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The acute toxicity of iron and copper: Biomolecule oxidation and oxidative damage in rat liver

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ABSTRACT

The transition metals iron (Fe) and copper (Cu) are needed at low levels for normal health and at higher levels they become toxic for humans and animals. The acute liver toxicity of Fe and Cu was studied in Sprague Dawley male rats (200 g) that received ip 0–60 mg/kg $FeCl_2$ or 0–30 mg/kg $CuSO_4$. Dose and time-responses were determined for spontaneous *in situ* liver chemiluminescence, phospholipid lipoperoxidation, protein oxidation and lipid soluble antioxidants. The doses linearly defined the tissue content of both metals. Liver chemiluminescence increased 4 times and 2 times after Fe and Cu overloads, with half maximal responses at contents ($C_{50\%}$) of 110 μg Fe/g and 42 μg Cu/g liver, and with half maximal time responses ($t_{1/2}$) of 4 h for both metals. Phospholipid peroxidation increased 4 and 1.8 times with $C_{50\%}$ of 118 μg Fe/g and 45 μg Cu/g and with $t_{1/2}$ of 7 h and 8 h. Protein oxidation increased 1.6 times for Fe with $C_{50\%}$ at 113 μg Fe/g and 1.2 times for Cu with 50 μg Cu/g and $t_{1/2}$ of 4 h and 5 h respectively. The accumulation of Fe and Cu in liver enhanced the rate of free radical reactions and produced oxidative damage. A similar free radical-mediated process, through the formation $HO^{\bullet}$ and $RO^{\bullet}$ by a Fenton-like homolytic scission of H_2O_2 and ROOH, seems to operate as the chemical mechanism for the liver toxicity of both metals.

1. Introduction

The transition metals Fe and Cu show the phenomenon of hormesis: they are needed at low levels for normal health in mammals (Recommended Daily Allowance for humans 10–15 mg Fe/day and 1–3 mg Cu/day) and at higher levels (20–30 mg Fe/kg and 8–10 mg Cu mg/kg) both metals become toxic for humans [1–7] and animals [2].

The cellular and tisular levels of Fe and Cu are homeostatically determined by regulatory proteins and metallochaperons that control metal capture, transport and storage. In the liver, reserve Fe is mainly kept as cytosolic ferritin [8,9], a specialized protein envelope of 450 kDa that reversibly holds up to 4500 Fe atoms [8] and that accounts for as much as 50–55% of liver Fe, with another 20–25% of Fe forming part of mitochondrial and endoplasmic reticulum cytochromes and iron sulfur centers. From the liver, Fe is mobilized to peripheral tissues by plasma transferrin. Similarly, liver Cu is mainly kept in metallothionein [10] and the ATOX1 chaperone [11] and mobilized to other tissues by plasma ceruloplamin.

Fe and Cu are stored in the liver after being absorbed in small intestine, and are transferred to the membranes of bile canaliculi to dissipate the toxic levels of both metals [12].

In individuals with the genetic disease of hemachromatosis, Fe is deposited in various internal organs, especially in the liver. The common symptoms and pathology of Fe-related toxicity are: hepatomegaly, steatosis, insulin resistance, subclinical inflammation [13], skin pigmentation, joint diseases and lethargy [14].

It is well known that Cu promotes oxidative damage in the conditions of increased Cu levels in the liver and brain. The best known disorder associated to Cu dyshomeostasis is Wilson's disease, an autosomal recesive disorder linked to the Cu translocase expressed in hepatocytes. This enzyme is critical in the distribution and elimination of excess Cu from the organism [12]. Cu toxicity has been linked to cancer progression, cardiovascular disease, atherosclerosis, diabetes and especially to neurological disorders [15].

In the *in vitro* model of phosphatidylcholine/phosphatidyserine (60:40) liposomes and hydrogen peroxide (H_2O_2), Fe and Cu promote lipid peroxidation, interpreted as the consequence of the homolytic scission of H_2O_2 and of endogenous hydroperoxides (ROOH) with the

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generation of hydroxyl ($HO^{\bullet}$) and alcoxyl ($RO^{\bullet}$) radicals, depending strictly of the redox properties of the two transition metals [16].

Spontaneous light emission from *in situ* mammalian organs is a physiological phenomenon that also provides an assay for the determination of the steady state concentration of singlet oxygen ($^1O_2$) and indirectly of the rate of oxidative free radical reactions [17–19]. *In situ* liver chemiluminescence has been recognized as a reliable indicator of oxidative stress and damage in rat liver upon hydroperoxide infusion [17], ischemia–reperfusion [20], and chronic [21] and acute alcohol intoxication [22,23]. The increases in photoemission observed were parallel to increased contents of indicators of lipid peroxidation (malonaldehyde and 4-HO-nonenal) but with a higher experimental/control ratio in organ chemiluminescence [24].

The chemical mechanism responsible for spontaneous organ light emission is provided by the Russell's reaction in which two secondary or tertiary peroxyl radicals ($ROO^{\bullet}$) yield $^1O_2$ and excited carbonyl groups ($=CO^*$) as products. In turn, two $^1O_2$, through dimol emission, lead to photoemission at 640 and 670 nm, whereas $=CO^*$ yields photons at the 460–470 nm band [17,18].

The aim of this work was to study the acute liver toxicity of Fe and Cu, considering that liver is the first organ for Fe and Cu deposition after intestinal absorption. Liver toxicity was approached by determining liver chemiluminescence, *i.e.*, the rate of free radical reactions, the oxidative damage to phospholipids and proteins, and the level of liposoluble antioxidants as a function of liver metal content.

2. Experimental methods

2.1. Experimental animal model

Sprague Dawley male rats (200 g) received increasing doses of ferrous chloride ($FeCl_2$, 0–60 mg/kg, ip, n = 24) and of cupric sulfate ($CuSO_4$, 0–30 mg/kg, ip, n = 24) with control rats receiving 0.9 % NaCl. In separate experiments, rats received 30 mg $FeCl_2$ (n = 24) or 10 mg $CuSO_4$ (n = 18) and were followed for 48 h. Rats were anesthetized with 15% (w/v) urethane at 1.5 g/kg, ip. Liver chemiluminescence was determined 15 min after anesthesia, and after stable photoemission readings, usually 5–10 min, animals were sacrificed for the determinations of oxidative damage. Animal care was given in compliance with Argentine regulations (ANMAT) and with the Guidelines for Ethical Treatment in Animal Experimentation of the American Physiological Society (Bethesda, MD, USA).

2.2. In situ liver chemiluminescence

The whole procedure followed a previously used protocol [17, 19–22]. The abdomen of the anesthetized rats was open and washed with 0.9% NaCl to remove blood from the peritoneal cavity. Liver was exposed and the animal was covered with aluminum foil, in which a 2–3 cm^2 window allowed liver exposure. Liver chemiluminescence was measured with a Johnson Foundation photon counter (Johnson Research Foundation, University of Pennsylvania, Philadelphia, PA, USA) [17]. Photoemission was expressed as counts per second (cps/cm^2) of exposed liver surface.

2.3. Liver homogenate preparation

Liver were rapidly excised, weighed and homogenized in a medium consisting of 120 mM KCl, 30 mM phosphate buffer, pH 7.4, at a ratio of 1 g liver/9 mL of buffer at 0 °C. The homogenates were centrifuged at 600 *g* for 10 min to discard nuclei and cell debris. The supernatant, a suspension of organelles and plasma membranes was used as tissue homogenate [25,26].

2.4. Lipid peroxidation measurements

These measurements were performed by the determination of thiobarbituric acid-reactive substances (TBARS), as described by Fraga et al. [27]. The reaction mixture was of liver homogenate, 120 mM KCl, 30 mM phosphate buffer, pH 7.4, 4% w/v buthylhydroxytoluene in ethanol, 20% w/v thrichloroacetic acid and 0.7% w/v thiobarbituric acid. The deproteinized supernatant was heated at 100 °C for 20 min, the absorption of the pink solution determined at 535 nm ($\varepsilon = 156\ mM^{-1}\ cm^{-1}$) and expressed as nmol TBARS/g organ.

2.5. Protein oxidation measurements

These was done spectrophotometrically at 340 nm, determining protein carbonyl groups in the proteins of liver homogenates by conjugation with 2,4 dinitrophenylhydrazine [28].

2.6. Oxidative damage index

The index: [(TBARS-metal/TBARS-control) + (carbonyl-metal/carbonyl-control)] 0.5 was calculated for each metal content and time point with the data of the homogenates from metal-treated and control rats [29].

2.7. Content of lipophilic antioxidants

The *tert*-butyl hydroperoxide (*t*BuBOOH) initiated chemiluminescence of tissue homogenates provides an assay to determine the tissue content of lipophylic antioxidants [26]. Samples from control and treated tissue homogenates are processed in parallel at the same adjusted protein content, which implies a similar content of unsaturated fatty acids and hemoproteins. The initiation reaction is given by the hemoprotein-catalyzed scission of *t*BuBOOH and the propagation reactions are the lipoperoxidation of the unsaturated fatty acids.

The level of chemiluminescence in the assay reflects, with an inverse relationship, the level of non-enzymatic lipophilic endogenous antioxidants (mainly α − tocopherol). Lipophilic antioxidant content was determined in a Packard Tri-carb model 3355 liquid scintillation counter in the out-of-coincidence mode at 30 °C, at 0.1–0.2 mg protein/mL of liver homogenate in 120 mM KCl, 30 mM phosphate buffer, pH 7.4, with the addition of 3 mM *tert*-butyl hydroperoxide. Counting was followed until a maximal level of emission, usually after 15–20 min, was reached. The results are expressed as cpm/g organ [26].

2.8. Liver metal content

Liver metal total content was measured in an atomic absorption spectrometer (Buck model 200 A, East Norwalk, Connecticut, USA). Samples were incinerated for 4 h in a graphite muffle at 500 °C. Calibration was made by using standard solutions of 0.1 to 3 mg/L of Fe and Cu. Results are expressed in μg metal/g liver.

2.9. Protein determination

Protein contents were measured by the Folin reagent using bovine serum albumin as standard.

2.10. Chemicals

Chemicals were purchased from Sigma-Aldrich Chemical Co (St Louis, Mo).

2.11. Data analysis

Values in figures and table are means ± standard error of the mean (SEM). Data was statistically analyzed by the Tukey–Kramer multiple comparison test, and the Pearson's correlation coefficient calculated with a standard statistical package. Significance was given to $p<0.05$.

3. Results

3.1. Dose and liver content of iron and copper

The liver content of Fe and Cu increased up to 1.6 and 18 times, respectively, after acute metal overloads (Fig. 1A), in samples taken 16 h after metal overload. A quantitative linear correspondence was found between metal dose and metal accumulation with correlations of $r = 0.93$ and 0.95 for Fe and Cu, respectively ($p<0.01$).

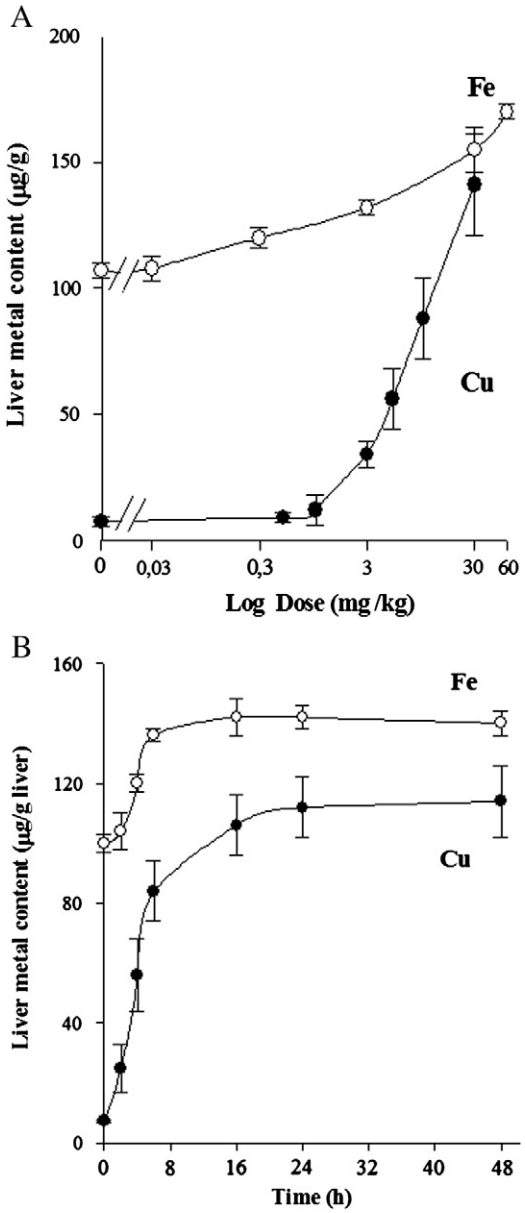

Fig. 1. Dependence of liver metal content on dose and time in the acute Fe and Cu overloads. (A) Doses of Fe (○) and Cu (●) and metal content in rat liver. (B) Metal content as a function of time after acute Fe and Cu overloads ($p<0.01$).

3.2. Time course of the liver accumulation of Fe and Cu after metal overload

This was followed after administration of 30 mg/kg of Fe or 10 mg/kg of Cu. The time for half maximal effect ($t_{1/2}$) in metal accumulation was about 4 h for both metals (Fig. 1B).

3.3. In situ liver chemiluminescence after metal overload

The dose–effect profile after Fe overload increased up to 4 times the spontaneous liver chemiluminescence with a half maximal effect ($C_{50\%}$) at 110 µg Fe/g liver (Fig. 2A) whereas the dose–time profile showed a $t_{½}$ of 4 h (Fig. 2B). For the Cu overload, there was an enhanced photoemission of 2 times, with a $C_{50\%}$ at 42 µg Cu/g liver (Fig. 2A) and a $t_{½}$ of 4 h (Fig. 2B) (Table 1). Small and moderate increments over the

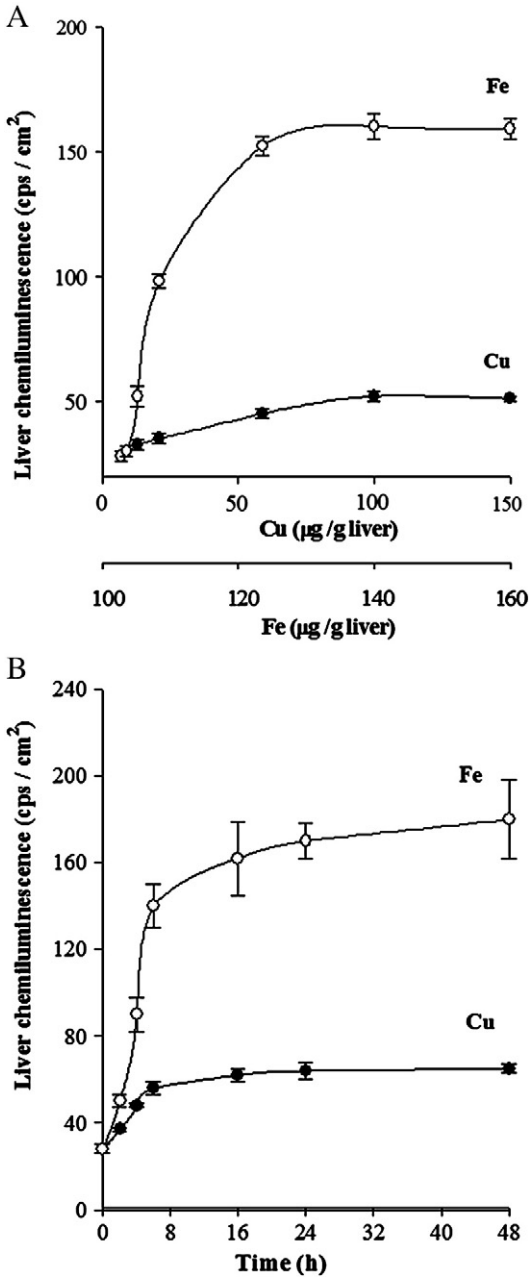

Fig. 2. Rat liver chemiluminescence as a function of metal content and time of metal overload. (A) *In situ* liver chemiluminescence of Fe (○) and Cu (●) overloads as a function of the metal contents in the liver. (B) Rat liver chemiluminescence of Fe (○) and Cu (●) as a function of time after metal overload.

basal levels in metal liver content, about 10% for Fe and 23% for Cu, were sufficient to produce a significantly increases in liver chemiluminescence. Metal contents and liver chemiluminescenece, at the different doses and times, showed correlations of r = 0.89 and 0.91 for Fe and Cu ($p<0.01$).

3.4. Liver lipid peroxidation

The content of lipid peroxidation products, measured as TBARS, increased in liver homogenates after Fe and Cu overloads. In Fe-treated rats, lipid peroxidation products increased 4 times (Fig. 3A), with a $C_{50\%}$ of 118 μg/g liver (Table 1) and a $t_{½}$ of 7 h (Table 1 and Fig. 3B). For the case of Cu, the increase in lipid peroxidation products was of 1.8 times (Fig. 3A), with a $C_{50\%}$ of 45 μg Cu/g liver and a $t_{½}$ of 8 h (Table 1 and Fig. 3B). Metal contents and lipid peroxidation products, at the different doses and times showed the significant correlations of r = 0.90 and 0.84 for Fe and Cu ($p<0.01$).

3.5. Liver protein oxidation

The level of protein oxidation products, measured as protein carbonyl groups, markedly increased after Fe and Cu accumulation in the liver. In Fe-treated animals, the increase was of 50 % (Fig. 4A) with a $C_{50\%}$ of 113 μg Fe/g liver and a $t_{½}$ of 4 h (Table 1). In Cu-treated rats, protein oxidation was enhanced by 15% (Fig. 4A), with a $C_{50\%}$ of 50 μg Cu/g liver and with a $t_{½}$ of 5 h (Table 1 and Fig. 4B). The homogenate content of the metals and of protein carbonyl groups, at various doses and times, were significantly correlated, r = 0.88 and 0.93 for Fe and Cu doses; and r = 0.87 and 0.42 respectively for the $t_{½}$ times ($p<0.01$).

3.6. Oxidative damage index and metal accumulation

The oxidative damage index combines two indicators, TBARS and protein carbonyls, and gives an extended quantification of the free radical-mediated damage to cells and tissues [29]. Liver chemiluminescence was linearly related to the oxidative damage index in both Fe and Cu treatments, with correlation of r = 0.80 for Fe and 0.96 for Cu (Fig. 5A), and a subjacent correlation between oxidative damage and metal content of 0.99 for Fe and 0.97 for Cu ($p<0.05$). Oxidative damage shows a $t_{½}$ of about 8 h for both metals (Fig. 5B).

3.7. Content of lipophilic antioxidants

The assay showed an increase of about 50% in tBuBOOH-initiated chemiluminescence after metals overloads, which indicates a quantitatively similar decrease in lipophilic antioxidants (α-tocopherol, ubiquinol, carotenoids, etc.). The $C_{50\%}$ were 117 μg Fe/g liver and 54 μg Cu/g liver, with a $t_{½}$ of 4 for both, Fe and Cu (Table 1). The correlation of metal content and tBuBOOH-initiated chemiluminescence was r = 0.81 and 0.90 for Fe and Cu ($p<0.01$; data not shown).

3.8. Liver metal contents and times that produce half maximal effects

The liver metal contents ($C_{50\%}$) and the times ($t_{½}$) that produced half maximal effects in the measured indicators are compared in Table 1, in an attempt to describe by this kinetic approach, the characteristics of the chemical pathways involved and the sequence of their occurrence. The $C_{50\%}$ for Fe and Cu in the various processes indicates that a unique metal-promoted process explains the observed effects in the fours indicators. Moreover, the $t_{½}$ of the processes indicate that the four of them are simultaneous, in each of two conditions either Fe or Cu overloads. The lipid peroxidation process seems slower, likely due to an increasing mass of oxidizable unsaturated fatty acids due to organelle disruption in the hepatocytes.

Table 1
Liver metal content ($MC_{50\%}$) and time ($t_{1/2}$) necessary for generating the 50% of the maximal oxidative damage.

Oxidative stress/damage	$C_{50\%}$ Fe (μg/g liver)	$t_{1/2}$ Fe (h)	$C_{50\%}$ Cu (μg/g liver)	$t_{1/2}$ Cu (h)
Liver chemiluminescence	110	4	42	4
Phospholipid oxidation	118	7	45	8
Protein oxidation	113	4	50	5
Lipophylic antioxidant content	117	4	54	4

4. Discussion

The transition metals Fe and Cu are involved in the basic biochemistry of aerobic life because these atoms participate as part of the active center of vital proteins and enzymes. Concerning Fe atoms, they participate in the reversible binding and transport of O_2 and in the electron transfer reactions of cytochromes and iron–sulfur centers of the mitochondrial respiratory chain. In the case of Cu atoms, they are

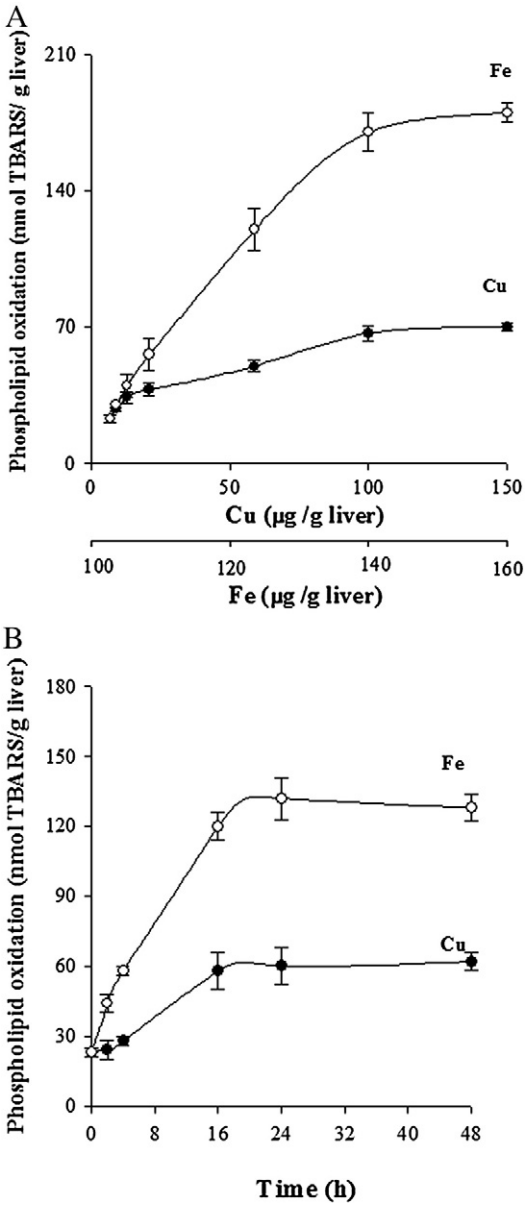

Fig. 3. (A) Phospholipid peroxidation, measured as TBARS in liver homogenates, as function of Fe (○) and Cu (●) contents. (B) Phospholipid peroxidation as function of time after acute metal overload.

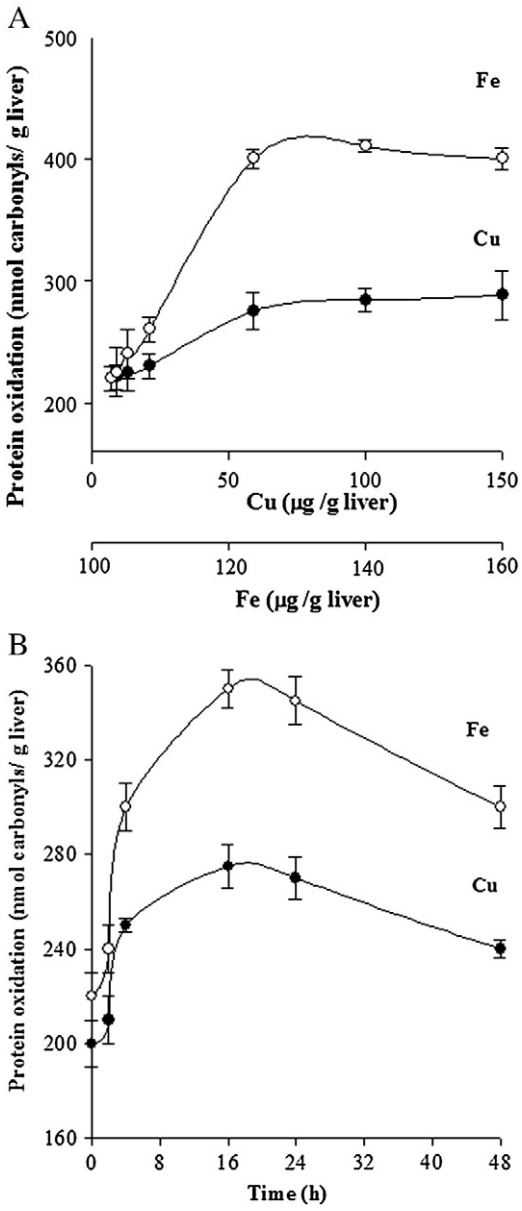

Fig. 4. (A) Protein oxidation, measured as protein carbonyl groups in liver homogenates, as function of Fe (○) and Cu (●) contents in rat liver. (B) Protein oxidation, as function of time after acute metal overload.

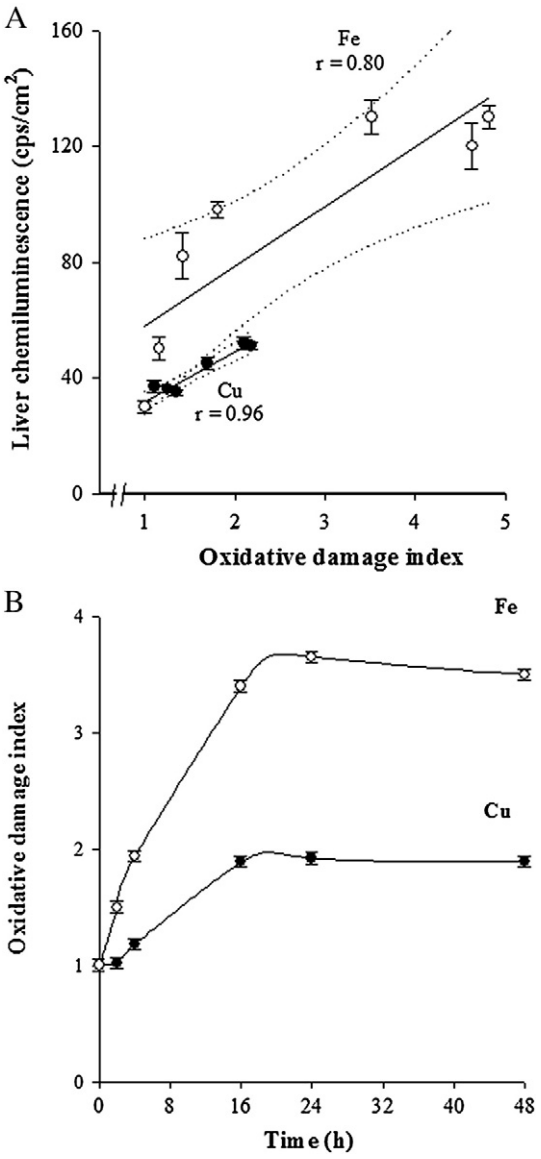

Fig. 5. Liver chemiluminescence as a function of oxidative damage index. (A) Oxidative damage index for Fe (○) and Cu (●) after metal overloads ($p<0.05$). (B) Oxidative damage index as a function of time after metal overload.

a half part of the binuclear center of cytochrome oxidase that accommodates and reacts with O_2. Moreover, Cu redox changes are the basis of the dismutation chemistry catalyzed by Cu,Zn-superoxide dismutase in which two superoxide radicals ($O_2^{\cdot-}$) yield hydrogen peroxide (H_2O_2) and O_2.

Besides that, relatively high levels of the two transition metals are toxic to aerobic cells, a fact that implies that both metals are kept by homeostatic mechanisms in narrow ranges of concentrations and content. Two main concepts, not necessarily exclusive, are being considered for transition metal toxicity in the liver: either an enhancing of the endogenous free radical chain reactions or an inactivation of essential thiol groups in enzymes and in protein regulatory factors.

The liver toxicity of Fe and Cu occurs when the metal overloads overwhelm the transport and liver storage capacities, which mainly consist of ferritin [8,9] and mitochondrial ferritin [30] for Fe and of metalloproteins and ATOX1 for Cu. The two metals lead the hepatocytes initially to reversible oxidative stress, then become cytotoxic with oxidative damage and finally lead to apoptosis and liver dysfunction.

Concerning the toxicity of transition metals, it is accepted that metal ions tightly bound to proteins are not toxic, in contrast with the free or weakly-bound ions that are cytotoxic at sub-micromolar concentrations. It is known that the liver storage proteins keep Fe and Cu sequestered in their higher oxidation states, Fe(III) and Cu(II). In this way, the free ions that are active in the Fenton-type reactions, Fe(II) and Cu(I), are kept at very low levels in the cytosol. We have calculated the concentrations of free Fe^{2+} and Cu^{+} ions in liver cytosol as 10^{-12} M Fe(II) and 10^{-14} M Cu(I). In the case of Cu, this level corresponds to one to few atoms per cell [31].

The critical question of how Fe^{3+} and Cu^{2+} ions that are kept in the storage proteins are reduced and released as free Fe^{2+} and Cu^{+} ions, has no answer but a couple of hypotheses, considering that the metals are tightly bound inside the protein and loosely bound at the surface. One of the hypotheses is that the redox state of the cell, *i.e.*, the NADH/NAD and reduced glutathione/oxidized glutathione couples (GSH/GSSG), equilibrate with the redox state of Fe and Cu in the storage proteins. Under the normal situation of high cellular reduction, with high levels of NADH and GSH, a slow metal release occurs [32,33]. The second hypothesis is that metal reduction is caused

by cytosolic $O_2^{\cdot-}$, which is normally kept in hepatocyte cytosol at a steady state of 10^{-11} M by the action of Cu,Zn-superoxide dismutase [34]. The poorly reactive radical $O_2^{\cdot-}$ is a good reductant ($E° = 0.15$ V) [35] for Fe(III) ($E° = 0.77$ V) and for Cu(II) ($E° = 0.15$ V) and cytosolic $O_2^{\cdot-}$ would be able to reduce Fe(III) and Cu(II) in the storage proteins and release Fe^{2+} and Cu^{+} ions [36–38].

The Fenton and the Fenton-like reactions are simple chemical processes in which the transition metal ion with charge n, transfers one electron to the –O–O– group of H_2O_2 or ROOH (organic hydroperoxide), resulting in an ion charge of n + 1 and the scission of the –O–O– group with formation of HO^- (hydroxyl anion) and hydroxyl (HO•) or alcoxyl (RO•) radicals. There is a long standing discussion on how this occurs in biological conditions, however the explanation of the Fenton or the Fenton-like reactions fits the experimental data and explain the biological effects [16,18].

The association between free radical-mediated reactions, increased phospholipid oxidation and pathological states was early recognized [18]. In human liver, the changes may affect organ structure and function, as it is the case for the bile canaliculi damaged in liver transplanted patients; a fact interpreted as consequence of the oxidative damage of ischemia–reperfusion [39–41].

The classical concept of oxidative stress is defined as an umbalance situation in which oxidants predominate over antioxidants [42,43]. In terms of the free radical-mediated biochemical chain reactions in cells and organs, oxidative stress is understood as an increased rate of the endogenous free radical reactions, with increases in the steady-state concentrations of the intermediates and in the content of oxidation products [44].

Concerning the mechanism of the oxidative stress and damage that follows to liver Fe and Cu overloads, the four indicators of the processes show an almost identical $C_{50\%}$ (Table 1). This indicates that there is a process with a common biochemical mechanism, with identical dependence on the rate limiting factors, *i.e.* the Fe and Cu liver contents. Moreover, the kinetic comparison of the $t_{1/2}$ of the effects of Fe and Cu overloads also indicates a simultaneous free radical-mediated process with three indicators: liver chemiluminescence, protein oxidation products and liposoluble antioxidants, exhibiting a similar time-course (a $t_{1/2}$ of about 4 h). The observation is consistent with the classic view of oxidative stress and damage in which antioxidants decrease and oxidized products increase progressive and simultaneously. The delay observed in the $t_{1/2}$ of phospholipid oxidation, the fourth indicator, is explained by the increasing amounts of free phospholipids associated to the disruption of cell membranes during the process. An integrated view of the biochemical liver reactions and oxidative processes is presented in Fig. 6.

There is a quantitative difference in the oxidative effects of Fe and Cu: the Fe-stimulated effects are more marked. The ratio of maximal Fe-effect/Cu-effect was 4.2–6.3 for liver chemiluminescence (Fig. 2A and B) and of 2.7–3.4 for TBARS contents in liver homogenates (Fig. 3), which are similar to the similar Fe/Cu ratio 2.7–4.0 for TBARS production in the *in vitro* model of phospholipid liposomes supplemented with H_2O_2 and transition metals [2]. This agreement indicates that the reactions involved in the *in vitro* system and in the *in vivo* liver are similar and that metal-mediated lipid peroxidation occurs in the liver after metal overloads. This final remark contrasts with the report that iron loading does not cause oxidative liver damage by Frei's group working

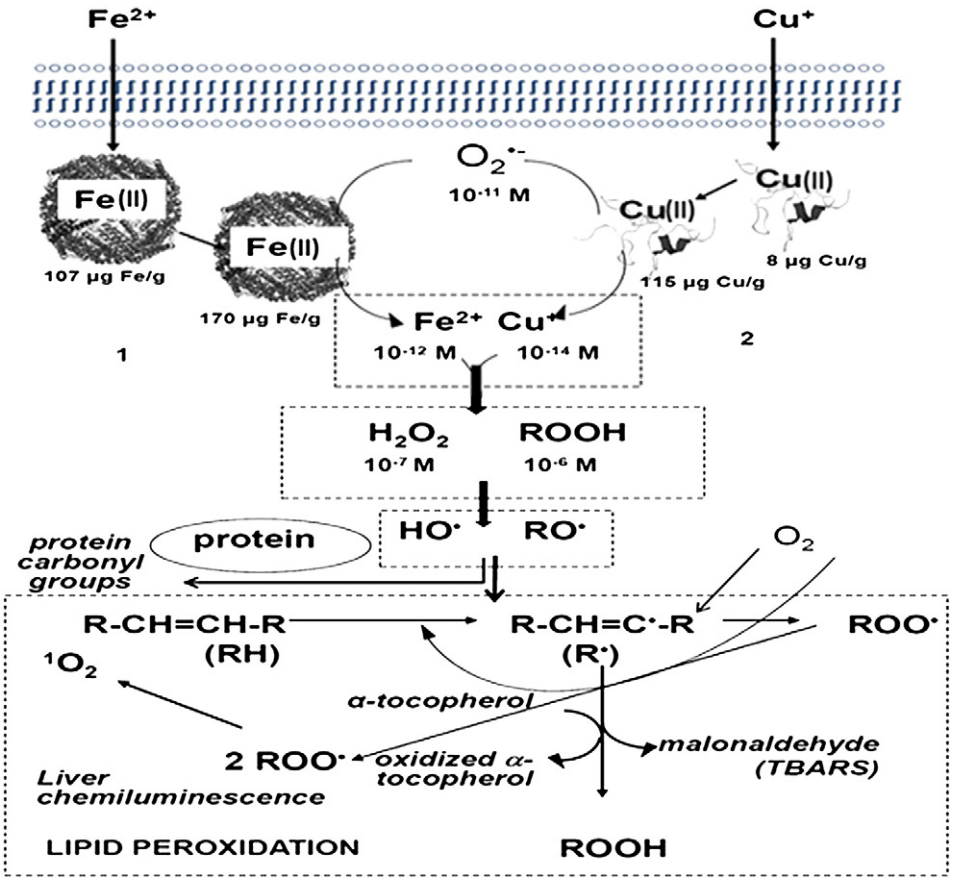

Fig. 6. Scheme of events in Fe and Cu liver overloads. The normal liver content of Fe and Cu in the liver is mainly deposited and bound to ferritin (1) and to methallothionein (2) without considering other metal-containing proteins. The increased liver content of both metals is more prone to $O_2^{\cdot-}$ reduction and to release free Fe^{2+} and Cu^{+}. These ions lead to increased formation of $HO^{\cdot}$ and $RO^{\cdot}$ radicals, which, in turn, lead to increases in protein oxidation, liver chemiluminescence, α-tocopherol consumption, and malonaldehyde production, which are considered consequences of a free radical-mediated processes in the hepatocytes.

with guinea pigs [45] but agrees with another report by the same group in rat liver [46].

5. Conclusions

Liver oxidative damage is produced by higher than normal Fe and Cu contents which are linearly related to metal intakes. Increased liver Fe and Cu contents increase liver surface chemiluminescence and phospholipid and protein oxidation and decrease the content of lipophilic antioxidants. The observed effects are produced at similar Fe and Cu $C_{50\%}$ for each of the two metal-promoted processes and indicate a common biochemical mechanism with a similar free radical-mediated toxic process in Fe and Cu overloads.

Abbreviations

$^1O_2$	singlet oxygen
*t*BuBOOH	*tert*-butyl hydroperoxide
$C_{50\%}$	metal liver content that produces 50% of the maximal oxidative response
cpm	counts per minute
cps	counts per second
ip	intraperitoneal
$O_2^{\cdot-}$	superoxide anion
ROOH	lipid hydroperoxides
SEM	standard error of the mean
$t_{1/2}$	time that produces 50% of the maximal oxidative response
TBARS	thiobarbituric acid-reactive substances

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The authors declare that they have no conflict of interest.

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