Confirmation of an acute no-observed-adverse-effect and low-observed-adverse-effect level for copper in bottled drinking water in a multi-site international study[☆]

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Abstract

In a double blind, 3×3 factorial (volume $\times$ dose) study, 70 adult females (18–60 years of age) at four different international sites (total pooled $n = 269$) were given 100, 150, or 200 ml of bottled drinking water with 0.4, 0.8, or 1.2 mg of copper (Cu) as the sulfate salt once each week. Two additional doses (0 and 1.6 mg Cu) were added at the 200 ml volume to determine a dose–response relationship and corroborate previously reported results. All subjects completed a questionnaire at 0, 0.25, and 1 h post-dosing that screened for positive gastrointestinal (GI) effects (nausea, vomiting, abdominal pain, and diarrhea). Nausea was the most prevalent symptom reported and was generally reported within the first 15 min (water volume, $p < 0.032$; copper dose, $p < 0.0001$; and water volume $\times$ copper interaction, $p < 0.97$). As volume increased, the effect of Cu-induced nausea decreased; as Cu dose increased, the incidence of nausea increased. At 200 ml, a significant increase in reported incidence of nausea at 0.25 h occurred at 1.2 mg Cu (6 mg Cu/L), indicating a NOAEL of 0.8 mg Cu (4 mg Cu/L) for adult females. These data confirm a previously determined human acute NOAEL for Cu added to distilled water, and provide additional, controlled human data for determining safe concentrations of Cu in drinking water.

Keywords: Copper; NOAEL; Bottled water; Nausea; Risk assessment; Dose–response; Acute exposure; Upper Safe Limit

1. Introduction

It has been reported that an acute human no-observed-adverse-effect level (NOAEL) and low-observed-adverse-effect level (LOAEL) for copper as the sulfate salt in water are 4 and 6 mg Cu/L (0.8 and 1.2 mg Cu in 200 ml). The study measured gastrointestinal effects from copper ingestion in water in an international study population of males and females aged 18–60 years (Araya et al., 2001). Consistent with other reports of high oral copper ingestion, the appearance of nausea is a primary manifestation of acute adverse copper effects,

especially following a fasting state (Center for Disease Control, 1975; Fitzgerald, 1998; Hopper and Adams, 1958; Knobeloch et al., 1994; Le Van and Perry, 1961; McMullen, 1971; Olivares et al., 2001; Pizarro et al., 1999; Ross, 1955; Semple et al., 1960; Spitalny et al., 1984; Wyllie, 1957). The results reported by Araya et al. (2001) (and heretofore referred to as Phase I) were based on copper as the sulfate salt in a single 200 ml bolus of distilled–deionized water once per week in a double-blind controlled study. The study was conducted with a laboratory generated water source that could be consistently duplicated at each of three international sites (Grand Forks, ND, USA; Santiago, Chile; and Coleraine, Northern Ireland). These copper doses were administered in addition to natural background intakes provided by drinking water and food, and the NOAEL was identified based on statistical significance. Estimates of dietary intakes of trace elements generally do not include the amounts in drinking water. Copper intakes from water in comparison to diet have been reviewed (Klevay, 1980, 1984) and range from trivial to substantial (Sharrett et al., 1982). Most frequently, copper intakes from water tend to be small in comparison to diet (Pang et al., 2001).

The goals of the study reported herein (referred to henceforth as Phase II) were to: (1) use a natural water supply, i.e., bottled water, rather than distilled–deionized water as used in Phase I, (2) corroborate the results of Phase I, (3) increase cultural and geographic diversity by adding a fourth study site (Shanghai, China), (4) increase statistical sensitivity by utilizing only female subjects, and (5) determine the effects of dose and volume as independent and interacting variables.

In Phase I, both male and female subjects at the three sites (approximately 30 males and 30 females at each site) consumed a 200 ml bolus of copper as copper sulfate in distilled–deionized water in concentrations of 0, 2, 4, 6, or 8 mg Cu/L. The subjects reported gastrointestinal (GI) effects on a questionnaire at 0, 0.25, 1, and 24 h following the ingestion of the drinking water. The GI effects (primarily nausea) were reported within the first 15 min and were not influenced by geographic site or age of the volunteers. Building on the results of the first phase of this study, the investigators endeavored to define further the effects of copper in acute ingestion scenarios.

Phase II attempted to corroborate the dose-response seen in Phase I, and was designed to examine the effects of dose and volume and their possible interaction on the acute effects of copper as measured by the onset of nausea. In Phase I, a statistical increase in the reporting of nausea occurred at the 6 mg Cu/L concentration (1.2 mg Cu, absolute). Rather than the distilled–deionized water used in Phase I, the natural drinking water used in Phase II was well-characterized and more typical of drinking water sources with respect to pH, hardness,

and organoleptic qualities. A natural spring, bottled water was selected over ordinary tap water delivered through public utilities because it provided a more consistent quality.

2. Materials and methods

2.1. Subjects, sample size, and design

Four sites were selected to carry out this protocol (the original three Phase I sites plus the addition of an Asian site): (1) the Institute of Nutrition and Food Technology, University of Chile, in Santiago, Chile; (2) the Grand Forks Human Nutrition Research Center, North Dakota, United States; (3) the Northern Ireland Centre for Food and Health (NICHE), University of Ulster, Coleraine, Northern Ireland; and (4) the Department of Environmental Health, School of Public Health, Fudan University (formerly Shanghai Medical University), Shanghai, China. The study protocols, questionnaires, data sheets to record the results, and operational definitions of the outcome variables were standardized over the four sites based, in large part, on the guidelines to conduct such studies published by the US Department of Health and Human Services, Food and Drug Administration (1997). Ethical approval for testing of human subjects was provided by each site's Institutional Review Board or Ethics Committee. All subjects gave their informed consent before inclusion in the study.

At present, the World Health Organization (WHO, 1998) has defined acute GI symptoms as the criteria to establish the safe limits of copper in drinking water. As previously described in Phase I, nausea is the main and earliest symptom to be detected. In Phase I, a target sample size of 60 individuals per site equally distributed among sex and age was sufficient to detect statistically significant differences in the combined site data analysis. The sample size estimate was based on unpublished preliminary data collected in Santiago (Araya, personal communication) demonstrating a basal prevalence of GI symptoms of 5%, the ability to detect significant differences for a $\geqslant 15\%$ change in gastrointestinal morbidity rate, and a drop-out rate (based on collective experience) of up to 20%. Analysis of the Phase I results indicated a biological trend of female subjects tending to be more sensitive in reporting GI symptoms, primarily nausea. Thus, in order to detect a more sensitive NOAEL, the investigators chose to recruit females exclusively and to increase the recruitment class at each site to 70 women, for a potential of 280 total subjects. As in the previous study, the sample size was calculated based on the expected total incidence of GI symptoms.

At each site (Coleraine, Santiago, Grand Forks, and Shanghai) a target group of 70 females was recruited by local advertisements. All subjects were informed of

Table 1
Copper (as sulfate) acute study 3×3 factorial design with addition of two 200 ml dose cells[a] (Units of concentration in mg Cu/L)

Volume (ml)	Dose (mg Cu)				
	0	0.4	0.8	1.2	1.6
100		4	8	12	
150		2.6	5.3	8	
200	0	2	4	6	8

[a] Shaded area shows 3×3 factorial design range.

study details and signed a written consent. Each subject answered a health and lifestyle questionnaire (the same used in Phase I). Individuals who were heavy drinkers or smokers, taking prescription drugs, or were pregnant were excluded from the study. As in Phase I, the sample was targeted to be stratified to include approximately 50% under and over 40 years of age; however, the median ages for the four sites were Santiago—37 years, Shanghai—31 years, Coleraine—27 years, and Grand Forks—37 years. All subjects received monetary compensation at the completion of study based on local customs and practices.

The core study design is a 3×3 two-way factorial design (volume × dose) with doses of 0.4, 0.8, and 1.2 mg Cu and volumes of 100, 150, and 200 ml bottled drinking water. An additional two doses (0 and 1.6 mg Cu) were added at the 200 ml volume in an attempt to corroborate the results of the Phase I study, by matching the doses and volume tested in that study. The final concentrations of copper sulfate utilized in the study are presented in Table 1.

2.2. Solutions

A single lot (PRO 03 AUG 99 08 26) of bottled, natural spring water was obtained from a water bottler (Naya, Que.,Canada) and distributed in sufficient quantity to each of the four sites. The source spring finished water product was analyzed by the bottler for inorganic (including copper) and organic contaminants and for other physical factors (including, but not limited to alkalinity, hardness, pH, total dissolved solids, turbidity, color, corrosivity, and odor threshold). No remarkable contamination or unusual physical factors were found. Copper contamination was not detected at a minimum detection level of 0.002 mg/L. As in Phase I, a single lot of copper sulfate pentahydrate (USP) pro analysis grade was obtained (Fisher Scientific, USA) and distributed among the four sites. A stock solution of 80 mg Cu/L was prepared daily at each study site and its concentration confirmed by atomic absorption spectrophotometry. The stock solution was serially diluted to the daily administered bolus of copper in bottled drinking water and the concentration was confirmed by atomic absorption spectrophotometry. Chemical analysis confirmed that the actual prepared copper solutions

were ±3% of the target concentration for the duration of the study at the four sites. In order to verify that the copper in the solution was completely ingested, the cups were retrieved and washed after the volunteers consumed the test solutions at two of the test sites. At the Grand Forks site, the retrieved cups were washed with dilute acid; analysis of the solutions by inductively coupled plasma atomic emission spectroscopy revealed that less than 0.6% of the dose had been retained in the cup on the average. At the Shanghai site, the emptied cups were washed with the stock bottled water. Analysis of the solution at the Shanghai site by atomic absorption spectrophotometry revealed that less than 0.4% of the dose had been retained in the cups.

2.3. Protocol

The protocol used in Phase II was exactly as that used in Phase I. Subjects fasted overnight and came to the test facility one morning each week for 11 successive weeks or until all 11 administrations were completed. Each subject was to complete ingestion of the bolus within 3 h of rising. Additional dietary restrictions included no alcohol on the evening prior to the test and no medicine or dietary supplements on the morning of the test. Immediately prior to ingesting the copper, individuals were asked to complete a written questionnaire containing a list of symptoms and signs (Araya et al., 2001), indicating whether these were present or absent on that day. The list of symptoms included the primary outcome variables: nausea, abdominal pain, vomiting, and diarrhea, as well as several symptoms not thought to be initiated by copper ingestion, but evaluated to distract the subject (e.g., backache, sweating, palpitations, heartburn, headache, feeling of anxiety, dizziness, and salivation). When it was confirmed that the subject was asymptomatic, she was given one of the 11 test solutions, assigned to each subject in a random order, and blinded to the experimental subject and the clinical investigator. The volunteers were asked to consume the solution as quickly as possible. Typically the solution was completely ingested in no more than 2 min from study initiation. After 15 min, subjects completed the same questionnaire again. Asymptomatic subjects were brought into a lounge provided with magazines, where they spent the remainder of the hour, while subjects reporting any symptoms remained under supervision. At the end of 1 h of direct observation, all subjects again completed the same questionnaire before leaving the study facilities. Instructions given to the subjects were carefully planned and coordinated prior to the beginning of data collection in order to make them constant and comparable at the four sites. Total number of individuals responding at 15 and 60 min is reported, as well as the total number of individuals responding at either time point. Note that the 60-min response reflects

symptoms between 15 and 60 min; it is not a cumulative measure from time 0 to 60 min.

2.4. Response evaluation and analysis of results

Consistent with Phase I, the threshold dose for a symptom was defined as the lowest dose at which a statistically significant increase of the symptom incidence over controls was observed. Thus, nausea, vomiting, diarrhea, and abdominal pain were defined as outcome variables, with nausea defined as the imminent desire to vomit, either mildly or intensely, as reported by the study participant. This definition of nausea was carefully explained to the participants at the time of incorporation to the protocol.

Analyses were done for two outcome variables, nausea and GI symptoms, as separate analyses. The outcome variable, GI symptoms, was defined as the occurrence of one or more outcome symptoms (nausea, vomiting, diarrhea or abdominal pain) after consuming a given copper dose. Outcome data for nausea and GI symptoms were modeled using logistic regression within the generalized estimating equation procedure (Proc Genmod) in SAS/Stat (SAS Institute, Cary, NC, USA). The repeated measures structure of the design was incorporated into the logistic regression models. Included as potential covariates in the models were test location, age group of the subject (less than or greater than 40 years of age), and the order in which the doses were administered. Three indicator variables were used to test for differences among the four test locations. Covariates that did not significantly improve the model fit were omitted from the final model. Dose–response estimates and odds ratios were generated using the parameter estimates obtained from the final model for nausea and GI symptoms.

The dosing regimen was designed to be incorporated into a 3 × 3 two-way factorial design with three different copper doses (0.4, 0.8, and 1.2 mg Cu) and three different bolus volumes (100, 150, and 200 ml). The nine administration cells resulted in copper concentrations ranging from 2 to 12 mg Cu/L (Table 1). This experimental design allowed for two independent variables (dose and volume) to be efficiently analyzed both independently from one another and dependently (interaction between the variables, i.e., copper dose × bolus volume interaction). As part of the study design, two additional 200 ml bolus volume cells were added for 0 and 1.6 mg Cu that allowed for the linear analysis of this bolus volume to independently corroborate the results from Phase I and to provide a similar dose–response analysis.

2.5. Quality control, monitoring, and coordination

Each of the four research institutions obtained ethical approval from their respective Institutional Review Board or Ethics Committee, and was responsible for maintaining internal quality control and strictly adhering to the protocol. In Coleraine, a 5-ml blood sample was taken from each volunteer at the insistence of the University of Ulster Ethical Committee to insure that liver function tests were within the normal range. Those who refused to give a blood sample or had abnormal liver function tests were excluded from the study. Toxicology Excellence for Risk Assessment (TERA), a non-profit risk assessment research organization, managed the study. This work included conducting a site inspection for each location, providing study monitoring and coordinating each location's study protocol, and insuring compliance for human exposure ethical approval protocols.

3. Results

A total of 269 women finished the trial at the four sites (70 in Santiago, Chile; 68 in Grand Forks, ND, USA; 58 in Coleraine, Northern Ireland, and 73 in Shanghai, China). The screened respondents were a healthy population with no apparent medical problems during or after completion of the protocol. The success of population recruitment and dropout rates varied among sites, and this variability was reflected in the final sample size at each site.

Our first objective was to corroborate the results for nausea (the earliest and most frequent response to copper) obtained in Phase 1 and assess their reproducibility. As the Phase I study (Araya et al., 2001) evaluated a 200 ml volume, results for the 200 ml volume are presented here first. Other studies on acute copper effects (Araya et al., 2003; Gotteland et al., 2001; Olivares et al., 2001; Pizarro et al., 1999) also tested a 200 ml volume. A 200 ml volume is also a portion size frequently ingested when fluids are consumed. Because the effects measured were related to copper concentration, which varies widely during the course of the day and is influenced by volume, all the potential variations could not be taken into account in a controlled design; the volumes chosen in this study were calculated to represent fluid portions consumed in real life situations.

Consistent with Phase I, for the 200 ml bolus volume, nausea was the earliest and most prevalent symptom observed. Nausea was most frequently reported within the first 15 min after ingestion and the prevalence decreased with time after the dose. Within 15 min, 24.3, 41.1, 25.9, and 50.0% of the subjects at each of the four sites, Santiago, Shanghai, Coleraine, and Grand Forks, respectively, reported at least one occurrence of nausea at any dose. The average prevalence of nausea among all subjects (at all sites) was 35.7% at 15 min. The overall prevalence of subjects reporting nausea decreased from 15.6% at 15 min to 6.3% at 60 min in the 6 mg Cu/L

Table 2
Incidence and generalized linear model results of nausea at 15 and 60 min by location for linear dose–response at 200 ml volume ingested

Location	Sample size	Copper (mg)				
		0	0.4	0.8	1.2	1.6
Incidence of nausea induced by copper in 200 ml bolus at 15 min						
Santiago	($n = 70$)	0	0	1	8	11
Shanghai	($n = 73$)	0	2	5	14	24
Coleraine	($n = 58$)	0	1	4	6	11
Grand Forks	($n = 68$)	1	4	10	14	24
Total	($n = 269$)	1	7	20	42	70
Overall prevalence (%)	($n = 269$)	0.4	2.6	7.4	15.6	26
Incidence of nausea induced by copper in 200 ml bolus at 60 min						
Santiago	($n = 70$)	0	0	0	1	4
Shanghai	($n = 73$)	1	1	6	10	2
Coleraine	($n = 58$)	0	1	2	3	2
Grand Forks	($n = 68$)	1	4	5	3	13
Total	($n = 269$)	2	6	13	17	21
Overall prevalence (%)	($n = 269$)	0.7	2.2	4.8	6.3	7.8
Total people reporting nausea induced by copper in 200 ml bolus						
Santiago	($n = 70$)	0	0	1	9	13
Shanghai	($n = 73$)	1	3	8	19	25
Coleraine	($n = 58$)	0	2	5	7	11
Grand Forks	($n = 68$)	1	7	12	15	28
Total	($n = 269$)	2	12	26	50	77
Overall prevalence (%)	($n = 269$)	0.7	4.5	9.7	18.6	28.6

Generalized linear model (repeated measures) ANOVA results for linear dose–response at 200 ml volume ingested

	Results at 15 min			Results at 60 min		
	DF	χ^2	*p* value	DF	χ^2	*p* value
Test #	1	0.10	0.75	1	0.52	0.47
Location	3	14.07	0.003	3	10.88	0.012
Copper dose (mg)	4	79.39	0.0001	4	25.97	0.0001

(1.2 mg Cu in 200 ml) group (Table 2). Similarly, for those consuming 8 mg Cu/L (1.6 mg Cu in 200 ml), the overall prevalence decreased from 26.0% at 15 min to 7.8% at 60 min. The difference in the occurrence of nausea at 15 and 60 min is also illustrated by the difference in the probability of a positive response at the two highest bolus doses using the 200 ml volume, as estimated using the linear regression model. The probability of an occurrence of nausea was elevated over control at both time points, but the incidence rates at 60 min post-dosing were considerably lower than at 15 min (Table 3). The results obtained and the statistical significances for the analysis based on all four outcomes (nausea, abdominal pain, vomiting, and diarrhea) were similar to the analysis for nausea alone. Fig. 1 presents the incidence of nausea at each copper dose at 15 and 60 min, for the 200 ml volume.

Table 2 also presents the total number of people reporting nausea (at either the 15 minute or the 60 minute time points). The total number reporting nausea is close to the number reporting nausea at 15 min. This means that most of those who reported nausea at 60 min had also reported nausea at 15 min, although there were

Table 3
Probability of all GI symptoms (confidence range) at 15 and 60 min by location and dose for linear dose–response at 200 ml volume ingested

	15 min	60 min
Probability of a positive response (confidence range)		
Location		
Santiago	0.03 (0.01, 0.05)	0.01 (0.01, 0.03)
Shanghai	0.06 (0.04, 0.11)	0.04 (0.04, 0.10)
Coleraine	0.04 (0.02, 0.07)	0.02 (0.01, 0.04)
Grand Forks	0.08 (0.05, 0.14)	0.06 (0.04, 0.12)
Copper (mg)		
0	0.00 (0.00, 0.02)	0.01 (0.00, 0.02)
0.4	0.02 (0.01, 0.05)	0.02 (0.01, 0.04)
0.8	0.07 (0.04, 0.10)	0.04 (0.02, 0.07)
1.2	0.14 (0.11, 0.19)	0.05 (0.03, 0.09)
1.6	0.25 (0.20, 0.30)	0.07 (0.04, 0.10)

some new responders at 60 min. (If there were no new responders at 60 min, then the total number of responders would equal the number of responders at 15 min.)

Using the statistically significant increased incidence compared with control as the criterion to ascertain an adverse effect level and the 200 ml linear dose–response

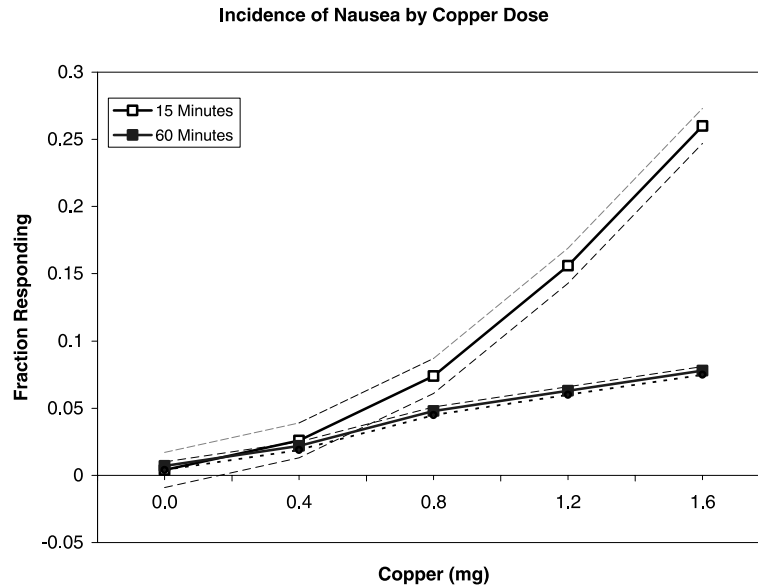**Incidence of Nausea by Copper Dose**

Fig. 1. Dose–response curve with 95% confidence interval for incidence of nausea at 15 and 60 min, for copper dosed in 200 ml bottled drinking water.

data at 15 min for nausea effects at all four sites, the generalized linear model analysis data indicate that for this dosing volume a LOAEL occurs at 1.2 mg Cu (6 mg Cu/L) and a NOAEL occurs at 0.8 mg Cu (4 mg Cu/L). Adjusting for multiple comparisons of the generalized linear model and using Bonferroni's adjustment, *p*-value comparisons of control (0 mg Cu/L) versus 0.4 mg Cu (2 mg Cu/L), 0.8 mg Cu (4 mg Cu/l), 1.2 mg Cu (6 mg Cu/L), and 1.6 mg Cu/L (8 mg Cu/L) were 0.266, 0.06, 0.0004, and 0.0004, respectively, for nausea at 15 min. These results are comparable to those found in the Phase I three-site mixed-sex study using distilled–deionized water. Similar temporal effects (i.e., a higher response incidence at 15 min than at 60 min) were observed for nausea symptoms at 15 and 60 min post-dosing (Table 4) and were especially noticeable at the higher concentrations, e.g., 12 mg Cu/L (1.2 mg Cu in 100 ml bolus). Table 4 also presents the total number of people reporting nausea (at either 15 or 60 min). As for the 200 ml volume, most people reporting nausea at 60 min had also reported it at 15 min, but there were some people who first reported nausea at 60 min.

Table 4
Incidence of nausea at 15 and 60 min by volume × dose (3 × 3)

Water volume (ml)	Location	Sample size	Copper (mg)								
			Incidence of nausea (15 minutes)			Incidence of nausea (60 Minutes)			Total people reporting nausea		
			0.4	0.8	1.2	0.4	0.8	1.2	0.4	0.8	1.2
100	Santiago	(*n* = 70)	1	5	15	1	2	5	1	6	15
	Shanghai	(*n* = 73)	3	10	16	2	6	8	4	13	18
	Coleraine	(*n* = 58)	0	9	12	1	1	3	1	10	12
	Grand Forks	(*n* = 68)	7	11	21	6	10	6	11	14	23
	Total	(*n* = 269)	11	35	64	10	19	22	17	43	68
150	Santiago	(*n* = 70)	1	3	10	1	0	2	2	3	10
	Shanghai	(*n* = 73)	3	4	21	2	3	6	4	6	24
	Coleraine	(*n* = 58)	1	3	6	1	1	2	1	4	7
	Grand Forks	(*n* = 68)	4	14	13	3	5	4	5	14	14
	Total	(*n* = 269)	9	24	50	7	9	14	12	27	55
200	Santiago	(*n* = 70)	0	1	8	0	0	1	0	1	9
	Shanghai	(*n* = 73)	2	5	14	1	6	10	3	8	19
	Coleraine	(*n* = 58)	1	4	6	1	2	3	2	5	7
	Grand Forks	(*n* = 68)	4	10	14	4	5	3	5	14	14
	Total	(*n* = 269)	7	20	42	6	13	17	12	27	55

Table 5
Generalized linear model (repeated measures) analysis of variance results and probability of a positive response for nausea at 15 and 60 min in a 3×3 factorial design (water volume $\times$ copper dose)

	Nausea at 15 min			Nausea at 60 min		
	DF	χ^2	p value	DF	χ^2	p value
Generalized linear model (repeated measures) analysis of variance results for nausea						
Test #	1	12.53	0.0004	1	1.95	0.16
Location	3	10.91	0.012	3	15.34	0.0015
Water (volume)	2	6.86	0.032	2	4.95	0.08
Copper (dose)	2	63.80	0.0001	2	14.80	0.0006
Volume $\times$ copper	4	0.50	0.97	4	1.33	0.86

Probability of a positive response (confidence range)

		Probability of Outcome (CR) at 15 min	Probability of Outcome (CR) at 60 min
Location			
Santiago		0.05 (0.03, 0.08)	0.02 (0.01, 0.03)
Shanghai		0.10 (0.07, 0.14)	0.06 (0.04, 0.10)
Coleraine		0.07 (0.04, 0.11)	0.02 (0.01, 0.04)
Grand Forks		0.13 (0.09, 0.18)	0.07 (0.04, 0.12)
Water (volume)			
100		0.11 (0.08, 0.14)	0.05 (0.04, 0.07)
150		0.08 (0.06, 0.11)	0.03 (0.02, 0.05)
200		0.07 (0.05, 0.09)	0.03 (0.02, 0.05)
Copper (mg)			
0.4		0.03 (0.02, 0.05)	0.02 (0.01, 0.03)
0.8		0.09 (0.07, 0.12)	0.04 (0.03, 0.06)
1.2		0.18 (0.15, 0.22)	0.05 (0.04, 0.08)
Volume $\times$ copper			
100	0.4	0.05 (0.02, 0.07)	0.03 (0.01, 0.05)
100	0.8	0.12 (0.09, 0.17)	0.06 (0.04, 0.09)
100	1.2	0.22 (0.18, 0.28)	0.07 (0.04, 0.11)
150	0.4	0.03 (0.02, 0.06)	0.02 (0.01, 0.04)
150	0.8	0.08 (0.06, 0.12)	0.03 (0.02, 0.05)
150	1.2	0.18 (0.14, 0.23)	0.04 (0.03, 0.07)
200	0.4	0.02 (0.01, 0.05)	0.02 (0.01, 0.04)
200	0.8	0.07 (0.05, 0.11)	0.04 (0.02, 0.07)
200	1.2	0.15 (0.11, 0.20)	0.05 (0.03, 0.08)

In addition, a benchmark dose (BMD) was calculated for the 200-ml bolus linear dose–response data at 15 min for nausea effects at a benchmark response (BMR) of 0.10. The U.S. Environmental Protection Agency's BMD Software Version 1.3 was used to evaluate these dichotomous data, using a multistage model with extra risk (U.S. EPA, 2001; U.S. EPA, 2000). The goodness of fit p-value for this model was $p = 0.94$ with a BMD of 0.94 mg copper (4.8 mg Cu/L). The corresponding 95% lower confidence limit on the BMD (BMDL) was 0.84 mg copper (4.2 mg Cu/L).

As was found with the 200 ml volume, nausea was also the earliest and most prevalent symptom at 100 and 150 ml. This study was not designed to identify the NOAEL/LOAEL at these other volumes, as there was no control group for the 100 and 150 ml volumes. However, estimates of the NOAEL/LOAEL for these volumes were made for comparison purposes, and are addressed in Section 4.

Table 5 summarizes the probability of outcome of the 3×3 dosing for nausea at both 15 and 60 min by city,

water volume, copper dose, and water volume $\times$ copper interaction. The 3×3 factorial analysis of variance demonstrates that at 15 min for nausea effects, there is a significant effect of water volume ($p = 0.032$) and copper dose ($p = 0.0001$). As copper dose increases or as water volume decreases (thereby increasing copper concentration for a given dose) there is an increased probability of experiencing nausea in female subjects (Fig. 2). Likewise, the highest incidence of symptoms was reported within the first 15 min. As with the linear 200 ml dose–response analysis, inclusion of all GI symptoms did not appreciably change the response incidence from nausea alone.

The interaction of volume by dose was not statistically significant ($p = 0.97$). This means that the effects of volume and dose were additive, i.e., volume and dose acted independently, not synergistically or antagonistically. The probability of a positive response increased significantly as the copper dose increased, regardless of water volume. Similarly, the probability of a positive response *decreased* significantly as the water volume increased,

Incidence of Nausea at 15 Minutes by Volume and Copper Dose

Incidence of GI Symptoms at 15 Minutes by Volume and Copper Dose
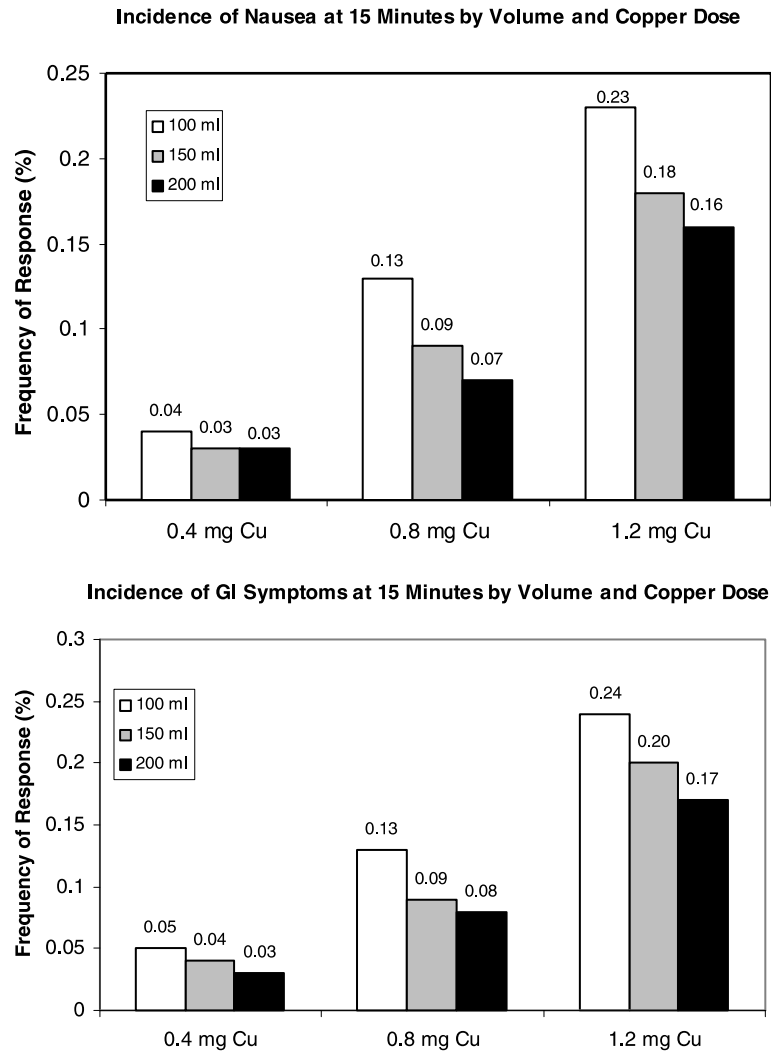

Fig. 2. Frequency of response of nausea and all gastrointestinal symptoms by water volume and copper dose within or at 15 min of ingestion of water/copper bolus (all sites combined).

regardless of copper dose. The lack of interaction of these terms is not informative regarding whether concentration is an appropriate explanatory variable. This study was designed to evaluate dose–response, rather than concentration–response, so formal analyses based on concentration are not possible. However, as shown in Fig. 3, there is a visually apparent concentration–response, overlaid on top of the dose–response. Nonetheless, based on the results of Figs. 2 and 3, neither dose nor concentration fully predicts the response; both units of exposure are relevant in determining the response.

As shown in Table 5, there is a significant effect of location at both 15 and 60 min. The probability of a significant response (across all doses) was greater in Grand Forks than in Santiago at 15 min. At 60 min, the probability of a significant response was greater in Grand Forks than in Santiago and Coleraine. The apparent location differences may be due to cultural

differences related to defining and reporting nausea and other GI symptoms; some cultural groups may be reluctant to "complain" of any symptoms to an authority figure running the test, regardless of how careful the instructions were. Alternatively, the differences among locations could be related to the composition of the water that each group of subjects was used to drinking. For example, if a subject is used to drinking water that tastes metallic, one might speculate that adding copper to the bottled water would not be as bothersome. Nonetheless, the interactions between location and copper, and between location and volume, were not significant, and so these interactions were not included in the final generalized regression model. This lack of interaction means that it is more appropriate to identify the NOAEL and LOAEL based on the combined group, for which the statistical power is greater, rather than identifying separate NOAEL/LOAELs for each location; identification of a statistical difference at the other

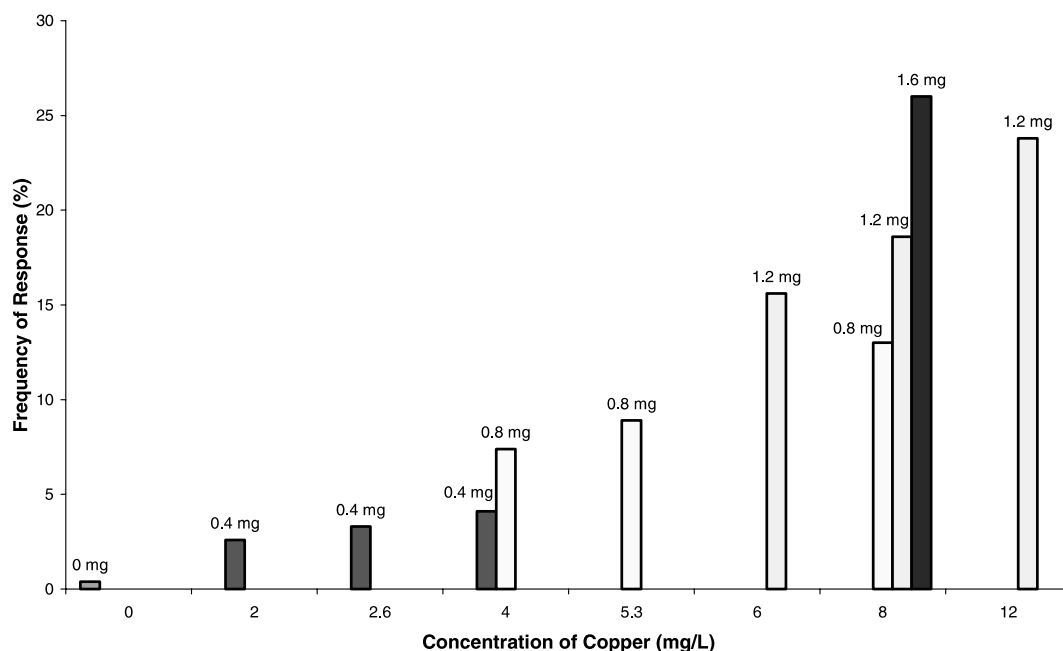**Incidence of Nausea at 15 Minutes by Concentration and Copper Dose**

Fig. 3. Frequency of response of nausea by concentration and copper dose within or at 15 min of ingestion of water/copper bolus (all sites combined).

locations would be limited by the small sample sizes, and resulting lower power.

4. Discussion

The US National Research Council (NRC) recently investigated the validity of the science upon which the U.S. EPA has set its maximum contaminant level goal (MCLG) of 1.3 mg/L for copper in drinking water (NRC 2000). At the present time, the MCLG for copper in drinking water is based on the acute effects of excess copper, although the U.S. EPA is currently reassessing the health risks from exposure to copper. The NRC recommended that the MCLG for copper be re-evaluated when data are available for copper-sensitive populations.

Both Phase I and Phase II of this copper drinking-water study have shown that with the ingestion of a single, 200 ml bolus of either distilled–deionized water or bottled drinking water that nausea is the primary adverse effect reported by fasted human volunteers and that this gastrointestinal effect occurs primarily within the first 15 min of ingestion. Furthermore, both studies have consistently indicated with a multicultural and multinational population that, based on extrapolation from doses in a 200-ml volume, the NOAEL for copper in water is 0.8 mg Cu (4 mg Cu/L) and the LOAEL is 1.2 mg Cu (6 mg Cu/L). These results are similar to those reported by others (Olivares et al., 2001). Early adverse effects of acute copper exposure in humans are quite

specific to the stomach. Recent studies on apparently healthy adult volunteers that tested 10 mg Cu/L of water showed that acute copper exposure would delay early gastric emptying (Araya et al., 2003) and that gastric, but not intestinal permeability, significantly increased 15 min after copper ingestion (Gotteland et al., 2001). The current results also show that the majority of responses were within 15 min and that the prevalence of a response decreased with time; however, we have no explanation for the few individuals who reported symptoms only at 60 min. Additional analysis of the Phase II data by the benchmark dose further confirms these observations with a calculation of a BMDL of 4.2 mg Cu/L, based on a dose of 0.84 mg copper in a 200 ml volume.

This study was not designed to identify the NOAEL/ LOAEL at volumes other than 200 ml, as there were no control groups for the 100 and 150 ml volumes; it is possible that the control response may vary with the water volume. However, it is possible to estimate the NOAEL/LOAEL for these volumes, by comparing the response at these volumes to the response in the 200 ml control group. Such estimates can only be considered approximate, but provide perspective on how the threshold varies for different dose/volume combinations. Using the generalized linear model and statistically significant increases over control as the criterion, the LOAEL for the 150 ml volume occurs at a dose of 1.2 mg Cu (corresponding to 8 mg Cu/L), with a NO-AEL of 0.8 mg Cu (corresponding to 5.3 mg Cu/L). For the 100 ml volume, the LOAEL was 0.8 mg Cu and the

NOAEL was 0.4 mg Cu, corresponding to concentrations of 8 and 4 mg Cu/L, respectively. Although these calculations are only approximations, they show a remarkably consistent picture, within the constraint that the NOAEL approach is limited to the doses tested. The NOAEL was consistently in the range 4–5 mg Cu/L, over a 2-fold range of total dose (i.e., over a range of 100 to 200 ml volumes). However, as illustrated in Figs. 2 and 3, neither dose nor concentration fully predicts the observed response.

Pizarro et al. (1999) determined the acute gastrointestinal effects in 60 adult Chilean females, who consumed at home 0, 1, 3, or 5 mg Cu/L in a public (tap) drinking water source. This was the sole drinking water source for these individuals for multiple 2-week periods. Mild gastrointestinal disturbances (nausea, abdominal pain, vomiting and diarrhea) were recorded at least once in 35% of the subjects. Nausea, abdominal pain and vomiting were significantly related to copper concentrations, with a recorded incidence of 5, 2, 17, and 15% in the individuals consuming 0, 1, 3, or 5 mg Cu/L, respectively. These data suggested that copper concentrations greater than or equal to 3 mg Cu/L can be associated with these gastrointestinal symptoms. Differences in design (repeated, variable exposure during the day registered in diaries versus single daily controlled exposure) and the way data were collected (asking collectively about the presence of any of four symptoms versus a list asking individually for each symptom, at specific times) impede further comparison of the two studies. In addition, results by Olivares et al. (2001) and Araya et al. (2001) and this study do not support the conclusion that 3 mg Cu/L is a LOAEL. From the mean daily consumption of drinking water in the study by Pizarro et al. (1999), 1.6 L/day, it can be calculated that the average copper intake from water was 4.8 mg/day. This threshold for acute gastrointestinal effects is consistent with the data reported here and the previously reported Phase I data (Araya et al., 2001). Thus, consumption of drinking water greater than 4 mg Cu/L will significantly increase the chance of experiencing these same mild gastrointestinal symptoms.

Results obtained are indeed relevant when used in a regulatory paradigm. Phase I and Phase II data represent a worst-case scenario for risk assessment of the acute gastrointestinal adverse effects of copper because the copper solution was administered on an empty stomach (likely to be free of food remnants) and after a long fast (likely to provide a low pH, which may efficiently solubilize copper); therefore it may overestimate the risk somewhat. The influence of volume on the appearance of GI symptoms poses an interesting situation for a regulatory paradigm, which usually addresses only concentration. Finally, the finding that the NOAEL identified for women in this study at the 200 ml volume is the same as the NOAEL identified for men and women combined in Phase I (Araya et al., 2001) is also relevant for regulatory purposes. The U.S. EPA currently has promulgated a risk assessment value in drinking water (the Maximum Contaminant Level Goal, MCLG) of 1.3 mg Cu/L (U.S. EPA, 1994). The World Health Organization supports a provisional guideline value of 2 mg Cu/L as a safe value of consumption of copper in drinking water (WHO, 1998) based on the acute effects of copper. The body of information now available indicates that a NOAEL of 4 mg Cu/L as a highly soluble sulfate salt can be established for the onset of acute gastrointestinal effects (i.e., nausea). The similar findings obtained both in Phase I and II, in an internationally and culturally diverse population, suggest that this NOAEL can be safely used globally. Although location was a significant variable, the interaction between location and copper was not significant, indicating that the approach used of identifying the NOAEL for all of the populations combined was the correct one. In summary, results of this study and information generated in recent years in controlled trials conducted in asymptomatic adult populations permit better understanding of the early effects induced by acute copper exposure.

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