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Genotoxicity and mutagenicity of iron and copper in mice

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Abstract The toxicity of trace metals is still incompletely understood. We have previously shown that a single oral dose of iron or copper induces genotoxic effects in mice in vivo, as detected by single cell gel electrophoresis (comet assay). Here, we report the effect of these metals on subchronic exposure. Mice were gavaged for six consecutive days with either water, 33.2 mg/kg iron, or 8.5 mg/kg copper. On the 7th day, the neutral and alkaline comet assays in whole blood and the bone marrow micronucleus (MN) test were used as genotoxicity and mutagenicity endpoints, respectively. Particle induced X-ray emission was used to determine liver levels of the metals. Females showed a slightly lower DNA damage background, but there was no significant difference between genders for any endpoint. Iron and copper were genotoxic and mutagenic. While copper was more genotoxic in the neutral version, iron was more genotoxic in the alkaline version of the comet assay. Copper induced the highest mutagenicity as evaluated by the MN test. Iron was not mutagenic to male mice. Iron is thought to induce more oxidative lesions than copper, which are primarily detected in the alkaline comet assay. Treatment with iron, but not with copper, induced a significant increase in the hepatic level of the respective metal, reflecting different excretion strategies.

Keywords Transition metals · Ferrous sulfate · Cupric sulfate · Comet assay · Micronucleus test

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Introduction

Lack of specific information makes it difficult to reach firm conclusions on the hazards of dietary

metals (Rojas et al. 1999), specially for iron and copper, which are among the most important.

Iron is a micronutrient required for almost all organisms because of its key role in biological systems, including oxygen transportation, oxidative metabolism (i.e., in several enzymes of the tricarboxylic acid cycle and oxidative phosphorylation), DNA homeostasis (ribonucleotide reductase), antioxidant defenses (peroxidases) and immune system function (myeloperoxidases). However, iron can be toxic because of its role in oxidative stress (De Freitas and Meneghini 2001).

Iron deficiency is a worldwide problem (Beard 2001) and is, in general, more frequent than its excess. Iron overload has been associated with several pathological conditions, including liver and heart disease, cancer, neurodegenerative disorders, diabetes, hormonal abnormalities and immune system abnormalities (Valko et al. 2005). Moreover, iron can damage biomolecules mainly through Fenton and Haber-Weiss chemistry, leading to the production of hydroxyl radicals and other reactive oxygen species (ROS) (Halliwell and Guterridge 2000). Iron compounds have been reported to be mutagenic in mammalian culture cells, as detected by Syrian hamster embryo cell transformation/viral enhancement assay (Heidelberger et al. 1983), base tautomerization in rat hepatocyte cultures (Abalea et al. 1999) and genetic alterations in the mouse lymphoma assay (Dunkel et al. 1999). However, negative results have also been reported, including from recombinational assay in DNA repair-deficient *Bacillus subtilis* (Leifer et al. 1981), sister chromatic exchange in hamster cell culture (Tucker et al. 1993), sex-linked recessive lethal gene mutation in *Drosophila melanogaster* (Lee et al. 1983), gene mutation in *Glycine max* (Vig 1982) and tryptophan reverse gene mutation in *Escherichia coli* (Brusick et al. 1980).

Copper is also an essential metal that can be hazardous at high levels. It is required as a co-factor for many enzymes that catalyze oxidation/reduction reactions, including those of the electron transport (cytochrome c oxidase) antioxidant enzymes (Cu/Zn superoxide dismutase and ceruloplasmin), melanin and collagen biosynthetic pathways (tyrosinase and lysyl oxidase, respectively) and hormones (e.g., dopamine-monooxygenase and a-amidating monooxygenase). The accumulation of large amounts of copper in cells and organs can be destructive. Destruction is thought to be due to ROS whose formation is catalyzed by free copper ions (or certain complexes) that might occur when the ability of the cells to store excess copper in benign form has been exceeded (Linder 2001). The genotoxicity of copper compounds has been reported mainly in vitro. For instance, positive results were reported for the Syrian hamster embryo cell transformation/viral enhancement assay (Heidelberger et al. 1983) and for HL-60 cells (Ma et al. 1998). We have shown in vivo genotoxicity for freshwater planarian, using the comet assay (Guecheva et al. 2001). In the same study, copper inhibited DNA repair. However, negative results have been observed in the recombinational assay in DNA repair-deficient *B. subtilis* (Leifer et al. 1981), *G. max* gene mutation (Vig 1982) and mitotic recombination or gene conversion in *Saccharomyces cerevisiae* (Zimmermann et al. 1984). An in vivo study by Saleha Banu et al (2004) in mice showed that, although copper genotoxicity demonstrated a clear dose-dependent response pattern, it gradually decreased from 48 h post-treatment, returning to the control levels 2 weeks after treatment. Given the conflicting results, the question is whether the toxicity of copper in vitro and in theory is likely to occur in vivo, and, if so, under what conditions (Linder 2001).

Since the genotoxicity of iron and copper is controversial, further clarification is needed in order to address the potential toxicity of these compounds. In a previous study, we observed genotoxicity after acute exposure (24 and 48 h) of mice to iron and copper, as evaluated by the alkaline version of the comet assay (Franke et al. 2006). The aim of the present work was to evaluate the genotoxicity and the mutagenicity of these metals in mice in vivo with a subchronic exposure schedule of 7 days. Particle induced X-ray emission (PIXE) was used to evaluate the iron and copper content of the livers of the treated animals.

Materials and methods

Chemical reagents

Phosphate buffered saline (calcium- and magnesium-free), Tris [tris (hydroxymethyl) aminomethanehydrochloride], disodium ethylenediamine-tetra-acetate

(EDTA), dimethylsulfoxide (DMSO), ethidium bromide, cyclophosphamide (CP) (CAS 50-18-0), copper sulfate ($CuSO_4$, purity ≥ 98%) (CAS 7758-98-7), Triton X-100 and bovine calf serum were purchased from Sigma. Ferrous sulfate ($FeSO_4$, purity ≥ 99%) (CAS 7782-63-0) was obtained from Ducto (Brazil). Low melting point (LMP) agarose and normal agarose (electrophoresis grade) were obtained from Gibco-BRL. Heparin sodium was bought from Roche (Brazil) under the commercial name Liquemine®.

Animals

CF1 mice, aged 5–7 weeks and weighing from 27 to 32 g were obtained from the State Foundation for Production and Research in Health (FEPPS), Porto Alegre, RS, Brazil. The mice were acclimatized to laboratory conditions (22 ± 3°C and 60% humidity) for 7 days, during which they received a commercial standard mouse cube diet (Nuvilab, CR1, Moinho Nuvipal Ltda., Curitiba, PR, Brazil) and water ad libitum. After acclimatization, the mice were clustered in groups according to sex and identified as control and test groups. All procedures were carried out according to the international practices for animal use and care under the control of an internal committee of the Universidade Federal do Rio Grande do Sul.

Treatments and tissue sampling

Three groups of ten mice (five males and five females) were used in the experiments. Each group was gavaged (0.1 ml/10 g of body weight) with a single daily dose of either water, $FeSO_4$ (33.23 mg Fe/kg body weight) or $CuSO_4$ (8.25 mg Cu/kg body weight) for six consecutive days. In addition, one group of five male mice was gavaged with CP (25 mg/kg body weight) on the 5th day of exposure, for comet assay. The dose used for iron, copper and cyclophosphamide was equal to 10.86% (Budavari et al. 1996), 11.14% (Canton et al. 1989) and 18.2% (Lewis 1996) of the mice oral LD_{50}, respectively. All substances were prepared just before treatment and protected from light. For the comet assay, blood (about 15 µl) was obtained on the 7th day after the beginning of the experiment by means of a small incision on mouse tail tips and immediately mixed with heparin sodium (7 µl). On the same day, femur bones were dissected to prepare bone marrow smears for the micronucleus (MN) test and livers were removed for PIXE analysis.

Comet assay

The neutral comet assay was adapted from Singh et al. (2003). The alkaline version of the comet assay was performed according to Franke et al. (2005a, b, 2006). Slides were prepared and cells were lysed rigorously in the same manner for neutral and alkaline versions. Briefly, an aliquot of blood cells/heparin mixture were embedded in LMP agarose and spread (final concentration 0.7% w/v) over a pre-coated microscope slide and a cover glass was gently placed over the slide. The slides were placed at 4°C for 5 min to allow gel solidification, the coverslips were removed and the slides were put in lysis buffer (2.5 M NaCl, 100 mM EDTA and 10 mM Tris, pH 10.00–10.50, with freshly added 1% Triton X-100 and 10% DMSO) for 24 h, to allow cell lysis. Slides were removed from lysis buffer, cleaned and immediately placed in a horizontal electrophoresis box. Slides were exposed to either an equilibration solution (500 mM NaCl, 100 mM Tris, 1 mM Na_2EDTA, pH 8.0) or an alkaline solution (300 mM NaOH, 1 mM Na_2EDTA, pH > 13) for 20 min at 4°C, for neutral and alkaline comet assays respectively. Electrophoresis was performed in the same solutions at 250 mA (neutral) or 300 mA (alkaline) and 25 V (0.90 V/cm) for 15 min at 4°C. The slides were then neutralized (Tris 0.4 M, pH 7.5) and silver stained after washing, fixing and rehydration (Nadin et al. 2001).

One hundred cells per individual (50 cells per replicate slide) were scored at 400× magnification using a conventional light microscope. Cells were visually divided into five classes, according to DNA migration, from 0 (no tails) to 4 (maximally long tails). The Damage index (DI) was obtained by the sum of the individual cell classes, ranging from 0 (no damage: 100 cells × 0) to 400 (maximum damage: 100 cells × 4). International guidelines and recommendations for the Comet assay consider that visual scoring of comets is a well-validated evaluation method. It has a high correlation with computer-based image analysis (Tice et al. 2000; Hartmann et al. 2003).

Micronucleus test

Bone marrow preparations were made by using fetal calf serum; two smears were prepared from each mouse. Slides were stained in 5% Giemsa and coded for "blind" analysis. To avoid false negative results and as a measure of toxicity of the compounds to bone marrow, the polychromatic erythrocyte (PCE)/normochromatic erythrocyte ratio was scored in 1,000 cells. Data are presented as the frequency of micronuclei per 1,000 PCE. Historical lab data for mice treated with 25 mg/kg CP for 48 h were used as the positive control (MacGregor et al. 1987; da Silva et al. 2002).

Particle induced X-ray emission analysis

Quantification of Fe and Cu in the liver of treated animals was carried out using PIXE. This technique has a truly multielemental capability; that is, all elements with atomic number higher than 11 can be simultaneously detected in a single measurement on the same target (Johansson et al. 1995). The sensitivity is very good and varies smoothly as a function of atomic number. It is important to note that PIXE sensitivity depends on the sample being analyzed. Typically, sensitivity is of the order of a few parts per million. The analysis is relatively fast and the measuring time is a few minutes. Since this technique is non-destructive, it preserves the original samples, allowing extra measurements if required. Sample preparation in its solid form (for a variety of samples) does not require either sophisticated handling or chemical treatment, thus reducing drastically any chance of contamination. Nowadays, PIXE is widely used to characterize a variety of materials, including biological, geological and environmental samples (Kern et al. 2004; Braga et al. 2005; Franke et al. 2006).

For PIXE analysis the liver samples were deep frozen (–80°C), lyophilized and pelleted, as described by Franke et al. (2006). Measurements were carried out at the Ion Implantation Laboratory of the Physics Institute of the Federal University of Rio Grande do Sul. A 3 mV Tandetron accelerator provided a 2 MeV proton beam with an average current of 2 nA for the experiments. Details of the experimental set-up are described in Dias et al. (2002). The characteristic X-rays induced by the proton beam were detected with a lithium doped silicon detector with an energy resolution of 155 eV at 5.9 keV, which was positioned at an angle of 45°C with respect to the beam direction. The data was analyzed using the GUPIX code (Maxwell et al. 1989, 1995; Campbell et al. 2000) The standardization procedure was carried out using a bovine standard from NIST (SRM–1577b).

Quantitative PIXE analysis of a sample in a thick target approximation (pellets) requires a knowledge of its matrix composition. Therefore, another ion beam technique, Rutherford Backscattering Spectroscopy (Chu et al. 1978), was employed to obtain this information for the liver and bovine standard used to calibrate the PIXE analysis. The matrix composition consisted of approximately C (70%), O (15%) and H (15%).

Statistical analysis

Two-way ANOVA was used to compare the genotoxicity/mutagencity and the levels of iron and copper according to sex and treatment. One-way ANOVA with Bonferroni's multiple comparison test was used to compare the genotoxicity/mutagenicity within sexes. When data did not show homoscedasticity, data were Ln transformed. For the MN test, the Kruskal–Wallis with Dunn's post-hoc test was used to compare the mutagenicity within sexes. Student's *t*-test was used to compare the levels of iron and copper within sexes. Prior to analyses, homoscedasticity was tested by Bartlett's test and parametric or non parametric tests were used accordingly. The GraphPad Prism (Graph-Pad Software, San Diego, CA, USA) was used for statistical analyses. Significance was considered to be at $p<0.05$. Values are expressed as mean ± standard error.

Results and discussion

The group of male mice treated orally with 25 mg/kg CP showed DI values of 83.74 ± 25.95 and 98.51 ± 28.82 in the neutral and the alkaline comet assays, respectively. Laboratory data showed averages in the MN test of 12.25 ± 4.35 MN‰ for males and 11.25 ± 4.45 MN‰ for females after a single intraperitoneal dose of 25 mg/kg CP 48 h before death. These values show the validity of the experiments.

Iron and copper were generally genotoxic and mutagenic to both male and female mice in the treatment scheme. As for previous lab data, females always showed lower DNA damage than males, although not significantly. Data for genotoxicity, mutagenicity and hepatic iron and copper levels are presented for each sex as well as jointly, since there was no significant difference between sexes (Figs. 1, 2).

Copper induced more DNA damage than iron in the neutral comet assay and the MN test [about 58.6% (43.5 ± 4.5 versus 38.3 ± 6.5) and 13.5% (1.76 ± 0.31 versus 1.11 ± 0.16) higher, respectively]. On the other hand, the alkaline version of the comet assay was more sensitive to detect iron genotoxicity (Fig. 1). These data are in disagreement with a previous acute experiment (24 h after exposure) using alkaline conditions, which showed 20% more DNA damage for copper at the same dose, of similar toxicity (about 11% of the LD_{50}) (Franke et al. 2006). It seems that DNA damage can be different after chronic or acute exposure.

While the neutral comet assay is known to detect primarily double strand breaks (DSB), the alkaline version is sensitive to single strand breaks (SSB), excision repair sites (ERS) and alkali labile sites (ALS) (Tice et al. 2000). Both iron and copper seem to induce DSB at a considerable level. Double lesions consist of two modifications of the DNA in close proximity. There is general agreement that most double lesions arise from adjacent independent lesions. Nevertheless, some studies support the idea that these lesions can be generated by a single free radical-initiating event, often involving guanine (Box et al. 2001). The results indicate that iron may have induced more SSB and ALS than copper, since there was 41.5% more DNA damage caused by iron (45.3 ± 5.0) than by copper (32.0 ± 4.2) in the alkaline comet assay. Continuous treatment with iron probably led to an accumulation of oxidative lesions in DNA that act as ALS or ERS. Oxidative lesions and more specifically 8-OHdG [one of the most prevalent lesions induced by iron containing substances (Abalea et al. 1999)] are removed from the damaged DNA by a repair enzyme called hOGG1, one of the DNA glycosylases (Nakano et al. 2003). The action of these enzymes leads to excision of 8-OHdG and, therefore, to ERS and ALS.

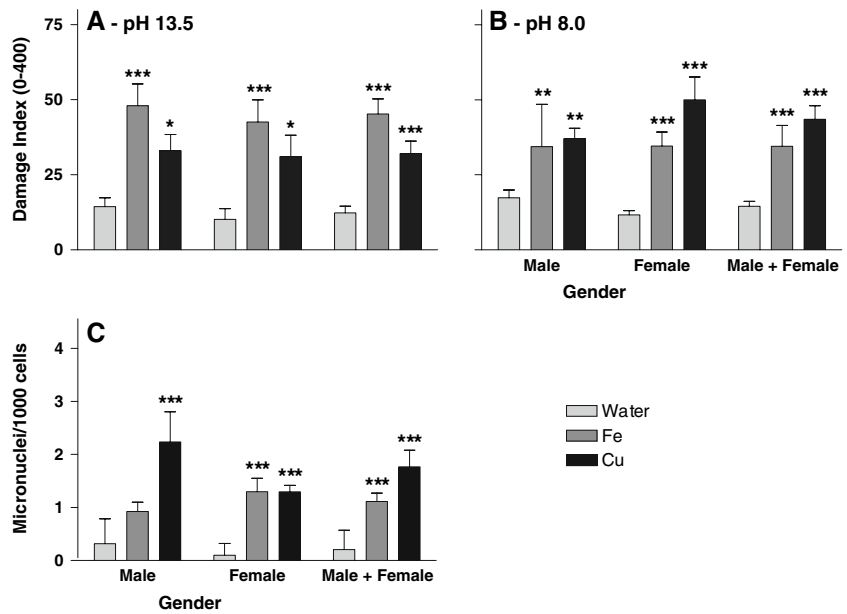

Fig. 1 Genotoxicity and mutagenicity of iron and copper to male and female mice, as evaluated by the Comet assay and the micronucleus test. (**A**) Comet assay under alkaline conditions (pH >13.5). (**B**) Comet assay under modified neutral conditions (pH = 8.0). (**C**) Bone marrow micronucleus test. Two-way ANOVA showed that DNA damage significantly differed between the treatments and controls and did not differ between genders for either the comet assay (alkaline and neutral) or the micronucleus test. *Asterisks* indicate significant differences from the group treated with water (control) using Dunn's post-hoc test at $*p < 0.05$, $**p < 0.01$ or $***p < 0.001$. Averages ± standard errors. $N = 5$ mice of each sex

Copper is more reactive in vitro than iron (Cai et al. 1995; Oikawa and Kawanishi 1998), possibly because of the ready cycling between cuprous and cupric states, which share the same genotoxicity (Tkeshelashvili et al. 1991). This can be enhanced by interaction with oxidants. For instance, Yoshino et al. (1999) showed that the interaction of copper with vitamin C led to five times more DNA damage than that with iron. On the other hand, Lloyd et al. (1998) showed that Fe(II) induced significantly more 8-OHdG than Cu(II) in vitro. While hydroxyl radicals are thought to be major intermediaries in iron induced base oxidation (Tkeshelashvili et al. 1991), copper compounds oxidize DNA in complexes formed with other molecules (Yamamoto and Kawanishi 1989; Fujimoto et al. 2005), usually produced within the biological system (Linder 2001).

DNA adducts formed from oxidative reactions mediated by metals may also be involved in the mutagenicity of transition metals. They can be formed by inter or intrastrand depurinations that have aromatic adduct-like characteristics (Lloyd et al. 1997). The results of a comparative study by Lloyd et al (1997), testing the induction of cross link-like lesions and strand breaks by several metals in vitro, showed that, while the copper(II)/hydrogen peroxide system produced by far the highest yield of bulky lesions and strand breakage, the iron(II)/hydrogen peroxide system induced a low proportion of DNA bulky lesions and an intermediate level of DNA strand breaks. The copper system also generated significant levels of bulky lesions in DNA that were unique to this system. Given the lack of correlation between DNA strand breaks and bulky adducts, the authors concluded that it was likely that the lesions arise via two different mechanisms, perhaps involving the association of transition metal ions with different regions of the double helix and different moieties of individual nucleotides (Lloyd et al. 1997). DNA cross-linking can be detected by the Comet assay, as demonstrated by retardation in the rate of DNA migration (Tice et al. 2000) and thus a reduction in DNA damage as evaluated by this method. Therefore, it is possible that cross-links induced by copper cause a detrimental reduction in DNA migration. However, further studies are needed to confirm this.

Although copper and iron share some common features in transport, most of their metabolism and storage is different. While iron is needed in much higher amounts and is stored within the cells either in ferritin and in the labile iron pool (Kruszewski 2003), an intracellular pool of free copper is almost non-detectable (Rae et al. 1999; Linder 2001). Thus, as expected, the hepatic level of iron was much higher in mice treated with iron than in controls. In the case of copper, the same trend was not seen, except for females treated with the metal, which showed a significantly higher hepatic level of copper (Fig. 2). This can be explained by the fact that excess copper is not stored in the liver. Analysis of the data of Uriu-Adams et al. (2005), who gavaged rats with a single dose of ^{67}Cu, revealed minor fluctuations in retention of the isotope in the liver up to 5 days after treatment. Conversely, a progressive increase in the percentage of retained ^{67}Cu was noticed in muscle, skin and

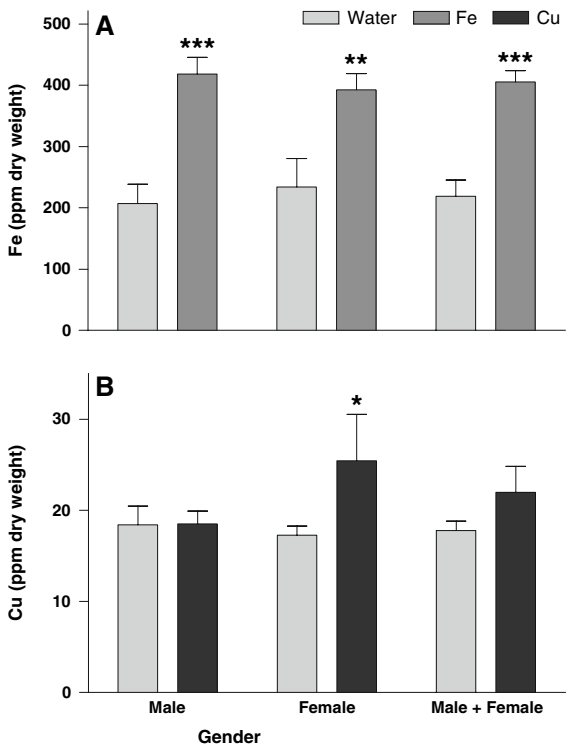

Fig. 2 Hepatic level of iron (**A**) and copper (**B**) of mice treated with iron and copper, respectively. Two-way ANOVA showed that the level of the metals differed significantly between the treatments and controls and did not differ between genders. *Asterisks* indicate significant differences from the group treated with water (control) using Student's *t*-test test at $*p < 0.05$, $**p < 0.01$ or $***p < 0.001$. Averages ± standard errors. $N = 5$ mice of each sex

bone. These tissues accounted for about 50% of all ^{67}Cu retained 5 days after treatment (Uriu-Adams et al. 2005). With respect to iron, the liver is the key storage organ and metabolism is through hepatocytes (major storage site) and mastocyte-like Kupfer cells (major recycler of iron derived from senescent red blood cells and storage in overload situations) (Anderson and Frazer 2005).

The Recommended Dietary Allowance (RDA), or the amount supposed to meet the nutritional requirement of 97.5% of the healthy population (Schumann 2001), is of 900 μg/day copper for adult men and women. For iron, the RDA is of 8 mg/day for all age groups of men and postmenopausal women and of 18 mg/day for premenopausal women. The tolerable upper intake level (UL) for adults is of 10 mg/day copper, based on protection from liver damage as the critical adverse effect, and of 45 mg/day iron, based on gastrointestinal distress as an adverse effect (Institute of medicine, IOM 2001). If we extrapolate the doses administered to mice in this study to a standard 65 kg human subject, the doses would account to about 0.54 g/day of copper and 2.16 g/day of iron, corresponding to about 50 times the UL for either copper or iron.

There are few studies addressing the harm of copper to human subjects as well as its average consumption as supplement. It has been shown that the daily drink of about 5 mg/day copper can lead to gastrointestinal distress and there were no liver-related adverse effects from daily consumption of 10–12 mg/day of copper in foods. Notwithstanding, it was shown that the chronic consumption of 30 mg/day copper as supplements can lead to liver failure (Institute of medicine, IOM 2001).

Accidental iron overdose is the most common cause of poisoning deaths in children under 6 years of age in the United State. Poisoning symptoms occur with doses between 20 and 60 mg/kg of iron, with the low end of the range associated primarily with gastrointestinal irritation while systemic toxicity occurs at the high end (Institute of medicine, IOM 2001) and dose between 10 and 20 mg Fe/kg are regarded as non toxic to humans (Schumann 2001). The dose used in this study was in the lower limit of this range. Regarding the dietary source of iron, Troppmann et al (2002) showed that adult Canadian individuals users of multivitamin supplementation have significantly higher iron intake than non users in all age group either females or males (40–85% more), although the average intake of iron in this group was lower than the UL.

The current results show significant genotoxicity for iron and copper at the high doses tested. The ingestion of trace nutrients is increasing as a result of the consumption of enriched foods, as well as of multiple vitamin–mineral supplement tablets and the consumption of high level of nutrients as supplements in not unusual (Troppmann et al. 2002). Many ideas about the physiology of metals have being questioned and there is growing evidence that copper and iron might be associated to DNA through in situ reactions, leading to genome damage (Tkeshelashvili et al. 1991; Meneghini 1997). Recent studies have even shown that phytochemicals can mobilize copper stores, leading to genotoxic effects (Azmi et al. 2006). In another had, iron accumulation and deficiency has been linked to several types of cancer (Ahmed 2004), as well as to cardiovascular (Cabantchik et al. 2005), neurodegenerative (Fredriksson et al. 1999) and other diseases (Oppenheimer 2001; Vyoral and Petrak 2005). Further studies on the physiology of copper are still needed before we can make more definitive statements. We need to know, for example, whether similar effects would be observed at lower doses than those tested. It is essential to elucidate the correct dosages of iron and copper to improve health without leading to noxious effects under various metabolic situations.

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