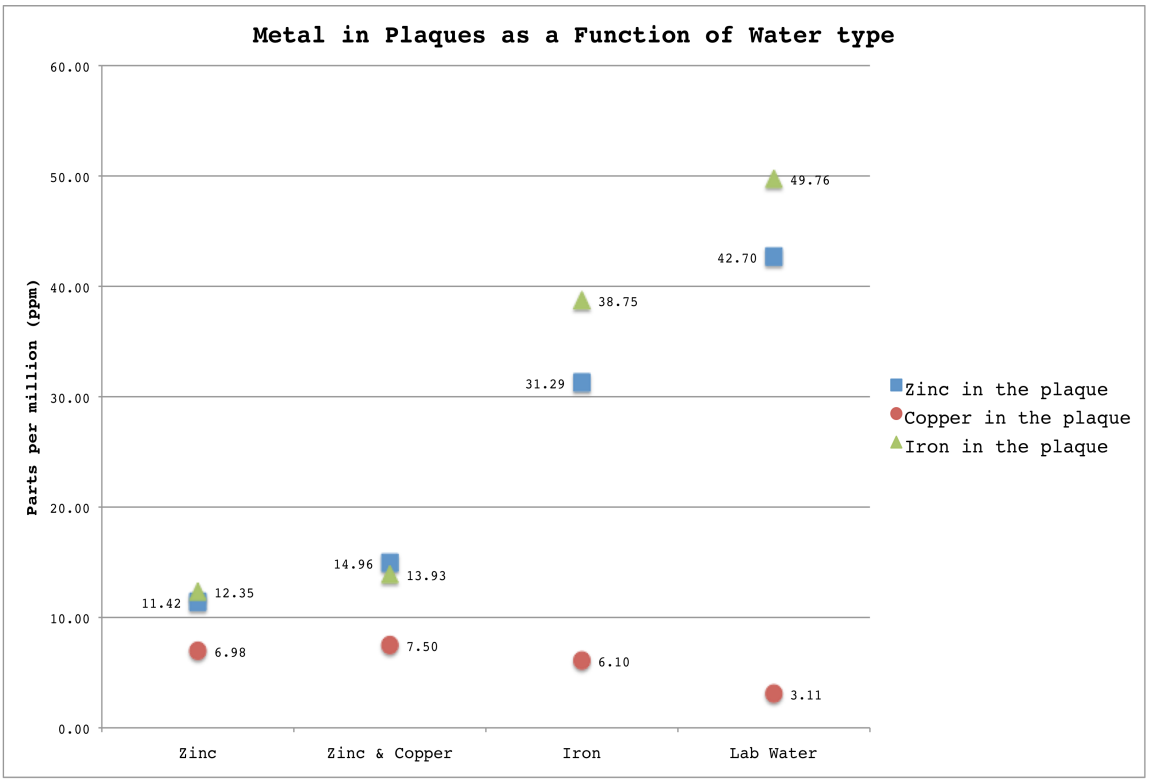

Fig. 1. Iron and zinc levels were significantly reduced in mice reared on Zinc and Zinc & Copper water, compared to Iron and Lab water. A significant difference for zinc levels was found for mice raised on iron water compared to lab water.

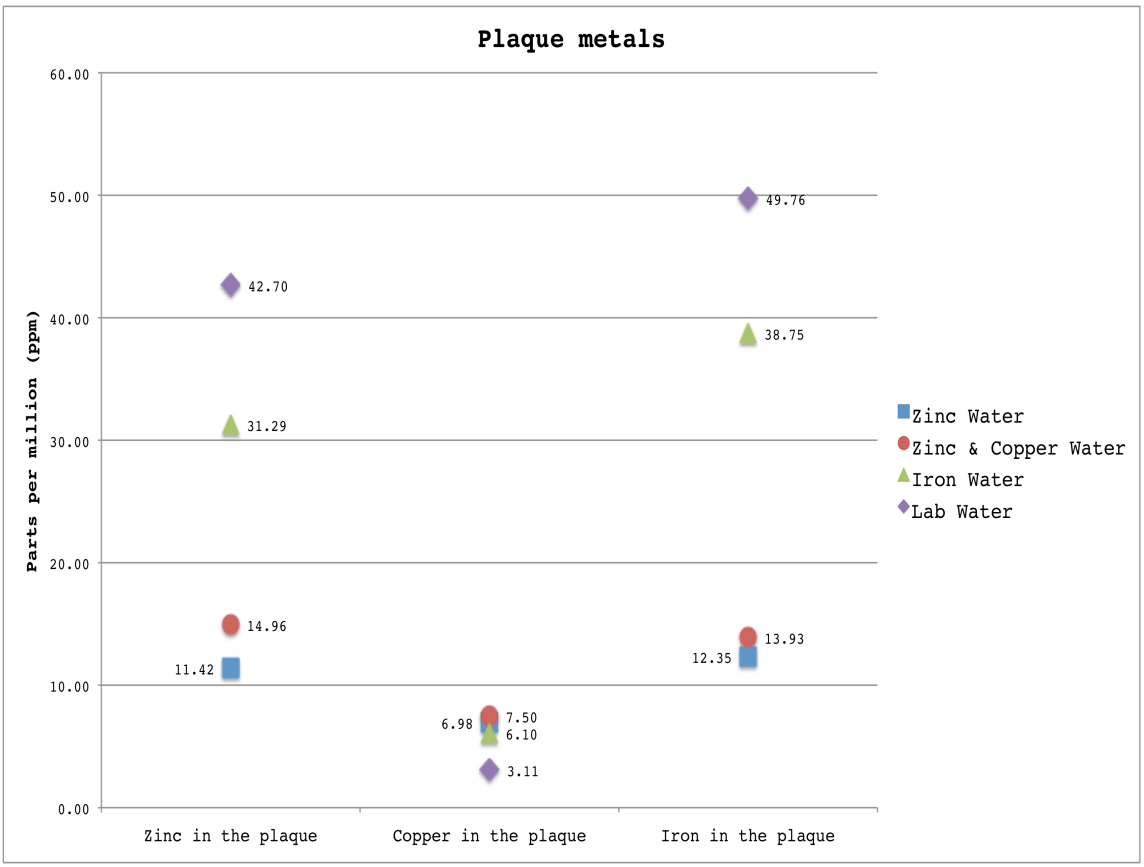

Fig. 2. For both zinc and iron in plaques dosing with iron water led to a smaller non-significant reduction in metal levels. However, dosing with zinc and zinc & copper in the water led to significant lowered levels of both metals compared to dosing with lab water ($p < .05$).

Zinc metal concentration for zinc in the plaque was significantly affected by water type ($H(3) = 30.42$, $p<.05$). Metal concentration for iron in the plaque was significantly affected by water type, ($H(3)=29.70$, $p<.05$). Metal concentration for copper in the plaque was not significantly affected by water type, ($H(3) = 4.22$, $p>.05$). Mann-Whitney post hoc tests using a Bonferroni correction were applied in order to examine which groups differed significantly from each other. This test used 6 comparisons requiring a new significance criterion $p<0.0083$ (see Table 3).

Zinc metal concentration in the plaques was found to be significantly higher in the lab water group compared to the zinc water group ($U=114.00$, $p<0.0083$) and the zinc & copper water group ($U=111.00$, $p<0.0083$). Zinc metal was not significantly different in the lab water group compared to the iron water group ($U=255.00$, $p=0.020$) and in the zinc water group compared to zinc & copper water group ($U=297.00$, $p=0.595$). Zinc metal was significantly higher in the iron water group compared to the zinc water ($U=213.00$, $p<.0083$) and to the zinc & copper water groups ($U=181.00$, $p<0.0083$). (See Table 3)

Table 3

Pairwise Comparisons of Zinc in the Plaques by Ranked Means

	Water Type	Mean Rank	U_1	*p*
Zinc	Lab Water	39.24	114.00	<0.0083*
	Zinc Water	19.84		
	Lab Water	30.76	111.00	<0.0083*
	Zinc & Copper Water	14.86		
	Lab Water	33.38	255.00	=0.020
	Iron Water	25.59		
	Zinc Water	27.39	297.00	=0.595
	Zinc & Copper	25.19		
	Zinc Water	25.48	263.00	<0.0083*
	Iron Water	39.28		
	Zinc & Copper	18.57	181.00	<0.0083*
	Iron Water	32.53		

*denotes significance at <0.0083

Iron metal concentration in the plaque was found to be significantly higher in the lab water group compared to the zinc water group (U=119.00, p<0.0083), and compared to the zinc & copper water group (U=81.00, p<0.0083). Iron metal in the plaques was not significantly different in the lab water group compared to the iron water group (U=291.00, p=0.080). Iron metal in the plaques was not found to be significantly different in the zinc water group compared to the zinc & copper water group (U=297.00, p=0.595). Iron metal was significantly higher in the iron water group compared to zinc water (U=263.00, p<.0083). Iron metal was significantly higher in the iron water group compared to the zinc & copper water group (U=159.00, p<0.0083). (See Table 4)

Table 4

Pairwise Comparisons of Iron in Plaques by Ranked Means

Iron	Lab Water	39.24	119.00	<0.0083*
	Zinc Water	19.84		
	Lab Water	30.76	81.00	<0.0083*
	Zinc & Copper Water	14.86		
	Lab Water	33.38	291.00	=0.081
	Iron Water	25.59		
	Zinc Water	27.39	297.00	=0.595
	Zinc & Copper	25.19		
	Zinc Water	25.48	263.00	<0.0083*
	Iron Water	39.28		
	Zinc & Copper	18.57	159.00	<0.0083*
	Iron Water	32.53		

*denotes significance at <0.0083

The results demonstrate an overall trend of higher levels of zinc metals and iron metals in mice raised on lab water, as compared to mice raised on zinc water. There was no significant difference found in copper metals among the mice raised on any of the types of water. Of note, mice raised on zinc water and zinc & copper water had the lowest amounts of zinc metal and iron metal in the amyloid plaque compared to mice raised on iron water and lab water.

4. DISCUSSION

The data from this study was analyzed using Kruskal-Wallis non-parametric test (K-W test). Non-parametric tests rely on fewer assumptions, and use the principle of ranking the data. When assumptions are violated, it is a reliable alternative to parametric testing (See Appendix 1).

In this study, we examined the effects zinc, zinc & copper, and iron to drinking water, and examined the levels of metal concentration in the plaques in the Tg2576 mice.

This study found water treated with various trace metals were significantly different among zinc and iron metal levels in the amyloid plaque, but came back non-significant for copper. It was hypothesized that adding trace metals to drinking water would have a significant effect on the metal concentration of the amyloid plaque. The results of this study support adding trace metals to the drinking water of Tg2576 mice does have an effect on plaque metal concentration.

Further interpretation of the post hoc analyses revealed mice treated with lab water were found to have the highest amount of zinc (M=42.70) among the groups, followed by iron treated water (M=31.29), zinc & copper treated water (M=14.96), and zinc treated water (M=11.42). (See Figure 2)

Examining iron concentration, the results found that mice given lab water had the highest amount of iron concentration in the amyloid plaque (M=49.76), followed by iron

treated water (M=38.75), zinc & copper treated water (M=13.93), and zinc treated water (M=12.35). (See Figure 2)

The zinc treated water group compared to the lab water group yielded a significant difference. Post hoc analysis revealed higher levels of zinc in the lab water group versus the zinc treated water group. It was hypothesized that, the additional zinc in the water would have led to higher amounts of zinc, instead the results showed an opposite trend. There were lower levels of zinc in the zinc & copper treated water when compared to lab water group. In conclusion, among the four water types, mice treated with lab water had the highest amount of zinc and iron metal in the amyloid plaques.

The results are surprising and unexpected. This inverse in results can be caused by a number of complex possibilities outside the scope of this study. Cuajungo et al. (2000) found an inverse correlation between amyloid plaque core density in Alzheimer's disease and oxidation of neocortical tissue. Observing the perplexing relationship between zinc metal and brain tissue, zinc has a role in amyloid plaque buildup, and then conversely acts as a protectorate in reducing ROS (subsequently reducing amyloid plaque buildup). This could provide an explanation to our results, as the zinc in the water is acting a protectorate, reducing the concentration of the amyloid plaque core, resulting in lowered zinc levels. Compared to lab water, which can be inferred as real world conditions, resulted in higher amounts of zinc in the amyloid plaque.

It is well understood that an AD brain is zinc deficient. Results from this study can speculate that the zinc treated water (as well as the zinc & copper treated water) can be acting to replenish the zinc-deprived brain. In previous literature, Maynard et al.

(2004), found exposure to zinc lead to a 5% increase in zinc in the brain. This can perhaps provide a clue in future directions of research about where the zinc went specifically. Our present study focused on the individual plaque itself, and not overall plaque load in the neocortex. Future studies can compare zinc metal levels inside the plaque to the levels in the non-plaque areas.

We examined the effects of adding zinc, copper & zinc, and iron to drinking water, and examined the effects of the metal levels of the plaques in the Tg2576 mice. There have been positive interventions towards slowing down Alzheimer's disease, but presently there is no cure. There is disagreement in the field about some of the underlying mechanisms relating to trace metals. Most concerns are whether these trace metals are harmful or beneficial to the AD brain. Since these three metals are found in amyloid plaques it is suggested that high levels of metals in amyloid plaques contribute to the higher amounts of oxidative stress in Alzheimer's pathology (Huang et al. 2004).

The present study uses an animal model to study Alzheimer's disease, as the methods involved in this study would be unethical if used on humans. The Tg2576 mice contain the Swedish mutation of human APP; they were genetically engineered to develop AD plaques by the addition of human APP with two mutations in order to accelerate amyloid plaque production (Hsiao, 1998). This paper analyzed metals in plaques of Tg2576 mice using X-ray spectroscopy. (George & McIntyre, 1994; Jenkins & DeVries, 1968).

Our findings in this study found that metals added to drinking water did have an effect on the metal levels in the plaque. This is supportive of suggestions about the

breakdown of the blood brain barrier in the Alzheimer's diseased brain, contrary to previous research stating an intact and highly regulated structure (Bush et al., 2008). Combined with increasing age, as the most dominant risk factor for most neurodegenerative diseases, understanding the mechanisms involved would further benefit treatment options for an aging population afflicted with Alzheimer's disease, as well as other neurodegenerative disorders. Possibly administering metals in the drinking water rather than in the food could allow for greater absorbtion in the brain.

It was surprising to see both zinc and zinc & copper treated water having the lowest amount of zinc found in the amyloid plaque overall. It can be speculated an unknown mechanism combined with the additional zinc to the system (via drinkable water) is causing the amount of zinc metal in the plaques to decrease. Similar research has found that zinc added to drinking water resulted in a 5% increase in zinc in overall brain tissue (Maynard et al., 2009). It would then be inferred that the zinc metal is going somewhere in the system, possibly the surrounding tissue and not internally within the amyloid plaque. A possible explanation of the decreased levels of zinc in the plaque, is that this study only focused on the plaque, and not any surrounding neocortical areas. The Lab water results add further intrigue, as the lab water group contained the highest amounts of zinc metal in the plaques. It can be predicted that the inverse is occurring; with the higher amounts of zinc metal in the amyloid plaque, the outside surrounding neocortical tissue would have lower amounts of zinc. This would be an interesting for future research to compare the concentration of metals between the inside of the amyloid plaque versus the surrounding neocortical tissue. The finding of this study poses further

questions to the behavior of the amyloid plaque. Future research can investigate the relationship between plaque load and water treatment. As this study has revealed, zinc treated water (including zinc & copper treated water), lead to significantly lower amounts of zinc in the plaque. As metals have been of great interest in terms of a therapeutic course of action, it would be of great importance to compare plaque loads versus water types, as it is well understood that zinc facilitates the oligomerization of amyloid beta (Barnham et al., 2004). Iron has been implicated in reactive oxygen species, causing oxidative stress, leading to further oligomerisation of Aβ. With significantly higher levels of iron found in lab treated water, versus zinc treated water, it can be predicated that more reactive oxygenated species in the system, would lead to additional/higher plaque loads.

Though we found significance from the statistical analysis, our preliminary intentions were to run a parametric statistic. The majority of the assumptions involved with a Mulitvariate analysis of variance (MANOVA) were in serious violations (See Appendix 1). While MANOVA can be robust it would simply make the results, with cumulative violations, unreliable to interpretations, leading to our use of the non-parametric Kruskal-Wallis and Mann–Whitney tests, which do not rely on assumptions for analysis. However, some subjects were lost due to data/computer corruption. The loss of subjects in any parametric testing leads to loss of power in the statistical model, contributing to false positives. In light of the significant results from the non-parametric statistic, it would add further weight to the outcome if the study were repeated with parametric assumptions preserved and the use of the MANOVA statistic. Investigating the preliminary data, we can see the overall trends of the metal levels in the plaque by

function of water type (See Appendix 2). For Zinc metal in the plaque, an overall trend of higher zinc metal in the mice raised on lab water and iron water, versus zinc water and zinc & copper water. There was no discernable trend across the scatter plot for copper metal in the plaque as function to water type, showing a similar pattern across groups. With iron levels in the plaque, there was an overall trend of higher amounts of iron in the mice raised on lab water and iron water, with the lowest amounts seen in the zinc water and zinc & copper water.

There is a limited amount of research focused solely on the amyloid plaque and the effects of trace metals in drinking water. In this study, we have seen an inverse pattern with zinc and zinc & copper treated water, with respect to the amounts of zinc and iron in the plaque. To our knowledge, there is no documented case of the of composition of an amyloid plaque in relation to trace metals added to drinking water. As inferred from the results of this study, it can be considered that lab water is analogous to real world conditions. Subsequently, those afflicted with Alzheimer's disease will have higher amounts of zinc in their amyloid plaques: in contrast to their counterparts with water with zinc (as well as zinc & copper treated water), it would be predicted they would have lower amounts of zinc in their amyloid plaques. Since the metal values for zinc and zinc plus copper enhanced water were very similar, this suggests that the zinc ins playing the more important role in influencing plaque composition.

The scope of this study focused on the amyloid plaque, excluding the surrounding areas and overall brain tissue. It is well documented that the Alzheimer's diseased brain is zinc deficient, and has been well documented on the behavioral effects. This study adds

to the complex dynamic of Alzheimer's disease by adding further information about the composition of the amyloid plaques when the Tg2576 mice are exposed to different types of water.

APPENDIX 1

The analysis focused on four water types and their effect on metal levels in the amyloid plaque. There were 3 dependent variables, each subject yielded three measurements from the amyloid plaques: zinc, copper, and iron measured using parts per million. The value for significance was set at .05 or below. If significant results were found, post hoc analysis were conducted. Data was analyzed using SPSS statistical software package. The mice used contributed to different amount of plaques in this study.

The intended statistical analysis was Multivariate Analysis of Variance (MANOVA), due to three dependent variables in the study. Before proceeding with a MANOVA, assumption testing was conducted to examine reliability of the data. Violations of assumptions would lead to unreliable test statistics, leading to misleading conclusions.

Preliminary analysis revealed the assumption of normality was violated; by examining the skewness and kurtosis values from preliminary statistics. With z scores greater than 1.96, the data for zinc, iron, copper were significant at $p < .05$. Positive skewness in the data indicated there was a significant amount of low scores in the sample distribution reflecting the data is not normally distributed. Subjects that exceeded three standard deviations above the mean were considered outliers and were removed from the data analysis.

Multivariate normality is an assumption of MANOVA. SPSS does not have the means to test for multivariate normality, in which case the alternative was to check the univariate normality for each dependent variable. Checking for univariate normality is useful and practical; but it does not guarantee multivariate normality. The Kolomogorov-Smirnov test for normality was significant ($p<.05$) indicating a deviation from normality. The Kolomogorov-Smirnov test indicated that the distributions of zinc, iron, and copper indicated the distributions were not normal. The zinc levels, $D(63) = 0.20$, $p < .05$, the iron levels, $D(63) = 0.20$, $p < .05$, and the copper levels, $D(63) = .31$, $p <.05$, were all significantly atypical. Robustness of the F statistic cannot be assumed in MANOVA because of unequal sample sizes across groups.

The following assumption tested was Homogeneity of variance. The assumption was tested using Box's M, if the test statistic came back non-significant, then the assumption was preserved. In our present study, Box's M came back significant ($p <.05$), which indicated that the covariance matrices are significantly different, violating the homogeneity of variance assumption. The intended test statistic, MANOVA, therefore was not used because of the violations of assumptions. A statistic was needed that did not rely on parametric assumptions.

APPENDIX 2: SCATTER PLOTS OF METAL CONCENTRATION IN PLAQUES BY WATER TYPE

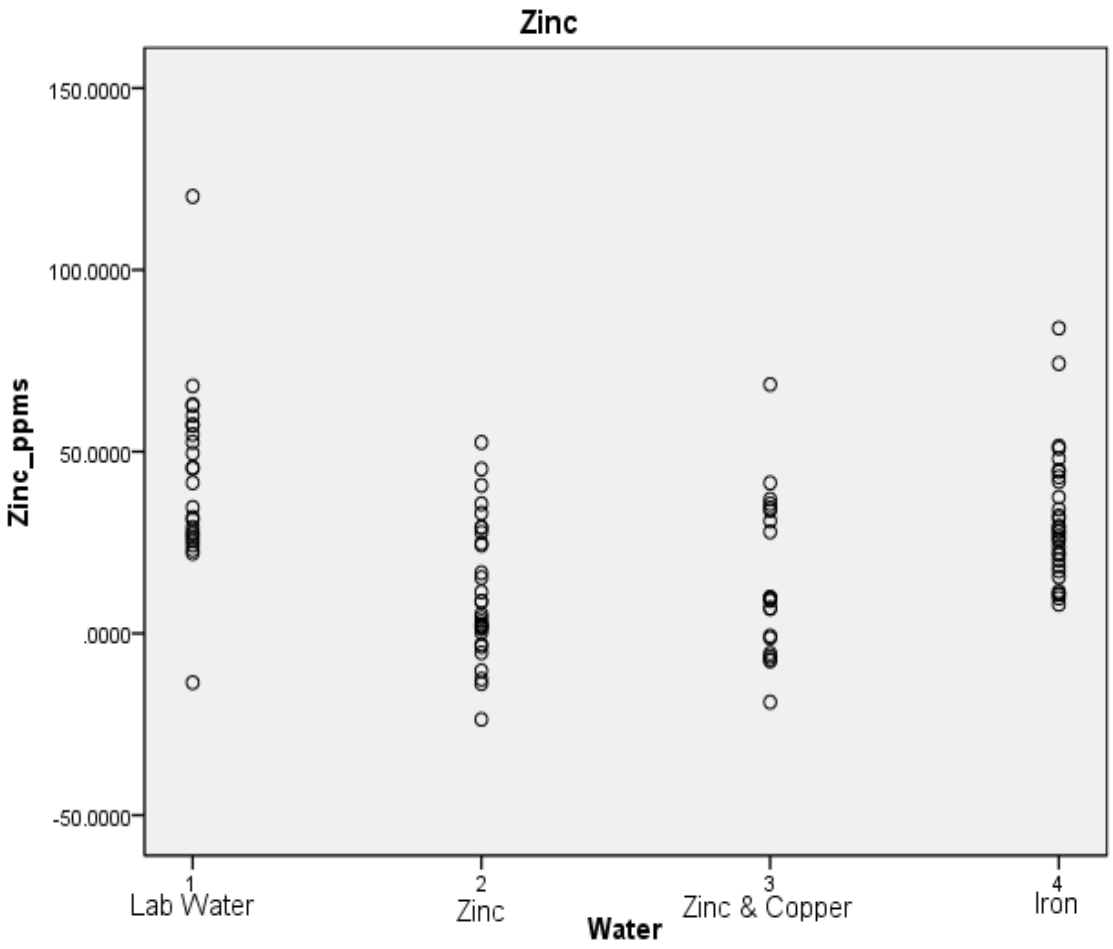

Fig. 3. Zinc metal in the plaques by water type. Zinc metal in the plaque was trending towards mice raised on lab water. Followed by mice raised on iron water, with similarities between zinc and zinc & copper water.

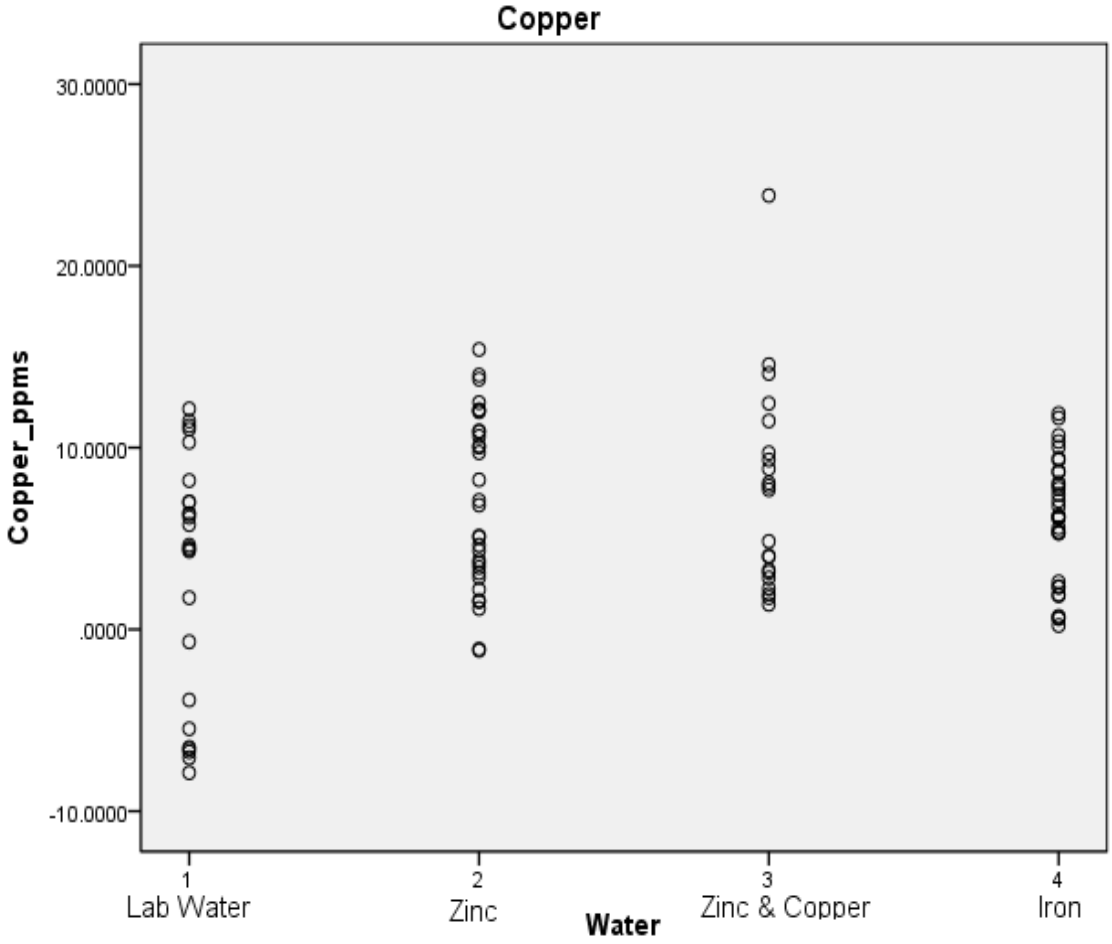

Fig. 4. Copper metal in the plaques by water type. Across water types, copper metal levels in the plaque appear to have similar trends.

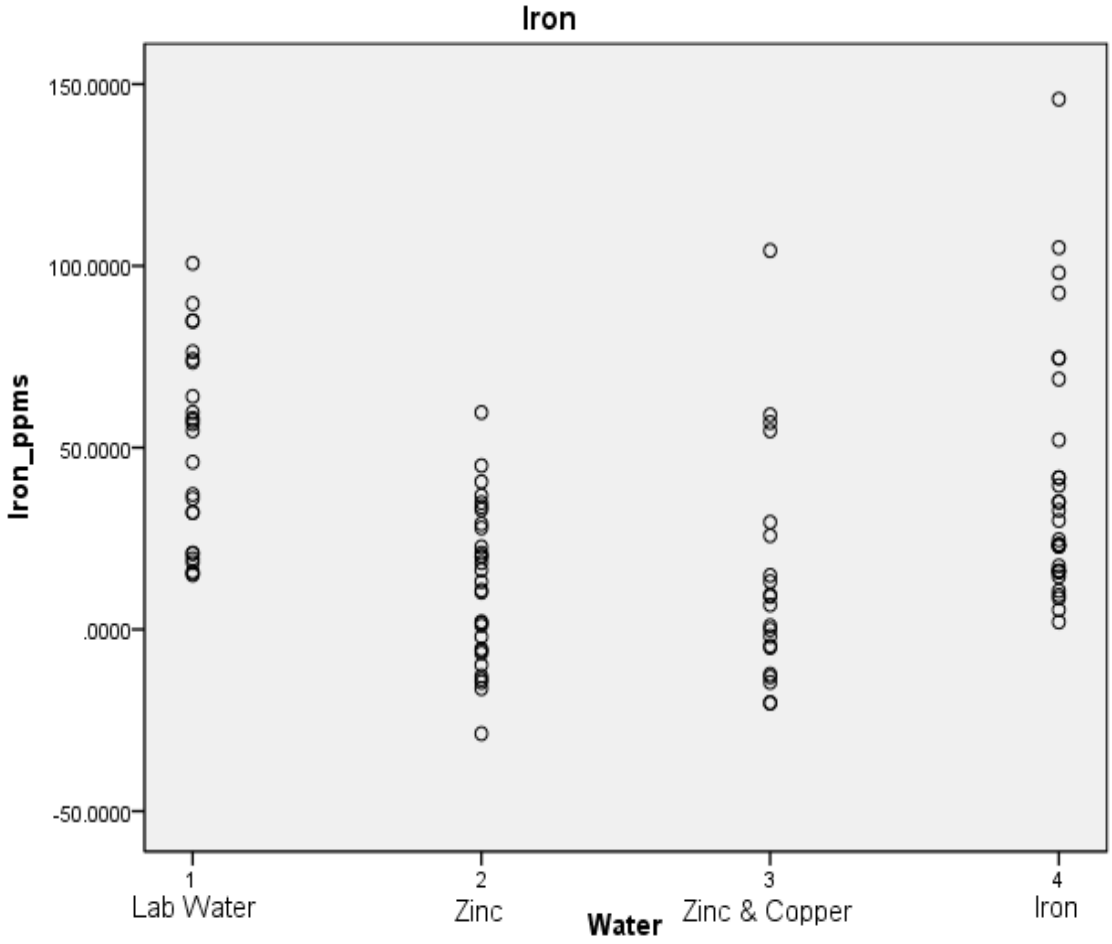

Fig. 5. Iron metal levels in the plaques by water type. Mice treated on Zinc and Zinc & Copper water showing a pattern of the lowest amounts of iron concentration in the plaques.

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