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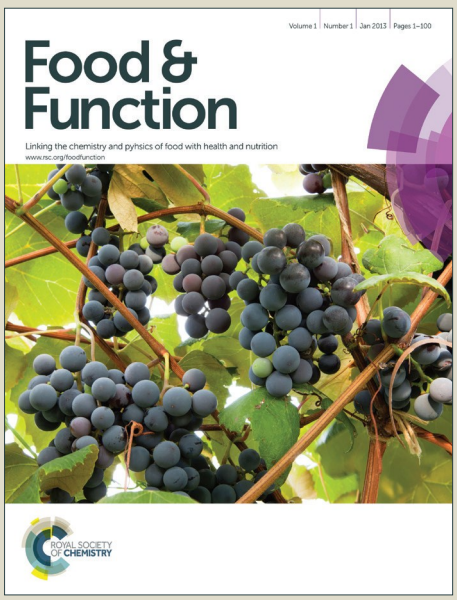

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Effect of copper from water and food: changes of serum nonceruloplasmin copper and brain's amyloid-beta in mice

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Copper is an essential element and also produces adverse health consequences when overloaded. Food and water are main sources of copper intake, however, few studies have been done to investigate the different between the ways of its intake in water and food in animals. In this study, copper was fed to mice with food as well as water (two groups: water and diet) for three months at concentrations of 6, 15 and 30 ppm . The copper concentration in water was adjusted for keeping the same amount during its intake in food. The experimental studies show slow growth rate, lower hepatic reduced glutathione (GSH)/ superoxide dismutase (SOD) activity and higher serum 'free' copper in water group. The brain's soluble Amyloid-beta 1-42 (Aβ42) of water group was significantly higher than that of the diet group at the levels of 6 and 15 ppm. In conclusion, copper in water group significantly increased the soluble Aβ42 in the brain while, the 'free' copper in the serum, decreased the growth rate and hepatic GSH/SOD activity. The research studies carried out suggest that the copper in water is more 'toxic' than copper in diet and may increase the risk of Alzheimer disease (AD).

Introduction

Copper is an essential trace element for normal body growth and development. It acts as a catalytic or structural co-factor of many enzymes and is required for functioning and development of the nervous and cardiovascular systems, skin, bones, etc. Many functions in the body, such as collagen production, formation of red blood cells, regulation of cholesterol levels, iron adsorption, oxidation of fatty acids, melanin production and control of intracellular energy levels are dependent on copper[1]. Excess copper can produce adverse health consequences. Copper can directly catalyze the formation of reactive oxygen species (ROS) via a Fenton-like reaction. The cupric ion [Cu (II)], in the presence of superoxide anion radical or biological reductants such as ascorbic acid or GSH, can be reduced to cuprous ion [Cu (I)]. The oxidation and reduction reactions are capable of catalyzing the formation of reactive hydroxyl radicals through the decomposition of hydrogen peroxide via the Fenton reaction. The production of ROS plays an essential role on the copper toxicity[2, 3]. Oxidative stresses occur when there is an imbalance between the production of ROS and the capacity of the antioxidant protection and repair systems.

The sources of copper for human are food and water. Dietary is the primarily absorb ways of copper, the concentration of copper in food range from 0.2-44 ppm by wet weight, the most relevant sources of dietary copper include seafood (especially shellfish), organ meats, grains, and legumes[4, 5]. Water contributes less copper than diet[4], the median concentration of copper in natural water is 4-10 ppb[5], , actual sampling data of drinking water from 180 homes all across N America showed that 1.8% were above 1.3 ppm, 71% were between 0.01 and 1.3 ppm, and 27% were below 0.01 ppm[6]. However, the copper pumping supply network may increase the ratio due to water characteristics like pH, hardness and the intake. Water left overnight in the corrosive copper fixtures may contain up to 60 mg Cu/L[5].

Dementia is one of the leading causes for dependence and disability in later life. In the year 2013, there were 44 million people with dementia worldwide, with this number expected to increase to 135 million by 2050[7]. Copper is thought to be responsible for AD which is the most common cause (50-75%) of dementia[8, 9]. The addition of 0.12-0.13ppm copper to the distilled drinking water can greatly enhance AD-type brain pathology and cognition loss in rabbit[10] and mice[11]. According to Squitti et al.[12-14], the amount of serum 'free' copper is positively correlated with the increasing mild cognitive impairment and the risk of AD. Brewer[15] has hypothesized that copper present in drinking water is toxic and can increase the mild cognitive impairment and the risk of AD.

To investigate the effect of different ways of copper intake, two modes (water and diet) with three levels were studied. According to Nutrient Requirements Guidelines of rodents given by United States National Research Council (NRC, USA), 6ppm is recommended as nutritional recommendation for mice; that indicate 6ppm is the physiological intake level of

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mice. 15ppm is the intermediate value given by Chinese standard laboratory animals-Nutrients for formula feeds (GB 14924.3-2010), while, 30ppm is tested as a high concentration.

The serum 'free' copper and the soluble $A\beta_{42}$ in the brain, mice grow rate, copper deposition and hepatic GSH and SOD activity were investigated.

Materials and Methods

Animals

Seventy two male ICR mice (Animal Experiment Committee of Hangzhou Normal University, Zhejiang, China), with an average initial body weight of 22.0±1.2g, were housed in standard polycarbonate cages with a controlled room temperature (21–23°C), relative humidity (50%), and a 12-h light/dark cycle (lights on at 07:00 AM). All animal care and experimental procedures were performed in accordance with protocols approved by the Animal Experiment Committee of Hangzhou Normal University (Permit Number: SYXK(Zhe) 2011-0157). All efforts were made to minimize their suffering.

Diet and water

Diet and water was supplied by ad libitum. After 10 days of acclimatization, mice were randomly assigned into 2 groups ensuring an even body weight distribution, including Water group (copper in water) and Diet group (copper in food). Each group had 3 levels (6ppm, 15ppm and 30ppm) of copper. Diet group drank ultra-pure water (Thermo science® NANOpure, USA) and fed with diet containing three levels of copper ($CuSO_4 \cdot 5H_2O$, Sigma Aldrich, Germany), water group drank copper solution ($CuSO_4 \cdot 5H_2O$, Sigma Aldrich, Germany dissolved in ultra-pure water) and ate purified feed without copper. The experiment lasted for 3 months and copper intake in diet and water was calculated every 3 days to maintain same amount of copper intake during the experimental studies. Detailed calculation process is shown in Supporting Information. The composition of the purified basal diet and copper content is shown in Table 1.

Weight gain and sample collection

Body mass was recorded as initial, at the beginning of experiment. Further measurements were carried out every 6 days throughout the experimental duration. Weight gain was calculated as:

$$\text{Weight gain} = ((\text{Final weight(g)} - \text{Initial weight(g)})/ \text{Initial weight(g)} \times 100\% \quad (1)$$

At the end of the experimental studies, blood was withdrawn from the retro-orbital and mice were sacrificed by manual cervical dislocation, and brain, liver, spleens and kidneys were removed immediately. The brain was put into liquid nitrogen and other tissues were rinsed with ice-cold physiological saline to remove excess blood, dry with filter paper and stored in -80°C freezer until analysis. The serum was separated and preserved at -80°C too.

GSH and SOD enzyme activity in liver

Frozen liver samples were homogenized by homogenizer (IKAT10®basic ULTRA-TURRAX®, Germany) with physiological saline in the ice-water bath, centrifuged at 1500×*g* for 15 min. at 4°C (SORVALL®Biofuge primoR, USA). The supernatant fraction was transferred to 1.5 mL Eppendorf tubes for antioxidant activity analysis. All the biochemical analyses were done during the same day. The GSH, SOD activity and total protein were measured using commercial assay kits (Jiancheng Institute, Nanjing, China). All results were corrected for the protein concentration of the tissues. The GSH was based on the reaction with disulfide generation of 2-nitro benzoic acid to produce 2-nitro-5-thiobenzoic acid, a yellow colored compound that can be detected at 412 nm. One unit of GSH was defined as 1 μmol of GSH /min/g protein at 37°C. The activity of SOD was determined according to the ability to inhibit the formation of nitrite at 37°C. The unit of enzyme activity was 50% inhibition of formation. SOD activity was expressed in arbitrary units per milligram protein.

Copper deposition

The serum was diluted 10 times with saline and measured by ICP-MS (Agilent 7700x, Australia) under standard operating conditions. The ceruloplasmin (Cp) was detected with an immunoturbidimetry assay (Jiancheng Institute, Nanjing, China). The Copper:Ceruloplasmin (Cu:Cp) ratio was calculated as reported in Twoney et al[16]. The calculation of nonceruloplasmin (non-Cp) copper followed as described by Walshe[17]. Precisely, based on the finding that Cp contains 0.3% copper, use the following formulae (formulae 2) to calculate the non-Cp copper:

$$\begin{aligned}\text{non} - \text{Cp copper}(\mu\text{g/L}) &= \text{serum copper}(\mu\text{g/L}) - \text{Cp}(\text{mg/dL}) \times 10(\text{dL/L}) \\ &\quad \times 0.3\% \times 1000(\mu\text{g/mg}) \\ &= \text{serum copper}(\mu\text{g/L}) - \text{Cp} \times 30(\mu\text{g/L}) \quad (2)\end{aligned}$$

The tissue samples (brain, liver, spleen and kidney) were digested with the help of a microwave oven cavity (Anton Paar Multiwave 3000). For the microwave digestion, 0.1-0.2g of the sample was used, which had been digested with the diluted oxidant mixture (1.0 mL of H_2O_2 and 5 mL of HNO_3). Precise heating program is shown in Supplementary Material. Acid digests were then made up to 10mL with ultra–pure water. Copper was measured by ICP-MS under standard operating conditions. Calibrations were based on 2.0M nitric acid aqueous standard solutions containing copper concentrations between 0 and 300 ppb.

Soluble $A\beta_{42}$ in the brain

Frozen brain samples were homogenized with physiological saline in the ice-water bath, centrifuged at 1500g for 15 min at 4°C. The supernatant fraction was transferred to 1.5 mL Eppendorf tubes for further analysis. The Soluble $A\beta_{42}$ was measured using ELISA kit (Jiancheng Institute, Nanjing, China),the proteins were measured using commercial assay kits (Jiancheng Institute, Nanjing, China).

Statistical analysis

The experimental data were expressed as mean ± standard error (SEM). Statistical significance was determined by one-way analysis of variance (ANOVA) followed by least significance difference (LSD) and multiple comparison tests using IBM SPSS22.0 software. A probability of $p<0.05$ was considered to be statistically significant.

Results

Feed, water and copper intake

Average feed, water and copper intake for each mouse during the experiment period were measured. There were no significant difference in the amount of feed and water intake (Fig. 1A, 1B). Copper intakes for all the 3 levels (6, 15, 30ppm) of the groups were almost identical (Fig. 1C).

Weight gain

The weight gains over the experimental period are presented in Fig. 2. Along with the different ways of intake of copper supplements, there was a significant difference between diet group and water group at all levels. At levels of 6ppm (Fig. 2A) and 15ppm (Fig. 2B), the difference was significant in the early experimental period. At level of 30 ppm (Fig. 2C), there was significant difference in the whole period.

Hepatic GSH and SOD enzyme activity

The hepatic GSH decreased with increased concentrations of copper in water group, however, at the same time, the diet group did not show significant difference between 6, 15 and 30ppm ($p>0.6$). With increasing concentrations of copper, the difference between water group and diet group became more and more significant, [(6ppm ($p=0.1$), 15ppm ($p=0.01$), 30ppm ($p=0.001$)].In water group, the hepatic GSH of 30ppm was significantly lower than 6ppm ($p=0.04$). At all levels of copper intake, the water group shown lower SOD activity than diet group (Fig.3B), the difference were significant [(6ppm ($p=0.007$), 15ppm ($p<0.001$), 30 ppm ($p=0.01$)].

Copper deposition

The amount of copper deposited was not significantly different in the spleen (Fig. 4B) and kidney (Fig. 4C). In the liver (Fig. 4A), only 15ppm concentration had some difference ($p=0.001$). However, in the serum, there was obvious difference between the different groups. The serum copper rose with increased concentrations of copper in water group, however, at the same time, the diet group did not show significant difference between 6, 15 and 30ppm (Fig. 4E). In water group, 30ppm was significantly higher than 6ppm ($p<0.001$) and 15ppm ($p=0.02$) and also, 15ppm was significantly higher than that of 6ppm ($p=0.006$) .There were significant differences between water group and diet group at 30ppm level ($p<0.001$).

The serum copper comprised of Cp copper (copper in Cp) and non Cp copper (serum 'free' copper). After the determination of Cp (Fig. 4G), no difference was found. In diet group, "free" copper in 6ppm was lower than 15 ($p=0.03$) and 30ppm ($p=0.02$) while 15 and 30ppm had no significant difference ($p=0.8$). In water group, free copper increased with the increased concentrations, significant difference between each concentration were found, 15ppm>6ppm ($p<0.001$), 30ppm>15ppm ($p = 0.04$) and 30ppm>6ppm ($p < 0.001$). At 30ppm level, the difference between water and diet group was significantly ($p< 0.001$), however, at 6ppm ($p = 0.7$) and 15ppm ($p= 0.09$), the difference was not so significant. As an additional copper index, Cu: Cp is a useful internal quality control verification of ceruloplasimin calibration[18]. In water group, the ratio rose with increased concentrations of copper, however, there wasn't any significant change in the diet group at all levels (Fig. 4H). There were significant differences between water and diet group at 30ppm ($p<0.001$).

Soluble $A\beta_{42}$ in the brain

The difference of brain's soluble $A\beta_{42}$ between water and diet group(Fig. 5) was significant in 6ppm($p =0.04$). In the diet group, with increasing concentrations of copper, the soluble $A\beta_{42}$ increased significantly, 30ppm group was higher than 6ppm ($p =0.002$) and 15ppm ($p = 0.02$). Meanwhile, the content between different concentrations of water groups had no significant difference ($p >0.2$). The soluble $A\beta_{42}$ in water group was significantly higher than that in diet group at 6ppm ($p =0.04$) and 15ppm ($p =0.02$), while, at 30ppm ($p=0.8$) there was no significant difference.

Discussion

The fact that excess copper is toxic has been identified and accepted for years, but, the studies on toxicity of different ways of intake are still rare. In this paper, 6, 15 and 30ppm concentrations and two modes of copper intake (water and diet) with three levels were studied. 6ppm in diet is the physiological intake level of mice, so the 6ppm in diet group can be seen as a control group. In water group, almost all the copper intake together with water (Tab. 1) and in diet group all of the copper is intake with diet since the mice drinking purified water. Experimental results showed significant difference between water and diet group.

The growth efficiency of the animal is considered as an important indicator of its health status. Experimental studies showed that the growth efficiency of water group was significantly lower than that of the diet group, which showed

that the copper intake with water may have adverse effects on mice. Liver is the "scheduling room" of copper. In order to prove the toxicity of copper in water, some oxidation indexes of liver were determined. The primary mechanism of copper toxicity is the reduction-oxidation (redox) cycles of oxidative stress, which produces H_2O_2 as a by-product and is catalyzed by transition metals (Fenton's and Haber Weiss Chemistry)[3, 19], decreases of GHS that was also found in the liver [20] and brain [21] of the copper overload mice. In our experimental studies, the GSH and SOD activity decreased significantly in water group, however, were stable in diet group. For GSH, some significant differences were found with the increase of copper concentration. As powerful cellular antioxidants, GSH can suppress copper toxicity by directly chelating the metal and keeping it in a reduced state making it unavailable for redox cycling. The high level of ROS will depleted the GSH, decrease their concentration[3]. The huge difference between diet and water group and concentration gradient in water group indicated that copper intake with water was easier to produce ROS, and with the increase concentration, the amount of ROS was also rising. In contrast, the copper in diet was more "safe". As another kind of antioxidant enzymes, SOD activity decreased significantly in water group at all levels.

The toxicity of copper is determined by its content and speciation. There is little difference between the depositions of spleen, kidney, liver and brain, however, some regular changes were found in the serum. As an important protein in copper transport and metabolism, CP contains 95% of copper in human serum, while the remaining amount binds to albumin, amino acids and low-molecular-weight complexes form called non-Cp copper ('free' copper). The result of Cp indicated that the difference of total copper content in serum is mainly due to differences in 'free' copper in serum. In diet group, the serum 'free' copper at 15 and 30ppm levels were significantly higher than that of 6ppm, however, there was no significant difference between 15ppm and 30 ppm. However, in water group, significant difference was found between all concentrations. Also, the serum 'free' copper at 15 and 30 ppm levels were significantly higher than that in diet group. Cu:Cp provides information about the actual stoichiometry between copper and ceruloplasimin in the specimens[18]. In water group, the ratio rose with increased concentrations of copper, however, in diet group there wasn't any significant change at all levels. The results showed that the copper intake with water has significant effect on serum 'free' copper and easily lead to the rise of serum 'free' copper. The serum 'free' copper is thought to be more toxic[12, 13, 18]. There is a hypothesis that copper in water is more toxic than it's in diet because it bypasses the liver metabolism and passes immediately into the blood, forming active 'free' copper in the serum[15, 22]. Higher serum 'free' copper brought by water indicates that the toxicity of copper in water is higher than it is in diet group.

According to the amyloid cascade hypothesis, amyloid-beta peptide accumulation is the primary event in AD pathogenesis, and soluble Aβ oligomers are the main neurotoxic species caused by cell membrane perturbation, oxidative stress, and other mechanisms[23]. The most commonly produced Aβ species is $Aβ_{40}$, however, $Aβ_{42}$ is more prone to aggregation and considered to be the most pathogenic[24]. Sparks et al. found the addition of trace amounts of copper to the distilled drinking water greatly enhanced AD-type brain pathology and cognition loss in a cholesterol-fed rabbit model of AD[10], another experiment confirmed the toxicity of copper in drinking water in mice models[11]. In our experimental studies, there was no significant difference found in brain's soluble $Aβ_{42}$ at the levels of 6, 15 and 30ppm in water group for they all at higher concentrations compare to diet group. However, in diet group, the soluble $Aβ_{42}$ in 6 and 15 ppm were significantly lower than 30ppm. It indicated that copper in water can increase the brain's soluble $Aβ_{42}$ and the risk of AD at physiological intake levels (6ppm); meanwhile, the brain's soluble $Aβ_{42}$ in diet group maintained a lower level at low and moderate concentrations.

While the copper in normal food is incorporated into proteins, the diet group in our experiment might not be the ideal control group (the copper sulphate was added in the diet). The differences might be even more extreme if the diet group was the ideal one. Exactly why copper in water is more 'toxic' of copper is not currently understood. Maybe the different effects are related to the different absorption rates of copper in water and diet, which is affected by copper's solubility, the chemical speciation of copper in gastrointestinal tract and the different transport pathways of copper, etc. Food and water are completely different; it is obvious that in the digestive tract, they will undergo different digestion processes. When copper reaches the absorption site, different chemical forms will exist. Despite limited understanding of their molecular mechanism, different forms of copper have different transport pathways, which lead to different targets[25].

Conclusions

Copper in water reduced the growth efficiency and the hepatic GSH and SOD activity, raised the serum 'free' copper. At low copper concentration (6ppm) in water group, after three months of experiment, the soluble $Aβ_{42}$ content in mice brain increased significantly. The results indicate that the copper in water is more 'toxic' than copper in diet and can increase the risk of Alzheimer disease (AD). Compare to the years of influence that water give us, the results of three month experiment prompted us to do more studies on the digestion and absorption of copper in water and diet.

Acknowledgements

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Figure legends

Figure 1

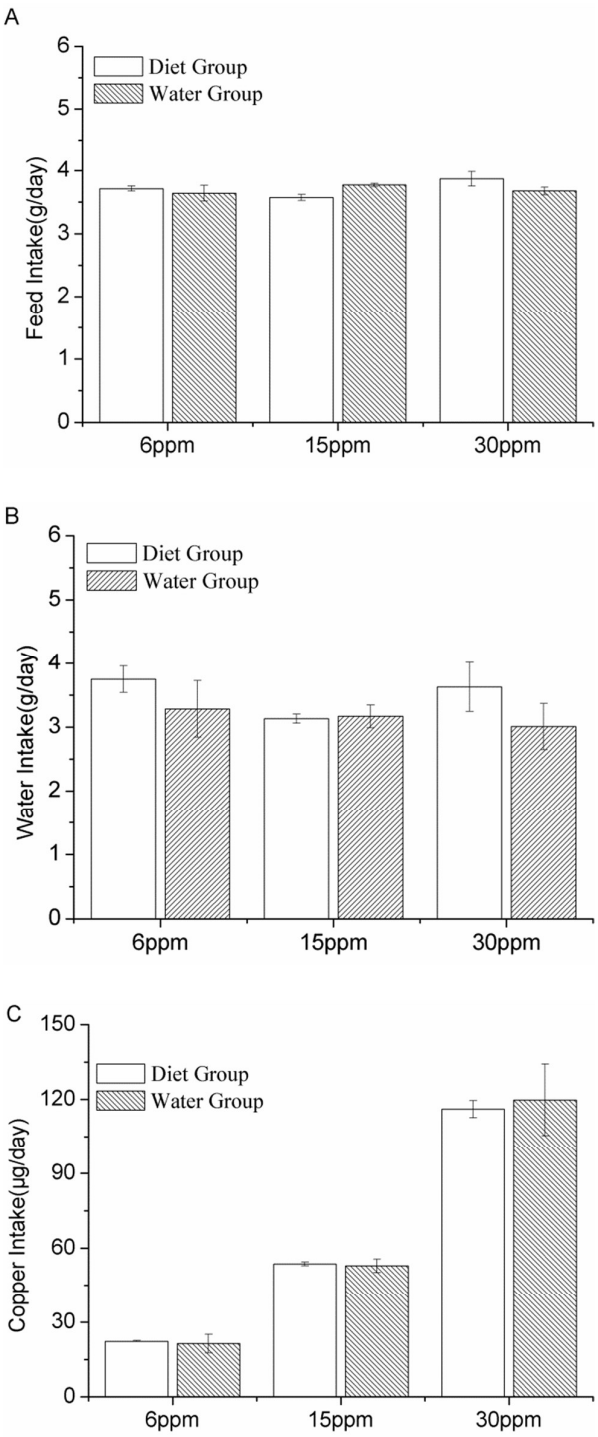


Fig.1 Average food (A), water (B) and copper(C) intake every day for each mouse. Values are shown as means ±SEM，**p＜0.05

Figure 2

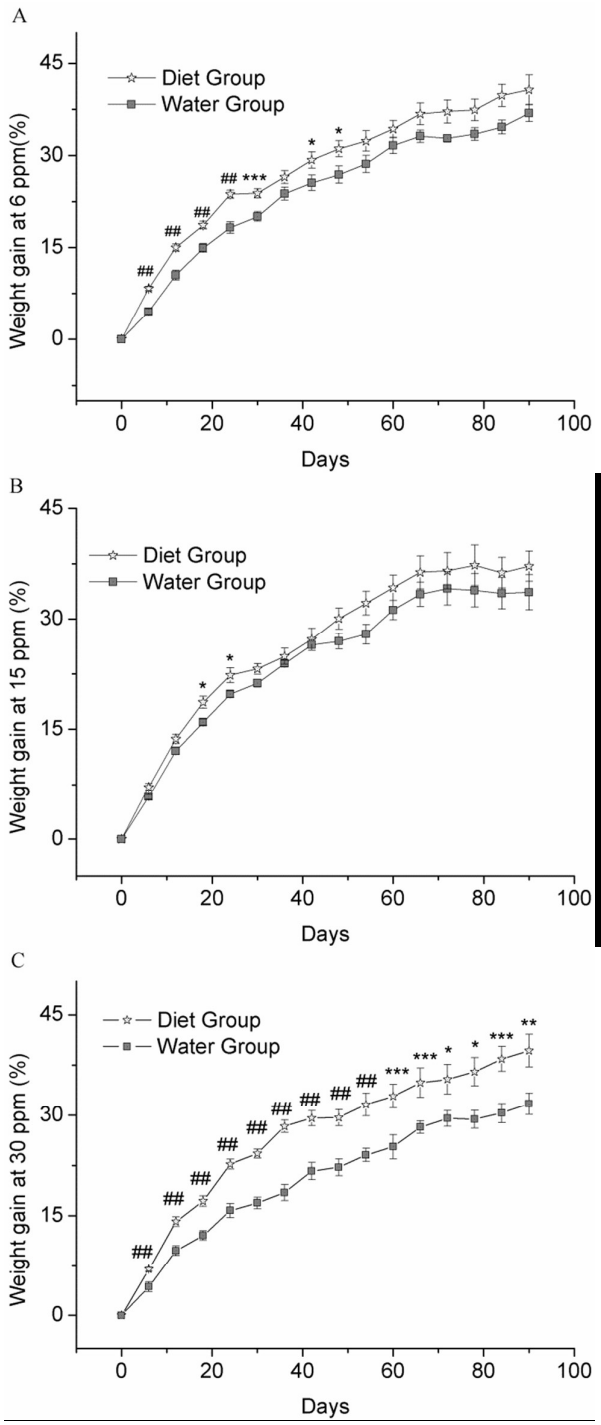
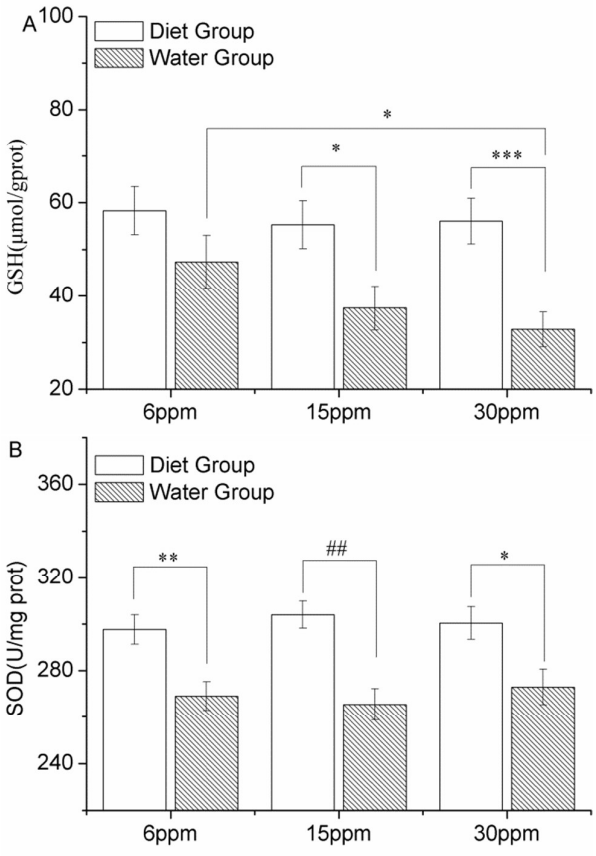


Fig.2 Body weight gain at level 6ppm(A), 15ppm(B) and 30ppm(C) during the experiment period. Values are shown as means ±SEM，*P＜0.05, **P＜0.01, ***P＜0.005, ##P＜0.001

Figure 3



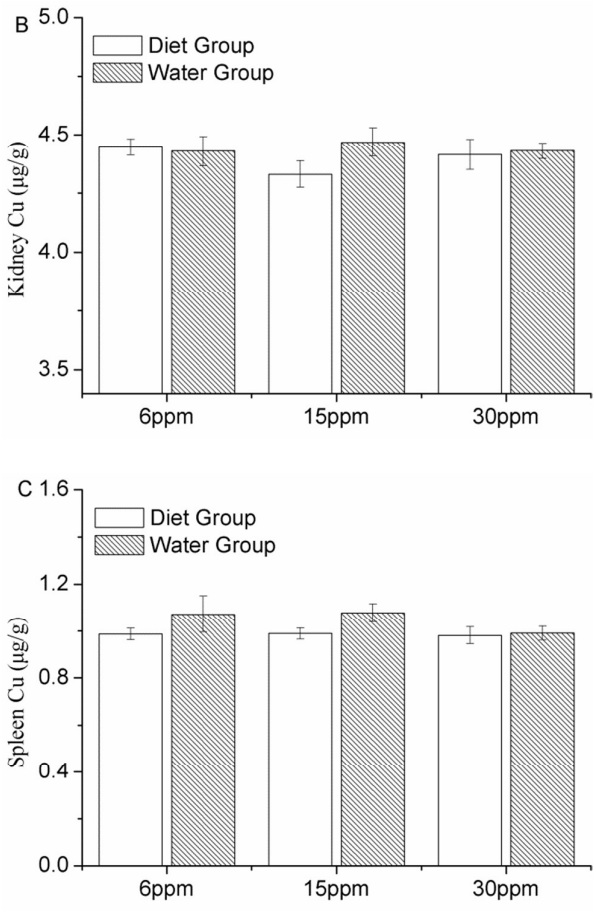
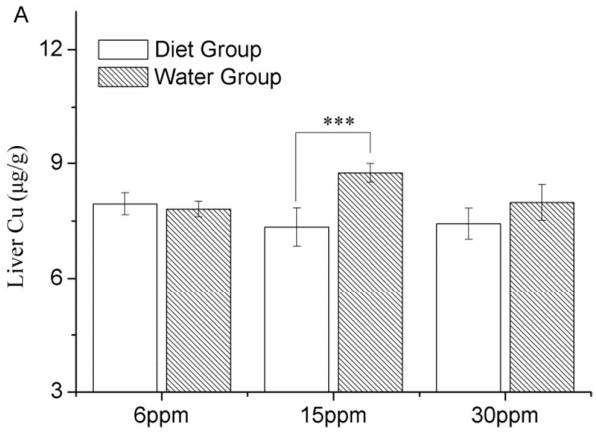
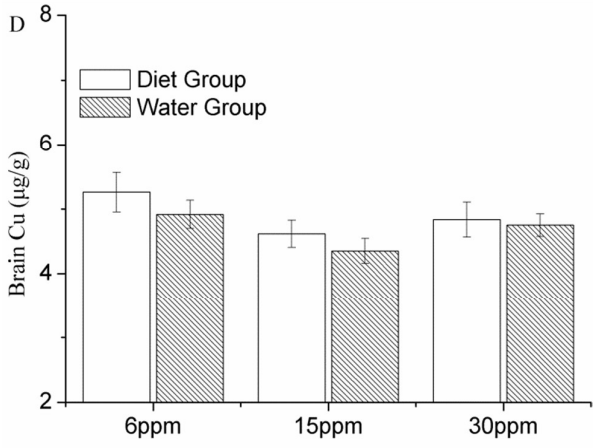
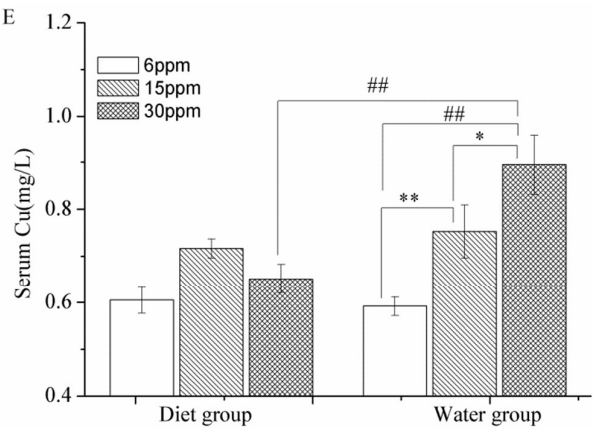


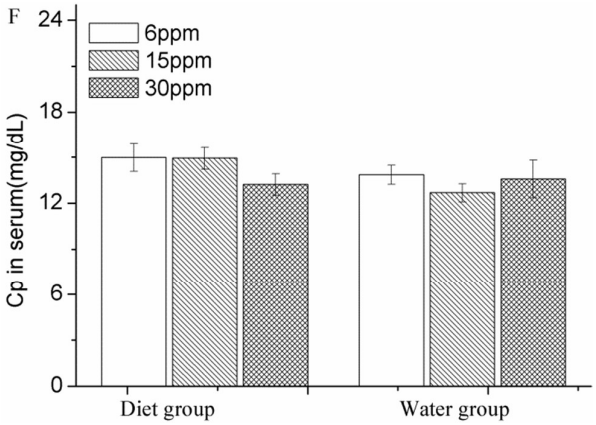
Fig.3 Hepatic GSH (A) and SOD (B) activity. Values are shown as means ±SEM，*P＜0.05, **P＜0.01, ***P＜0.005, ##P＜0.001

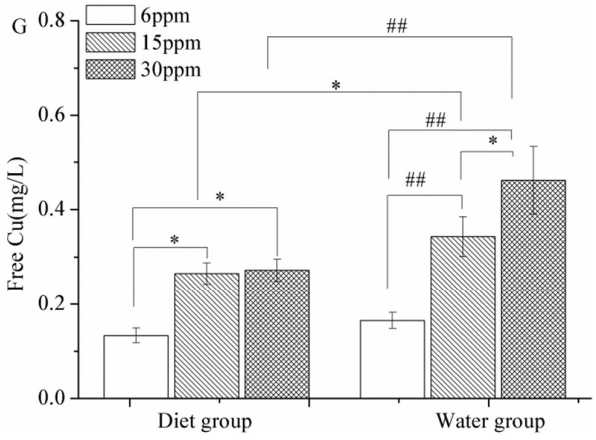
Figure 4











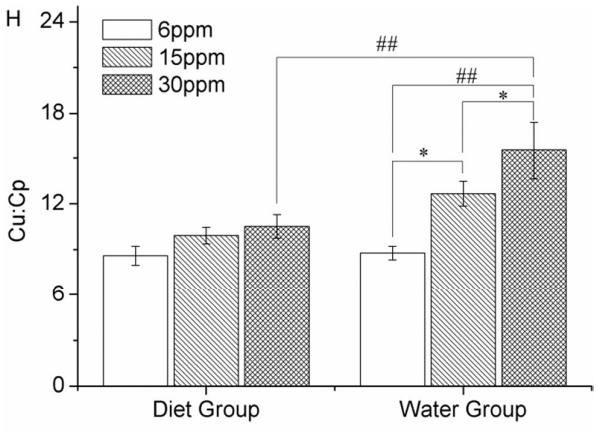


Fig.4 Copper deposited in liver (A), kidney (B), spleen (C) and brain (D). Serum copper (E), Cp (F), serum 'free' copper (G) and Cu:Cp (H). Values are shown as means ±SEM，*P＜0.05, **P＜0.01, ***P＜0.005, ##P＜0.001

Figure 5

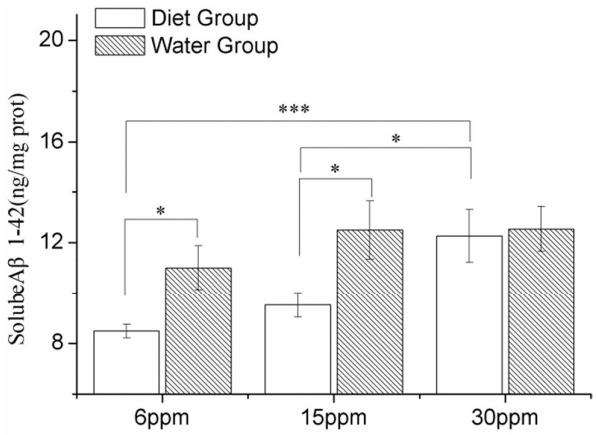


Fig.5 Soluble $A\beta_{42}$ in the brain. Values are shown as means ±SEM，*P＜0.05, **P＜0.01, ***P＜0.005, ##P＜0.001

Tables

Table 1. Ingredient composition of the basal diets [a)]

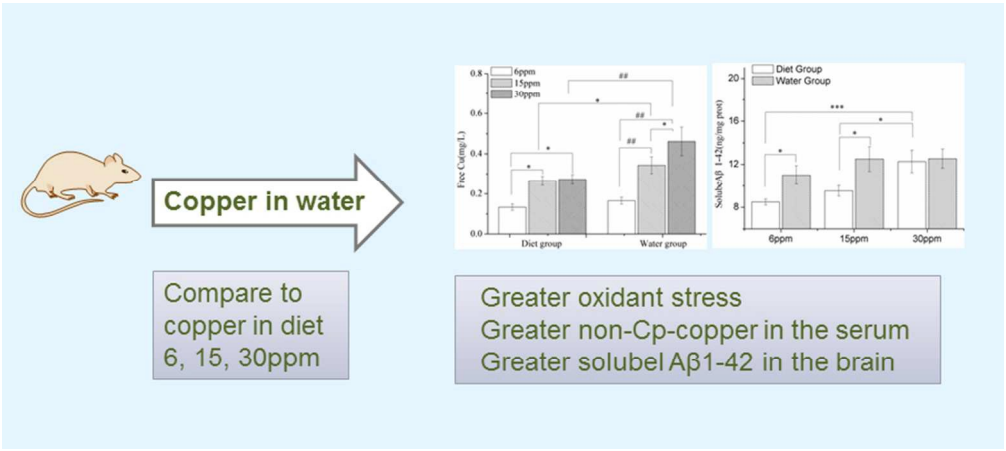
Ingredient	*g/kg diet*
Cornstarch	397.486
Casein (>85% protein)	200.000
Dextrinized cornstarch (90-94% tetrasaccharides)	132.000
Sucrose	100.000
Soybean oil (no additives)	70.000
Fiber	50.000
Mineral mix without Cu [b)]	35.000
Vitamin mix [c)]	10.000
L-Cystine	3.000
Choline bitartrate (41.1% choline)	2.500
Tert-butylhydroquinone TBHQ	0.014

a) Diets were purchased from Trophic Animal Feed High-tech Co., Ltd, China.

b) Upon analysis, the total copper concentration of the basal diets was 0.81ppm (for water group), different amount of copper was added, the final concentration in feed is 6.77, 15.81 and 30.69ppm (for diet group). The other ingredients were corresponding with the AIN-93 purified Diets for laboratory rodent.

c) Vitamin and Mineral mix provided corresponding the AIN-93 purified Diets for laboratory rodent.

Copper in water is toxic than copper in food for it raise the serum nonceruloplasmin copper and brain's amyloid-beta.



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