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Intestinal absorption of copper from drinking water containing fulvic acids and an infant formula mixture studied in a suckling rat model

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Abstract

The purpose of this study was to investigate if the intestinal absorption of copper in drinking water is altered in the presence of complexing agents from a fulvic acid mixture and an infant formula powder. Ten to twelve day old rat pups were given a single oral dose of radio-labeled Cu in deionized water (0.93 mg Cu/l), in water containing fulvic acids (10 mg/l), in infant formula mixed with deionized water, or in infant formula mixed with water containing fulvic acids. Six hours after dosage, radioactive Cu was analyzed in the mucosa of the small intestine, the liver and the remaining carcass (excluding the liver and gastrointestinal tract) by gamma counting. Dialysis and centrifugation experiments showed that Cu was complexed by components in the fulvic acid and formula mixtures, although the presence of fulvic acids in the water did not alter the Cu fractionation in the formula. The fractional Cu uptake (% of dose) from the intestinal lumen to the mucosa was not markedly changed by the presence of the chelating agents. However, the retention of Cu in the intestinal mucosa was increased by both fulvic acids and formula. Concomitantly, the absorption rate of Cd to the circulatory system was decreased. No interactive effect between fulvic acids and formula was found on the Cu absorption. These findings indicate that the water quality may be an important determinant of the rate of intestinal Cu absorption from drinking water. Moreover, in the future risk assessment of copper in drinking water, the possibility of alterations in absorption of drinking-water Cu has to be considered when the drinking water is used for cooking.

Introduction

Infants who are not breast-fed are highly dependent on formula diets. Since powdered infant formulas are mixed with water, it is important to have drinking water of good quality. Associations between elevated copper concentrations in drinking water/soft drinks and gastrointestinal upsets have been reported in several studies (Knobeloch *et al.* 1994; WHO 1998). Recently WHO (1998) proposed 2 mg Cu/l as a provisional guideline value for copper in drinking water, based on copper-induced gastrointestinal effects after ingestion of contaminated drinking water. In an experimental study on infants, published after the WHO guidelines, no acute or chronic adverse consequences of consuming water with a copper content of 2 mg/l were detected during the first year of life (Olivares *et al.* 1998). The researchers concluded that the results support the safety of WHO's provisional guideline value for copper in drinking water during infancy. However, as pointed out by Fitzgerald (1998), there are still uncertainties regarding possible acute health effects of copper in drinking water, and good research is required to improve our understanding of the threshold of copper's acute toxicity. Although Cu is toxic to animals and humans at high exposures (Bremner 1998), Cu is also an essential trace element, and in cases of low dietary intake of Cu, absorption from water may be of great importance for the copper balance. Still, little is known about the influence of water qual-

ity on the absorption of Cu, and how the absorption is affected when the water is used for food preparation.

Cu binds readily to humic acids (HA) and fulvic acids (FA) that are common in drinking water (Driscoll *et al.* 1988; Lövgren & Sjöberg 1989; Flaten & Bøviken 1991; Livens 1991; Alberts *et al.* 1992). HA and FA are very heterogeneous mixtures of compounds with carboxylic, phenolic and hydroxylic functional groups, but FA have a lower molecular weight than HA. Moreover, FA can be dissolved in both acids and bases whereas HA are more soluble at pH > 7. Similarly as in mixtures of humic compounds, infants formulas contain Cu-complexing agents (Lönnerdal *et al.* 1985). In this case it has been shown that the bioavailability of Cu differs depending on the composition of the formula (Lönnerdal *et al.* 1985).

This study was designed to determine whether the absorption of Cu from water is affected by complex binding of copper to components in a fulvic acid mixture and an infant formula. The suckling rat model was used since it has been shown to be a suitable model for studying the bioavailability of trace metals from milk and infant formulas (Sandström *et al.* 1983; Lönnerdal *et al.* 1985; Palminger Hallén & Oskarsson 1995). The concentration of copper in the experimental water used was 0.93 mg/l, which is close to the median concentration of copper in drinking water before flushing of pipes in some of the largest cities in Sweden (0.72 mg Cu/l) (Thuvander & Oskarsson 1998).

Materials and methods

Animals

Female rats (Sprague Dawley) from Möllegaard (Denmark) weighing approximately 200 g were mated with males for 3 days. The animals were maintained behind strict hygienic barriers at 23 °C with 50–60% humidity and a 12 h:12 h light:dark cycle. The rats were given free access to food, (R3 pellets, Ewos AB, Södertälje, Sweden) and drinking water (Uppsala tap water). About a week after birth the pups were divided among the dams, with 10 pups for each dam. The pups were used in the experiment at 10–12 days of age. Principles of laboratory animal care were followed and the animal experiments were approved by the Uppsala Ethics Committee of Animal Experiments (permission no. C243/93).

Table 1. Concentrations of trace elements in the feeding solutions

	Water mg/l	FA[a] μg/l	Formula[a] mg/l	Formula+FA mg/l
Ca	–	2.8	460	460
Fe	–	9	4	4
Zn	–	0.007	4	4
Cu	0.93[b]	0.93[b]	1.5	1.5

[a]Information about the concentration in the freeze dried fulvic acid preparation was obtained from Riise and Salbu (1989) and in the infant formula powder from the manufacturer.
[b]mg/l (N=1).

Chemicals

Radioactive Cu, as a mixture of the ^{67}Cu ($T^{1/2}$ = 61.92 h) and ^{64}Cu ($T^{1/2}$ = 12.7 h) isotopes in 0.01 M HCl, was obtained from the The Svedberg Lab, Uppsala, Sweden. The specific activity in the isotope mixture was 2.9 MBq/μg Cu upon the day of arrival. The fulvic acids came from the Nordic Reference Fulvic Acid (Riise & Salbu 1989). Infant formula (BabySemp 1, Semper AB, Stockholm, Sweden) was purchased at a local grocery store.

Feeding solutions

All feeding solutions contained both Cu from the isotope mixture (0.1 mg/l) and stable Cu (1.0 mg/l) added as $CuCl_2$ * 2 H_2O (analytical grade, Merck, Darmstadt, Germany). The final Cu concentration was determined to 0.93 mg/l by atomic absorption spectrophotometry (Table 1). The radioactive and the stable Cu was added to deionized water at the same time and the fulvic acids (10 mg/l) were added to one part of this solution. Cu solutions with and without fulvic acids were allowed to equilibrate for 24 h at 8 °C before use. The solutions containing infant formula were prepared according to the instructions on the package, that is, the equilibrated water was heated, the formula powder was mixed into it and the mixture was allowed to cool. This solution was prepared about one hour before the pups were fed. The final nominal Cu concentration was 1.5 mg/L in the solutions with infant formula. The amount of native Cu as well as other trace elements (Ca, Fe and Zn) in the fulvic acids was negligible (Table 1).

Experimental procedures

The experimental procedure basically followed that described by Lönnerdal *et al.* (1985) with some modifications. At the start of the experiment, the 10–12 day old pups (both males and females) were divided into four groups of 10. Care was taken that the mean body weights and age of the pups did not differ between the groups. The pups were separated from their dams five hours before dosage (0.3 ml), which was done by gastric intubation, and then for an additional six hours after dosage. The pups were kept warm by the use of heating lamps during the 11 hours of separation from their dams. Fifteen minutes after dosage the radioactivity of each animal was analyzed in a whole body gamma counter (NaI well crystal; diam. 80 mm; depth 120 mm, counting efficiency 17%) to check the dose given to the animals.

Six hours after dosage the pups were weighed and anaesthetized (Metofan). Blood was collected by heart puncture and the pups were killed by exsanguination. The gastrointestinal tract and the liver were removed. The small intestine was separated from the stomach and caecum/colon. The lumen of the small intestine was rinsed with 10 ml of saline (0.9% in 5 mM EDTA) to remove surface-bound Cu. The livers were weighed.

The liver, blood and rinsed small intestine were analyzed for radioactivity in an organ gamma counter (Nuclear, Chicago, Model 1185, counting efficiency 27%). The remaining carcass (without the liver and gastrointestinal tract) was analyzed in the whole body gamma counter.

The lower counting efficiency of the whole body counter was due to a larger well diameter in the whole body counter, resulting in higher losses of radioactivity through the opening of the well. Differences in the thickness of the metal coating of the NaI crystal also contributed to the differences in counting efficiency, as well as a higher absorption of low energy radiation by the larger tissue mass analysed in the whole-body counter. The results were corrected for differences in counting efficiency between the whole-body and organ counters.

Compensation for radioactive decay

Ten standards (mixture of ^{64}Cu and ^{67}Cu) were counted in the organ gamma counter and in the whole body gamma counter at the start of the experiment. These standards were then counted together with the samples and used for compensation. The results were corrected for differences in counting geometry by counting standards with the same volume and geometry as the samples.

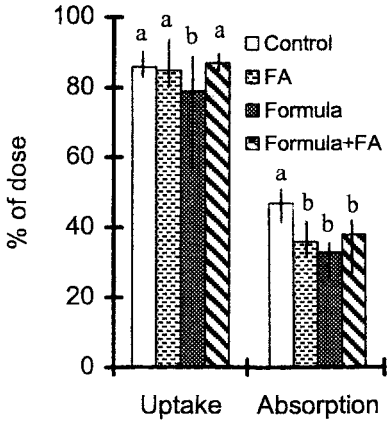

Figure 1. Intestinal uptake and absorption of drinking water copper (median (min-max)) in suckling rats in the presence of fulvic acids and/or infant formula in the water. Uptake and absorption was measured 6 h after a single oral dose of Cu in water (see MATERIALS AND METHODS for details about calculations of uptake and absorption). N=10 in control, FA and Formula+FA groups. N=9 in Formula group. Different letters above the bars denotes a statistically significant difference, $p<0.05$ (Mann–Whitney U-test).

Fractionation of Cu in the feeding solutions

The two solutions containing infant formula were centrifuged at 100 000 g for 2 h (4 °C). The fat layer was removed and the ultra supernatant (whey fraction) was filtered through a Sephadex G-75 column (2.5 × 60 cm, Pharmacia/LKB, Sweden) equilibrated with 50 mm Tris-HCl buffer (Kebo Lab AB, Sweden), pH 6.5 at 4 °C, and calibrated with Blue Dextran 2000 (void volume, Merck, Germany) and metallothionein (Merck, Germany) (Mw = 10 000). The flow was 60 ml/h and 3 ml fractions were collected. The pellet containing insoluble proteins, the fat layer, 200 μl of the whey fraction containing soluble proteins, and the 3 ml fractions were analyzed in the organ gamma counter.

The complex binding of copper in water solutions containing fulvic acids was studied by dialysis. Cellulose ester dialysis tubing with a cut-off of 500 (Spectra/Por® , CE, Houston, USA) was pre-treated with deionized water. The tubes were filled with pure deionized water or deionized water containing 10 mg FA/l. After that they were placed in plastic beakers filled with deionized water to which stable and radioactive Cu had been added to a final concentration of 1 mg Cu/l. The system was equilibrated for 72 h at room temperature on a rotating table. Samples from

the inner and outer solutions were analyzed for radioactivity in the organ gamma counter. The per cent of complexed Cu was calculated using the formula:

$$\%\,\text{complexed} = ((\text{cpm}^{*}\text{ml}_{\text{in}}^{-1} - \text{cpm}^{*}\text{ml}_{\text{out}}^{-1})/\text{cpm}^{*}\text{ml}_{\text{in}}^{-1}) \times 100. \quad (1)$$

Calculations and statistics

The fractional retention of Cu in the tissues (% of dose) 6 h after dosage was calculated from the formula:

$$\text{tissue}_{\text{frac}} = (\text{cpm}_{\text{tissue (6 h)}}/\text{cpm}_{\text{whole body (15 min)}}) \times 100), \quad (2)$$

where $\text{cpm}_{\text{tissue(6 h)}}$ is total cpm in the sampled tissue 6 h after dosage, and $\text{cpm}_{\text{wholebody(15 min)}}$ is the measured dose in the animal 15 min after dosage.

The absorptive process of copper is divided into two steps. Step 1 is the uptake of Cu from the intestinal lumen into the mucosa, step 2 is the absorption of Cu from the mucosa to the circulatory system. The copper taken up the small intestine during the 6 h after dosage was detected in the mucosa of the intestine, and in the blood, liver and carcass (gastrointestinal tract and liver excluded). The fractional intestinal uptake (% of dose) (step 1) was thus calculated using the formula:

$$\text{fractional uptake} = (\text{small intestine}_{\text{frac}} + \text{blood}_{\text{frac}} + \text{liver}_{\text{frac}} + \text{carcass}_{\text{frac}}). \quad (3)$$

The copper detected in the blood, liver and carcass (gastrointestinal tract and liver excluded) has completed both steps of the absorptive process. Thus, the fractional absorption (% of dose) (step 2) was calculated using the formula:

$$\text{fractional absorption} = (\text{blood}_{\text{frac}} + \text{liver}_{\text{frac}} + \text{carcass}_{\text{frac}}). \quad (4)$$

The Kruskal–Wallis one way analysis by ranks was used to compare results in all four groups. When significance occurred by this method the Mann–Whitney U-test was used to further compare results from two groups. The limit for significance was set at $p<0.05$ for both tests. The results are presented as median (min-max).

Results

Organ and body weights

The body and liver weight of the pups did not differ between the groups (median body weight 20.9–22.5 g and median liver weight 0.57–0.64 g).

Copper retention

The median fractional retention of Cu in the blood, liver and carcass (GI tract and liver excluded) was higher in the water group than in the fulvic acid and formula groups, whereas the fractional retention of Cu in the intestine (after removing Cu from the intestinal lumen and surface of the intestinal mucosa) was lower in the control group than in the other experimental groups (Table 2). As calculated from Equation (2), the median fractional intestinal uptake (step 1) was not markedly different between the groups, except for a slightly lower uptake in the formula group (Figure 1). The fractional intestinal absorption (step 2), on the other hand, was higher in the water group than in the other groups. There was no indication of an interactive effect between the fulvic acids and infant formula on intestinal uptake and absorption of Cu.

Fractionation of Cu

As calculated from Equation (1), at least 24.6% (median; min = 23.5%; max = 25.3%; N=3) of the Cu was complexed in the solution containing 10 mg FA/l, whereas no complexed Cu was detected in the pure water solution (N = 2). In the formula solutions with or without FA more than 70% of the Cu was found in the pellet, i.e., the Cu was associated to ligands with low water solubility (Table 3). The rest of the Cu was evenly distributed to the fat and whey fractions. In the whey fraction Cu was associated to compounds of both high and low molecular weights, and no indication of the presence of free copper ion was found (Figure 2). The presence of FA did not alter the Cu distribution in the infant formula solution.

Table 2. Retention of radioactive copper tracer (% of dose) in different organs 6 hours after oral administration to suckling rats. Results are presented as median (min-max)*

	Water $n = 10$	FA $n = 10$	Formula $n = 9$	Formula+FA $n = 10$
Small intestine	39 (37–41)[a]	48 (43–51)[b]	45 (21–55)[b]	49 (45–54)[b]
Blood	0.7 (0.6–0.8)[a]	0.6 (0.3–0.6)[b]	0.5 (0.3–0.7)[b]	0.5 (0.4–0.7)[b]
Liver	23 (21–24)[a]	15 (15–19)[b]	16 (14–18)[b]	17 (15–20)[b]
Carcass	23 (21–25)[a]	19 (18–22)[b]	18 (16–21)[b]	19 (17–22)[b]

*Different letters denote significant differences, $p<0.05$ (Mann–Whitney U-test).

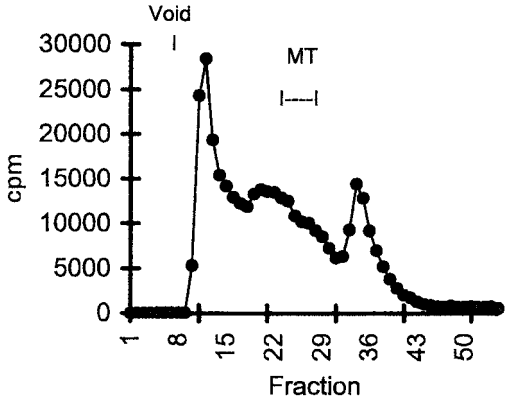

Figure 2. Distribution of copper in the water-soluble fraction (whey) of the infant formula after filtration through a Sephadex G-75 column (2.5 × 60 cm, Pharmacia/LKB, Sweden) equilibrated with 50 mM Tris-HCl buffer (Kebo Lab AB, Sweden), pH 6.5 at 4 °C. The flow was 60 ml/h and 3 ml fractions were collected. The column was calibrated with Blue Dextran (void volume, Merck, Germany) and metallothionein (Mw = 10 000, Merck, Germany).

Table 3. Distribution of Cu (%) in the feeding solutions containing infant formula[a]

	Formula (%)	Formula+FA (%)
Water insoluble	70	71
Water soluble (whey)	16	16
Fat	14	13

[a]N=1.

Discussion

Several studies have shown that fulvic acid mixtures have the capacity to complex-bind copper ions in water (Driscoll *et al.* 1988; Lövgren & Sjöberg 1989; Flaten & Bøviken 1991; Livens 1991; Alberts *et al.* 1992). As expected, in the ion-poor water used in the present study, copper was complexed by components in the fulvic acid mixture. The real degree of complexation was not possible to determine from the dialysis experiment, since it cannot be excluded that Cu complexes with a lower molecular weight than 500 may have been present.

When the copper-containing water was used to mix the infant formula, more than 70% of the Cu was detected in the insoluble pellet after centrifugation. This fraction has a high content of casein, which contains phosphoserine groups with a capacity to bind metal ions (Greenberg *et al.* 1976; McMahon & Brown 1984; Palminger Hallén & Oskarsson 1995; Danielsson *et al.* 1995). The rest of the copper in the solution of infant formula was evenly distributed between the fat and whey fractions. This distribution pattern was similar to that found in cows' milk formula by Lönnerdal *et al.* (1985). In the whey fraction Cu was complexed both to high- and low-molecular-weight components. No indication of free copper ions was found when the whey fraction was gel filtrated. The centrifugation experiment also showed that the presence of fulvic acids in the water did not affect the distribution of Cu in the formula solution. The complex binding of Cu to FA in the drinking water used to mix the formula was apparently not strong enough to prevent a redistribution of copper from the FA fraction to components in the formula mixture.

The fractional uptake (% of dose) of copper from the intestinal lumen to the mucosa, during the 6 h period after Cu dosage of the suckling rats, was not markedly affected by the fulvic acids or formula, despite the presence of fulvic acids and infant formula. This shows that the intestinal uptake of copper was not dependent on the form of copper in the feeding solution. Although not studied by us this could be due to a dissociation of the Cu complexes during passage through the acidic stomach, where both the acidic environment and peptic digestion may cause a release of copper from foodstuffs (Wapnir 1998). The unchanged uptake in the presence of fulvic acids and infant for-

mula may also be due to uptake by a non-specific uptake mechanism for Cu in the intestine. It has been shown that the ileum of the suckling rat has a high capacity of non-specific pinocytosis, and it has been indicated that copper and lead may be taken up by this mechanism in enterocytes lining the distal part of the small intestine (Jones 1978; Keller & Doherty 1980; Dinsdale *et al.* 1986). Which of these two mechanisms (or others) that contribute to the unchanged intestinal uptake of Cu in the presence of fulvic acids and infant formula has to be determined in future studies.

The median fractional uptake of copper in the suckling rat ranged from 79% to 87% of the dose. The rest of the copper dose (13% to 21%) may consist of copper that was not bioavailable for intestinal uptake or of excreted copper, for instance Cu excreted in the bile (Mohan *et al.*, 1995). We did not measure Cu retention in the whole body 6 h after dosage or the excretion of copper during the post-dosage period, due to a tight time schedule. It is thus not possible to give an estimate of the percentage of dose excreted by the animals.

In contrast to the intestinal uptake of Cu into the mucosa (step 1), the fractional absorption of Cu, 6 h after Cu dosage, was slower in the presence of FA and infant formula. Concomitantly, the fractional retention of Cu in the intestinal mucosa was higher in the FA and formula groups than in the control group. The same effect was seen in the group receiving infants formula mixed in water containing FA, although, as expected from the fractionation experiment, no interactive effect was seen between FA and the infants formula. The mechanism behind the higher retention in the intestinal mucosa and the slower absorption cannot be determined from our results. However, studies on rat pups have shown that the uptake of lead in the intestinal mucosa occurs more distally in the small intestine when high concentrations of complex binders, such as casein, are present in feeding solutions (Palminger Hallén & Oskarsson 1995). Thus, hypothetically, the presence of complex binders in the water may have caused a delay in the absorption of Cu due to a more distal uptake of Cu in the intestine. In the work of Dinsdale *et al.* (1986), more than 90% of the Cu in the small intestine was located in the ileum in 11 day old rat pups fed Cu in rat milk, which has a very high casein content.

Another possibility is that high concentrations of elements such as Zn, Fe and Ca in the fulvic acid and infant formula mixture affected the Cu absorption. Studies on adult rats have indicated an inhibitory effect of trace elements on Cu absorption (Wapnir *et al.* 1993). However, in the case of the fulvic acid preparation, the concentrations of Fe, Zn and Ca were low (Riise & Salbu 1989), thus making an inhibitory effect of these elements on the Cu absorption less likely. More studies are needed in order to determine the precise mechanism behind the slower absorption of drinking-water Cu in rat pups in the presence of FA and infants formula.

To our knowledge only one earlier study has tried to determine the absorption of Cu from water in suckling rats. Lönnerdal *et al.* (1985) found that 11% of a dose of ^{64}Cu in water was detected in the liver 6 h after dosage. The addition of different infants formulas increased the 6 h retention in the liver. In our study, which was performed in a similar way, the liver retention was 23% when Cu was administered in ion-poor water, and, in contrast to the former study, the retention of Cu was decreased when the formula was added to the water. However, a comparison of the results from the two studies is difficult, since Lönnerdal *et al.* did not report the water quality used and the concentration of Cu in the water. Moreover, the pups were older (14 days) and had been starved for a much longer time (18 h) than the pups in our study (10–12 days old and 5 h starving period). Our study shows that mixing of infants formula in ion-poor water made the absorption of drinking-water Cu slower in 10- to 12-d old rat pups. Moreover, the presence of fulvic acids in the water affected the absorption in a similar fashion, although no interactive effect on absorption was found when formula was mixed in water containing fulvic acids.

Conclusions

Our results show that the absorption of drinking-water Cu may both be affected by the water quality and by components in food stuffs mixed in the water during cooking. Although the knowledge about the influence of water quality and cooking on the absorption of drinking-water Cu is scarce, our study indicate that these factors may have to be considered in future risk assessments of copper in drinking water. Moreover, these factors may be important in cases of copper deficiency, when drinking-water Cu may give an important contribution to the Cu intake.

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