Journal of the American Association for Laboratory Animal Science
Vol 52, No 1
January 2013
Pages 22–27

Leaching of Heavy Metals from Water Bottle Components into the Drinking Water of Rodents

Elizabeth A Nunamaker,[1,*] Kevin J Otto,[2,3] James E Artwohl,[1] and Jeffrey D Fortman[1]

Providing high-quality, uncontaminated drinking water is an essential component of rodent husbandry. Acidification of drinking water is a common technique to control microbial growth but is not a benign treatment. In addition to its potential biologic effects, acidified water might interact with the water-delivery system, leading to the leaching of heavy metals into the drinking water. The goal of the current study was to evaluate the effects of water acidification and autoclaving on water-bottle assemblies. The individual components of the system (stainless-steel sipper tubes, rubber stoppers, neoprene stoppers, and polysulfone water bottles) were acid-digested and analyzed for cadmium, chromium, copper, iron, lead, magnesium, manganese, selenium, and zinc to quantify the metal composition of each material. In addition the amounts of these metals that leached into tap and acidified water with and without autoclaving were quantified after 1 wk of contact time. On a weight basis, sipper tubes contained the largest quantities of all metals except magnesium and zinc, which were greatest in the neoprene stoppers. Except for cadmium and selenium, all metals had leached into the water after 1 wk, especially under the acidified condition. The quantities of copper, lead, and zinc that leached into the drinking water were the most noteworthy, because the resulting concentrations had the potential to confound animal experiments. On the basis of these findings, we suggest that water-quality monitoring programs include heavy metal analysis at the level of water delivery to animals.

Abbreviation: ICP, inductively coupled plasma.

Drinking water quality is an important factor in experimental outcomes and in the daily care and maintenance of laboratory animals. Animal Welfare Regulations[1] and the *Guide for the Care and Use of Laboratory Animals*[26] both stipulate that research animals must be provided clean, potable water, but typically water is only monitored for microbiologic contaminants in the academic environment.[21,31,34] Good Laboratory Practice regulations require that animal drinking water be tested for potential confounding contaminants but do not identify specific agents.[20] The literature supports the use of acidified water as a means to control bacterial contamination of rodent drinking water, but this practice has several biologic effects, and evidence suggests that it can affect the water-delivery system.[28,33,46,49]

Water acidification has been reported to have multiple biologic effects.[48] When male CD1 mice ingested water acidified to pH 2.0, they had decreased water consumption, decreased weight gain, and decreased numbers of bacterial species in the terminal ileum.[22] Rats provided water acidified to pH 2.0 for 24 wk had extensive corrosion of both the enamel and dentin of the first 2 mandibular molars.[27,32,50] Mice provided water acidified to pH 2.0 for 120 d had reduced reticuloendothelial clearance rates, spleen weights, and spleen:body weight ratios, indicating a potential for alteration of the immune response.[24] Although these effects were attributed to the acidification of the water, they may have been potentiated by the effects of acidified water on the water-delivery system and the subsequent ingestion of leached metals.

In addition, acidifying drinking water can cause metals to leach from water-bottle stoppers. In one study, acidified–deionized water leached more metals from polymer stoppers than did nonacidified–deionized water; the authors suggested that other types of stoppers would be more appropriate for specific nutritional and toxicological studies.[28] Although 4 different types of polymer stoppers (rubber, neoprene, vinyl, and silicone) were evaluated, the study had several limitations that were never further examined.[28] For example, metal analysis was performed on only 2 of the stopper types (rubber and neoprene), and the authors evaluated only 2 samples of each.[28] Furthermore, the metal analysis of the stoppers was limited to 5 metals and the water analysis to 7 metals, with lead being the most notable metal that was absent from the analysis. Moreover, the study[28] did not assess the effects of autoclaving on the water-delivery system, nor did the authors perform metal analysis of sipper tubes or the water bottle.

As part of our own facility water-quality assurance program, we recently extended our analysis to include point-of-delivery (water bottle) assessment in addition to source assessment for both heavy-metal and microbiologic contaminants. As a result, the goals of the current study were to identify potential heavy-metal contaminants in the water-bottle assemblies and to evaluate the leaching of those metals into the drinking water by using inductively coupled plasma (ICP) spectrophotometry. Specifically, we evaluated neoprene and rubber water-bottle stoppers, stainless-steel sipper tubes, and polysulfone water bottles for contaminant heavy metals after acid digestion. We then evaluated the effects of water acidification and autoclave treatment on the leaching of heavy metals from the water-bottle assemblies into the drinking water after 1 wk of inversion on an empty rodent cage.

Materials and Methods

Acid digestion of materials. The individual components of a water bottle were digested in 1 N trace-metal HCl (Sigma,

Received: 30 May 2012. Revision requested: 03 Jul 2012. Accepted: 13 Jul 2012.
[1]Biologic Resources Laboratory, University of Illinois at Chicago, Chicago, Illinois; [2]Department of Biological Sciences and [3]Weldon School of Biomedical Engineering, Purdue University, West Lafayette, Indiana.
[*]Corresponding author. Email: enunamak@uic.edu

St Louis, MO) for 1 wk. Five samples of each constituent were digested and tested independently to identify variability within specific components. New double-seal rubber and neoprene water bottle stoppers (Ancare, Bellmore, NY) each were cut into 1 mm^3 pieces by using a razor blade to maximize surface area and then digested in a 50-mL polypropylene tube (BD Labware, Franklin Lakes, NJ) containing 10 mL HCl per gram of sample. We poured 10 mL HCl into each of 5 polysulfone screw-top water bottles (Ancare), which subsequently sealed and stored on their sides to maximize the contact area. Stainless-steel open straight drinking tubes (Ancare) were placed individually in 50-mL polypropylene tubes containing 10 mL HCl. To control for potential leaching from the tubes, we placed 10 mL HCl in each of 5 empty tubes and let them sit for 1 wk. At the time of sample analysis, we also analyzed 5 aliquots of fresh HCl, as an additional control for contaminants in the HCl.

After 1 wk, each sample was diluted with deionized water to decrease the acid concentration to less than 5 weight–percent. Samples were passed over syringe filters (diameter, 25 mm; pore size, 0.45 μm; Aerodisc, PALL Life Science, Ann Arbor, MI) and analyzed for cadmium, chromium, copper, iron, lead, magnesium, manganese, selenium, and zinc by using a spectrophotometer (Vista-MPX ICP, Varian, Walnut Creek, CA). Before analysis, standard curves for each metal were established over the range of 0.02 to 100 ppm.

Leaching into drinking water. Water-bottle components equivalent to those we used in the acid-digestion study were assembled to make complete water bottles that included either a rubber or neoprene stopper. The water conditions included: HCl-acidified (pH 2.2) water; autoclaved, HCl-acidified (pH 2.2) water; tap water; and autoclaved tap water. Samples were autoclaved in fully assembled water bottles. Water bottles remained inverted on an empty rodent cage for 1 wk to simulate the intended use. We assessed 5 replicates of stopper and water condition combination to accommodate intersample variation. At the time of analysis, fresh samples of tap and acidified water (n = 5 each) were included to serve as baseline controls for the leaching experiments.

Water samples (6 oz each) were submitted for analysis (Siemens Analytical Laboratory, Rockford, IL). Heavy-metal analysis was performed by using an ICP spectrophotometer (Ultima 2, Horiba Jobin Yvon, Edison, NJ) that was calibrated with a multielement standard prior to each sample run. The water samples were tested for cadmium, chromium, copper, iron, lead, magnesium, manganese, selenium, and zinc. In addition, all water samples were pH-analyzed by using an Accumet XL25 (Fisher Scientific, Waltham, MA) that was calibrated daily at pH 4 and 7.

Statistical analysis. All statistical analysis was performed by using MATLAB (MathWorks, Natick, MA). The results of the acid digestion were analyzed by ANOVA and a multiple-comparisons procedure using a Bonferroni correction to identify the significance associated with metal concentrations in the individual water-bottle components. The leaching data were evaluated by using 3-way ANOVA to test for the interaction effects of stopper type, water acidification, and autoclaving on metal concentrations in the water. For all statistical tests, significance was defined as a P value of less than 0.05.

Results

Acid digestion. The quantities of heavy metals in the individual water-bottle components and controls are reported in Figure 1. New rubber and neoprene stoppers, old rubber stoppers, water bottles, and sipper tubes weighed 22.9 ± 0.1 g, 25.9 ± 0.3 g, 16.7 ± 0.4 g, 74.5 ± 3.4 g, and 8.0 ± 0.1 g each, respectively. The sipper tubes contained significantly ($P < 0.05$) more heavy metal (as micrograms metal per gram material) for all tested metals except magnesium and zinc (Figure 1). Compared with sipper tubes, neoprene stoppers contained more ($P < 0.05$) magnesium and zinc. Compared with neoprene stoppers, rubber stoppers contained less ($P < 0.05$) of all tested metals. The quantities of heavy metals in the water bottles and controls were at or below the limit of quantitation for all tested metals (data not shown).

Leaching. As determined by ICP spectroscopy, heavy metals leached into the water; the pH for each combination of stopper and water treatment (acid and autoclave) are reported in Table 1. There was no detectable leaching of either cadmium or selenium into the water from any water bottle combination. Acidification and stopper type had significant ($P < 0.05$) main effects on the leaching of copper, iron, zinc, and lead, as did the interaction between acidification and stopper type. Acidification was the only variable to have a significant ($P < 0.05$) effect on chromium. There were significant ($P < 0.05$) 3-way interactions between acidification, stopper type, and autoclaving on both manganese and magnesium, and each individual variable had significant ($P < 0.05$) main effects. Acidified water samples had a pH of 2.23 ± 0.03; tap water samples had a pH of 7.89 ± 0.02. The main effects of acidification, stopper type, and autoclaving; the interaction between acidification and autoclaving; and the interaction between acidification and stopper type all had significant ($P < 0.05$) effects on pH.

Discussion

This study is the first to report the metal analysis for all of components of a water-bottle assembly. Although we used a very similar analysis method (ICP spectroscopy) to that used previously (atomic absorption spectroscopy), the composition of the water-bottle stoppers differed slightly between studies.[28] Rubber stoppers in the current study had less copper, iron, magnesium, manganese, and zinc per gram of polymer.[28] Neoprene stoppers had less copper, iron, and magnesium but comparable quantities of manganese and zinc per gram of polymer to those evaluated previously.[28] We also identified quantifiable amounts of lead, cadmium, chromium, and selenium in both the rubber and neoprene stoppers, which was not previously reported. We did not analyze any other stopper types, because they are not commercially available. The stainless-steel sipper tubes had large quantities of all tested metals, which were significantly greater than those in the stoppers for all metals except magnesium and zinc.

Similar to findings from the previous study,[28] leaching in the current study was increased under the acidified condition. Of the metals tested, magnesium, zinc, and copper reached the highest concentrations in the water after 1 wk. Acidified water leached lead from the neoprene stoppers—a finding that has not previously been reported. In the current study, acidified water leached more chromium and magnesium but less copper, iron, and zinc and comparable amounts of manganese and selenium from rubber stoppers, compared with previous results.[28] Similarly, acidified water leached more copper and magnesium but less iron and zinc and comparable amounts of chromium, manganese, and selenium from neoprene stoppers in the current study compared with the previous study.[28] Slight differences between the studies may be partially responsible for some of these discrepancies. In particular, water bottles in the current study were left inverted for 7 d instead of 6 d; deionized water was not used; pH was 0.3 units lower; and the source of the stoppers was different.

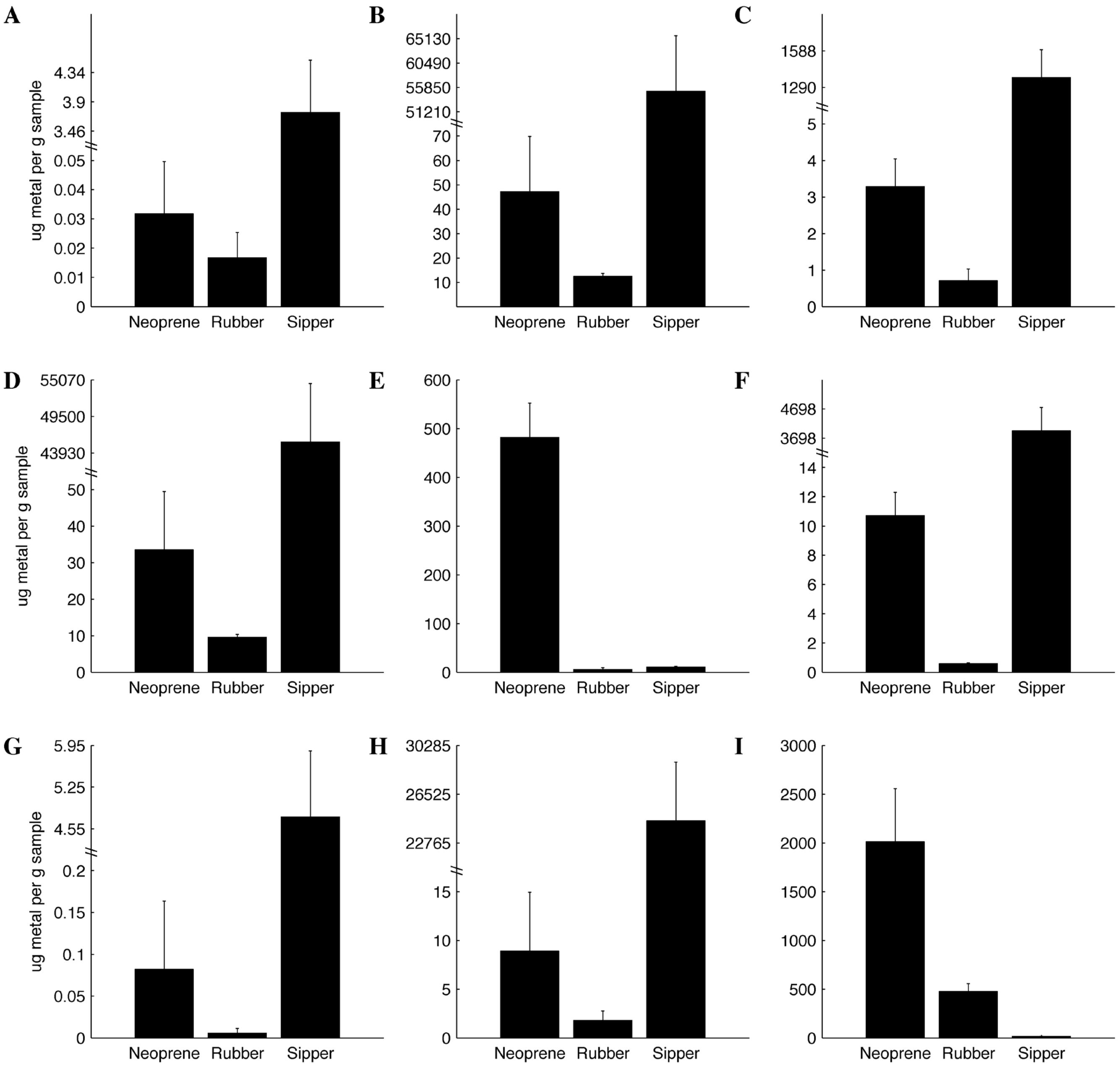

Figure 1. Heavy-metal composition (µg metal/g material; mean ± SEM; $n = 5$) of stoppers and sipper tubes as determined by ICP spectrometry. (A) Cadmium. (B) Chromium. (C) Copper. (D) Iron. (E) Magnesium. (F) Manganese. (G) Lead. (H) Selenium. (I) Zinc.

The results of the current study and those obtained previously[28] collectively suggest that water should be analyzed for contaminants at the level of delivery to animals. These studies further suggest that all materials used in water bottle assemblies should be analyzed for potential heavy-metal contaminants.[28] The differences in the metal content of the stopper types between the 2 studies highlights the potential variability in stopper materials due to differences in sources and manufacturing processes. To address the variability in material contents, facilities should periodically test these materials for potential contaminants. This suggestion is further supported by the recent reports of lead and melamine contaminants in children's toys and pet foods from foreign suppliers.[8,10,11,47,52]

Heavy-metal leaching into drinking water may be problematic due to the individual biologic effects of these components after exposure. Lead has been documented to cause a variety of toxic effects and, as a result, the Environmental Protection Agency has set the ideal water concentration of lead at 0 ppm and the level of action at 0.015 ppm.[51] In the current study, lead leached from neoprene stoppers into acidified drinking water. These lead levels reached 0.2 ppm, thus exceeding the human guidelines.[51] The primary toxic effects of lead are immunologic and reproductive in nature, but lead also can affect other body systems in rodents when administered at high concentrations.[7,9,13,14,23,39-42,45,53,54] Contaminant concentrations of dietary lead (0.4 µg/g wet weight of feed) have been associated with increased mortality rates in aged male CD1 mice.[44] The amount of lead that these previous mice[44] ingested over the course of their lives was comparable to the exposure (on a µg/g body weight basis) with that which theoretically would be ingested from acidified water in contact with neoprene stoppers. This finding suggests that animals on aging studies should not be

Table 1. Heavy-metal levels in drinking water after 1 wk of contact time with water-bottle components

Sample			ppm (mean ± SE; n = 5)									pH
Stopper	Water	Autoclaved?	Cd	Cr	Cu	Fe	Pb	Mg	Mn	Se	Zn	
Rubber	Acid	No	<0.002	0.015 ± 0.01	0.022 ± 0.008	0.095 ± 0.035	<0.02	12.10 ± 0.18	0.001	<0.05	0.901 ± 0.056	2.15 ± 0.17
Rubber	Acid	Yes	<0.002	0.009 ± 0.003	0.005[a]	0.053 ± 0.013	<0.02	13.26 ± 0.09	<0.001	<0.05	2.390 ± 0.225	2.29 ± 0.04
Rubber	Tap	No	<0.002	<0.002	0.030[a]	<0.002	<0.02	11.76 ± 0.04	<0.001	<0.05	0.119 ± 0.063	7.01 ± 0.17
Rubber	Tap	Yes	<0.002	<0.002	<0.002	<0.002	<0.02	12.88 ± 0.12	<0.001	<0.05	0.051 ± 0.012	8.12 ± 0.42
Neoprene	Acid	No	<0.002	0.016 ± 0.011	3.250 ± 0.612	0.147 ± 0.050	0.196 ± 0.034	13.74 ± 0.19	0.003 ± 0.001	<0.05	6.112 ± 1.823	2.21 ± 0.03
Neoprene	Acid	Yes	<0.002	0.013 ± 0.001	3.110 ± 1.253	0.105 ± 0.016	0.240 ± 0.057	14.40 ± 0.10	0.004 ± 0.001	<0.05	6.602 ± 2.474	2.22 ± 0.08
Neoprene	Tap	No	<0.002	<0.002	0.003[a]	<0.002	<0.02	11.88 ± 0.04	<0.001	<0.05	0.180 ± 0.005	7.39 ± 0.23
Neoprene	Tap	Yes	<0.002	<0.002	<0.002	<0.002	<0.02	13.48 ± 0.04	<0.001	<0.05	0.021 ± 0.003	8.14 ± 0.47
None	Tap	No	<0.002	<0.002	<0.002	<0.002	<0.02	12.16 ± 0.09	<0.001	<0.05	0.003 ± 0.000	8.26 ± 0.12
None	Acid	No	<0.002	<0.002	0.004 ± 0.0002	0.007 ± 0.000	<0.02	12.18 ± 0.02	<0.001	<0.05	0.415 ± 0.002	2.04 ± 0.13
EPA maximal contaminant level [51]			0.005	0.1	1.3	0.03[b]	0.015	—	0.05[b]	0.05	5.0[b]	6.5–8.5[b]

[a]Only a single sample had a quantifiable concentration of copper.
[b]This concentration is the national secondary drinking water regulation. Note that the Environmental Protection Agency (EPA) recommends these secondary standards but does not require compliance with these recommendations.

provided acidified water from water bottles with neoprene stoppers, to mitigate potential premature death associated with chronic low-level lead toxicity.

Copper was detected in the acidified water at a maximal average concentration of 3.25 ppm for neoprene stoppers and 0.02 ppm for rubber stopper. The limit for copper in drinking water is 1.3 ppm due to the development of gastrointestinal distress acutely and liver or kidney damage chronically in humans.[51] Although the amount of copper in acidified water exposed to a neoprene stopper exceeds this limit, the risk of rodents developing long-term liver or kidney damage as a result of long-term exposure under these watering conditions is minimal, given that they typically consume a larger quantity of copper from their diet (Table 2).

Zinc was detected in the largest concentrations in the neoprene stoppers and leached into the water that contacted these stoppers. When the water was acidified, the amount of zinc leached increased significantly to the maximal average of 6.6 ppm after 1 wk. Although acute zinc toxicity often is associated with the ingestion of nonfood items (for example, a penny or lead sinker), dietary zinc is relatively nontoxic.[18] However, when supplemented at 10 to 20 times the recommended dietary allowance of 15 mg/d in humans, clinical signs of zinc toxicity do develop.[18] The risk of rodents developing toxicity due to long-term exposure to zinc under these watering conditions is minimal because they normally consume a much larger quantity of zinc from their diet (Table 2).

Although the concentrations of copper and zinc that leached into acidified water in contact with neoprene stoppers is not expected to lead to acute toxicities, the potential to affect experimental outcomes remains. For example, an investigator studying the immune-modulating effects of copper and zinc might choose to feed their animals a diet low in copper and or zinc.[4,12,15,16,19,29,30,37,38] In these cases, the potential quantity of zinc and copper ingested from acidified water on neoprene stoppers would exceed that from the diet by 5-fold and 20-fold, respectively (Table 2). This situation likely would negate the effects of feeding the metal-deficient diets and potentially confound the results of the animal experiment.

The Environmental Protection Agency currently does not have a threshold concentration for iron in drinking water, because iron toxicity is exceedingly less common than is iron deficiency in humans. Iron deficiency increases the absorption, potentially leading to toxicity, of other divalent metals, including lead, cadmium, aluminum, and manganese.[55] The amount of iron in drinking water affects its palatability, which sharply declines at 0.3 ppm for humans.[51] Iron leached into the water in the current study, especially when the water was acidified and a neoprene stopper was used, but the maximal iron concentration reached was only 0.1 ppm. This finding indicates that leaching of iron is unlikely to affect water palatability or cause toxicity in rodents.

Magnesium often is found in high concentrations in tap water, especially when the source is groundwater, and tap water is considered a major source of the required dietary magnesium in humans.[55] As expected, magnesium was present in high concentrations in all water samples in the current study, including fresh tap water. Although additional magnesium leached from the neoprene stoppers exposed to acidified water, the potential quantity of magnesium ingested from drinking water remains far less that the amount consumed from the diet (2000 µg/g diet, Teklad 7912). These results suggest that the magnesium that leached from the neoprene stoppers is of minimal concern and not expected to result in toxicity.

Table 2. Amounts (μg) of zinc and copper potentially ingested daily by a 30-g mouse or 300-g rat according to average daily intakes for adult rodents

	Mouse		Rat	
	Zn	Cu	Zn	Cu
Harlan Teklad 7912	315	115	945	345
Teklad mineral–deficient diets	7.5	1.6	22.5	4.8
Acidified water, neoprene stopper	42	21	190	95
Acidified water, rubber stopper	6	0.2	30	0.7

Manganese and chromium were present in the sipper tubes and neoprene and rubber stoppers. Under all tested conditions, both metals leached at very low levels. Although manganese is a cofactor in several important enzymatic pathways and functions with vitamin K, leading to the formation of prothrombin, there are relatively few studies of toxicity, and deficiency is of a much larger clinical significance.[25] When administered intraperitoneally to rats at high concentrations (10 mg/kg body weight), manganese causes hyperglycemia and hypoinsulinemina, indicating an effect on carbohydrate metabolism through alterations in insulin release and gluconeogenesis.[25] Chromium is a required element in the diet and is important for normal growth and survival of rodents.[35,44] Due to the potential for toxicity, the Environmental Protection Agency has set the limit for chromium at 0.1 ppm for people.[51] The very small amounts of manganese and chromium that into the drinking water in the current study is not likely to pose as a significant source for toxicity.

Although neither cadmium nor selenium was detected in the water samples, both metals were present in the tested stoppers and sipper tubes, making ingestion a possibility. Excess dietary cadmium has immune-modulatory effects, is associated with an inability to thrive, and, over the long-term, can influence the absorption and distribution of essential elements and replace them in biologic enzymes.[56] Rodent diets high in selenium (3.0 mg Se/kg diet) have been associated with gestational diabetes and insulin resistance, which can be passed onto the pups.[36,43] Despite the potential for cadmium and selenium ingestion, toxicity is not anticipated.

Although few of the metal concentrations in the water reached individually toxic levels, quantifying the amounts of the metals in the materials and the amount leached into the water remains important. Rodents that are placed on diets deficient in specific micronutrients (for example, calcium, copper, iron) will be predisposed to toxicity from the nonessential metals ingested from the water due to the complex interaction between the metals and micronutrients.[2,3,5,6,17,43,44] Furthermore, when otherwise healthy animals are fed a regular rodent chow but exposed to high concentrations of a given metal, the metabolism and activity of other metals can be affected, leading to relative deficiencies or toxicities.

In addition, rodents potentially can ingest the stopper material. Old rubber stoppers that had evidence of gnawing activity weighed an average of 6.2 g less than did new rubber stoppers. Although it is highly improbable that a single animal ingested the entire 6.2 g, rodents are likely to ingest small amounts of the stopper material. Exposure of the stopper material to the acidic environment of the stomach may lead to leaching of heavy metals, putting the animal at potential risk of toxicity. Further evaluation of this hypothesis is warranted to identify the actual risk associated with this route of heavy-metal exposure.

Although autoclaving had minimal effects on metal leaching, with the exception of manganese and magnesium, it did have degradative effects on the material properties of the stoppers. Specifically, the neoprene stoppers began to crack the first time they were autoclaved, and they subsequently crumbled after repeated cycles of autoclaving, requiring their removal from circulation after just 2 or 3 cycles. The rubber stoppers were not observed to degrade during this brief experimental period.

As illustrated in the current study, water acidification has unintended consequences that should not be ignored. The effects of water acidification on the water-bottle assembly can become a confounding variable, especially in rodent immunologic and aging studies. In light of these findings, we suggest that water-quality monitoring programs should include periodic analysis for heavy metals in addition to microbiologic contamination at the level of water delivery to the animal. We also suggest establishing a program to routinely perform metal-analysis testing of water bottle stoppers to identify potential sources of water contamination. Additional studies may be necessary to identify the biologic effects of long-term exposure to the metals leached from water bottle stoppers or gaskets in automatic water systems into acidified water.

Acknowledgments

We thank Saman Shafaie and Sarah Saslow (Northwestern University) for their useful discussions and assistance with the ICP spectroscopy and Mike Smith and Volta Perkins for support with water bottle assembly and autoclaving samples.

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