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Redox dyshomeostasis in the experimental chronic hepatic overloads with iron or copper

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ABSTRACT

Male rats of 80–90 g were overloaded with either Fe(II) or Cu(II) for 42 days by high concentrations of $FeCl_2$ or $CuSO_4$ in the drinking water. The animals were fed with a commercial rodent diet of 2780 kcal/100 g. Both metal treatments led to a liver redox imbalance and dyshomeostasis with oxidative stress and damage and the concomitant enhancement of oxidative processes as indicated by *in vivo* surface liver chemiluminescence, the sensitive and organ non-invasive assay for oxidative free radical reactions, and by *ex vivo* determined processes of phospholipid peroxidation and protein oxidation. In parallel, marked decreases in the antioxidant defense were observed. Liver reduced glutathione (GSH) content and the reduced/oxidized glutathione ratio (GSH/GSSG) were early indicators of oxidative metabolic disturbance upon the metal overloads. Thus, GSH plays a central role in the defense reactions involved in the chronic toxicity of Fe and Cu. Chronic overloads of Fe or Cu in rats afford an experimental animal model of hemochromatosis and of Wilson's disease, respectively. These two animal models could be useful in the study and development of the beneficial effects of pharmacological interventions in the two human diseases.

1. Introduction

Currently, the toxicity of the transition metals Fe and Cu in mammals is explained by one prevailing hypothesis, which is based on the reversible redox properties of both metal ions and postulates that the reduced forms of the metal ions, Fe(II) and Cu(I), react to produce the homolytic cleavage of the covalent peroxide bond (−O−O−) in hydrogen peroxide (H_2O_2) and organic peroxides (ROOH) in a Fenton-type reaction in the aqueous phase of cell cytosol to produce the hydroxyl (HO·) and alkoxyl (RO·) free radicals. This initial reaction is followed by a free radical mediated process in the lipid domain of the phospholipid bilayer of cell membranes. There are two possibilities for the initial homolysis of the −O−O− bond: the classical one, where Fe (II) or Cu(I) directly on molecular collision produce the reaction and a second one, in which Fe(II) and Cu(I) bound to peptide or protein sites are then able to react with H_2O_2 and ROOH to generate HO· and RO·. These two free radicals are capable of initiating harmful cellular processes as phospholipid peroxidation in the bilayers of cell membranes and protein oxidation in the aqueous cytosol. In the cytosol, the free radicals HO· and RO· react immediately, in the next molecular collision, oxidizing neighbor amino acids with subsequent protein cross-linking, fragmentation and denaturation [1]. This molecular mechanism has been considered to describe β-amyloid protein oxidation in Alzheimer's disease [2].

In humans, the chronic overload of blood Fe occurs in the prolonged treatment of anemia and in hereditary hemochromatosis, a genetic disorder that affects Fe metabolism, with a prevalence of 0.3% [3]. There is also a secondary hemochromatosis, where Fe overload is caused by repeated blood transfusions [4,5], severe hemolysis or an excess of Fe in the diet.

The best-known example of chronic Cu toxicity is Wilson's disease, a rare autosomal recessive disease, with an incidence of 1:30000 to 1:100000. The genetic alteration takes place in the adenosine triphosphatase (ATPase) ATP7B pump, responsible for the excretion of the metal from hepatocytes to bile. Dysfunction or absence of ATP7B leads to an increase in the intracellular Cu content in hepatocytes. The

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subsequent events due to Cu overload seem to involve mitochondria. Different mechanisms have been proposed for this effect, among them the opening of the membrane transition pore that leads to cell death [6]. Although liver is the primary storage organ involved, the Cu overload ends up overcoming the organ storage capacity and excess metal passes into the bloodstream, from where it is able to reach brain and other organs. The signs and symptoms of Wilson's disease include liver disorders, as chronic hepatitis and cirrhosis, that end in liver failure and neurological alterations, such as Parkinsonism, seizures and psychiatric symptoms as personality changes, depression and psychosis. The Kayser-Fleischer sign is frequently observed as a brown-gold ring in the periphery of the cornea caused by Cu deposits.

The results reported in this study show that the chronic overloads of Fe(II) and Cu(II) in rats for 42 days lead to a liver redox dyshomeostasis with an enhancement of oxidative processes and an impaired antioxidant defense.

2. Material and methods

2.1. Experimental model of Fe and Cu chronic overloads

Male Sprague-Dawley rats of 80–90 g were fed with a commercial rodent diet of 2780 kcal/100 g. Experimental animals received for 42 days the standard diet *ad libitum* and drinking water with either 1.0 g/L of $FeCl_2$ or 0.5 g/L $CuSO_4$. The control group received the same diet and drinking water without metals. 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). The experimental protocol was approved by the Committee for the Care and Use of Laboratory Animals of the Faculty of Pharmacy and Biochemistry, University of Buenos Aires (Res. D 3685/16). Rats were obtained at the Central Animal House of the Faculty of Pharmacy and Biochemistry and were acclimatized to laboratory conditions for 7 days before the experiments. The animals were maintained under controlled conditions of temperature (23–25 °C) and humidity (50%) with an alternating 12 h light–dark cycle. Rats were sacrificed at the indicated times during the 42 days of treatment.

2.2. Liver metal content

The Fe and Cu contents in liver were determined by atomic absorption. After acid mineralization, samples of about 100 mg were incinerated for 4 h in a graphite muffle at 500 °C until the disappearance of brown coloured vapours. Standard solutions, 0 to 3 mg/L were used for calibration. The determination was made in an atomic absorption spectrometer (Bück model 200A, East Norwalk, CT, USA) and results were expressed in μg of metal/g of wet liver [4,5].

2.3. Liver chemiluminescence

Liver chemiluminescence *in vivo*, the sensitive and organ non-invasive assay for free-radical reactions, was determined following previously used protocols [7–9]. The photon counter used to measure liver photon emission was designed and constructed by Britton Chance at the Johnson Research Foundation of the University of Pennsylvania (Philadelphia, PA, USA) [7]. Liver chemiluminescence was expressed in counts per second per cm^2 (cps/cm^2) of exposed liver surface. The abdomen of 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 was cut to allow liver surface exposure to the detector. Liver surface chemiluminescence was measured after stable photoemission readings (usually 2–3 min). Liver chemiluminescence was recorded for 2–3 min. After that, liver was excised for the *ex vivo* determinations of oxidative damage.

2.4. Liver homogenate preparation

After chemiluminescence determination, rat liver was rapidly excised, weighed and cut into small pieces of 2–3 mm that were washed with ice cold saline solution. The homogenate was prepared in 120 mM KCl, 30 mM phosphate buffer, pH 7.40, at a ratio of 9 mL solution/g of tissue at 0 °C. The whole procedure was performed in an ice bath. The suspension was passed through a Potter-Elvehjem homogenizer and then centrifuged (Sorvall RC-2B, rotor SS34, 2500 rpm) at 600*g* for 10 min and at 0–1 °C. The supernatant, constituted by a suspension of organelles and cell cytosol, was considered as liver homogenate and was stored at −80 °C. The pellet, constituted by cell debris, was discarded [10–12]. For the determinations of reduced glutathione (GSH) content and reduced/oxidized glutathione ratio (GSH/GSSG), small pieces of 1–2 mm of liver were added with 0.5 M $HClO_4$ at a ratio of 50 mL $HClO_4$/g of liver. The suspension was passed through a Potter-Elvehjem homogenizer and centrifuged at 600*g* for 10 min. The pellet was discarded and the supernatant stored at −80 °C for glutathione determinations. The whole procedure was carried out at 0–1 °C.

2.5. Phospholipid peroxidation

Measurements were performed by the determination of thiobarbituric acid reactive substances (TBARS): 1.0 mL of liver homogenate prepared as described was added with 0.1 mL 4% w/v butylhydroxytoluene in ethanol and the samples were deproteinized by addition of 1 mL 20% w/v trichloroacetic acid and 10 min centrifugation in a table centrifuge. The supernatant was passed to a test tube and added with 1.0 mL of 0.7% w/v thiobarbituric acid. The tubes were vortexed and heated in a boiling water bath for 40 min. The absorbance of the pink solution was measured at 535 nm ($\varepsilon = 156\ mM^{-1}\ cm^{-1}$) and the results were expressed as nmol TBARS/g of liver [13].

2.6. Protein oxidation

Protein oxidation was measured by the product of the conjugation of protein carbonyl groups (> CO) with 2,4-dinitrophenylhydrazine (DNPH) spectrophotometrically at 360 nm. First, 1 mL of tissue homogenate was added with 4 mL of 10 mM DNPH prepared in 2.5 M HCl. Control determinations without liver homogenates were performed. Tubes were incubated for 1 h at room temperature in the dark and vortexed every 15 min. Then, 5 mL of 20% trichloroacetic acid were added to each tube and left in an ice bucket for 10 min. After that, samples were centrifuged in a tabletop centrifuge for 5 min to obtain the protein precipitates. The supernatant was discarded and 4 mL of 10% trichloroacetic acid were added to the pellet which was mechanically broken with a glass rod. The suspension was centrifuged again for 5 min and the pellet was washed 3 times with 4 mL of a mixture of 1:1 ethanol-ethyl acetate to remove free DNPH and lipid contamination. Finally, the protein pellet was dissolved in 2 mL of 6 M guanidine and incubated for 10 min at 37 °C. The absorbance at 360 nm was determined in a spectrophotometer (Hitachi, model U-2000). A calibration curve was used using serum albumin (0.2–0.6 mg) dissolved in guanidine (0.4 mL) as standard [14].

2.7. Oxidative damage index

The index: [(TBARS-metal / TBARS-control) + (protein carbonyls-metal / protein carbonyl-control)] × 0.5 was calculated for each sample at the different time points for liver homogenates from metal-treated and control rats [15,16].

2.8. GSH and the GSH/GSSG ratio

The content of GSH was determined by its reaction with the Ellman's Reagent, the 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) forming

2-nitro-5-thiobenzoate (TNB) which is a yellow-coloured compound that absorbs at 412 nm [17]. Homogenate samples (1 mL) were added with 1 mL 0.5 M perchloric acid and neutralized with 2 M KOH. GSH was measured by its reaction with 70 μM DTNB ($\varepsilon_{412} = 13.5\ mM^{-1}\ cm^{-1}$) in 100 mM phosphate buffer (pH 7.20). GSSG was measured by the nicotinamide adenine dinucleotide phosphate reduced form

(NADPH) consumed in its reduction by 0.2 mM NADPH and 0.2 U/mL glutathione reductase. Liver GSH content was expressed in mM, considering 1.0 g of liver as 0.8 mL.

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.

3. Results

3.1. Animal survival

The survival of the experimental rats was followed for 42 days, time at what survival was 98% upon Fe overload and 72% upon Cu overload (Fig. 1).

3.2. Animal growth

Rats were daily weighted during the experimental period, in which the animals showed a continuous and linear weight increase. At the end of the experimental period of 42 days rat weights were as follows: control rats 376 ± 4 g; rats with Fe overload 360 ± 3 g; and rats with Cu overload 328 ± 3 g. Fe-overloaded rats showed a slightly decreased growth rate which was more evident in Cu-overloaded animals.

3.3. Liver content of Fe and Cu

The chronic treatment with Fe and Cu led to a hyperbolic increase in their liver contents. At the end of the treatment, liver Fe was increased by 87% from the initial level of 107 ± 8 μg Fe/g liver and liver Cu showed a significant 450% increase from the initial 7.5 ± 0.3 μg Cu/g (Fig. 2). A linear correlation was found between liver metal contents and treatment time in the period of 2 to 42 days ($r = 0.88$ and 0.98 for Fe and Cu, respectively, $p < 0.05$).

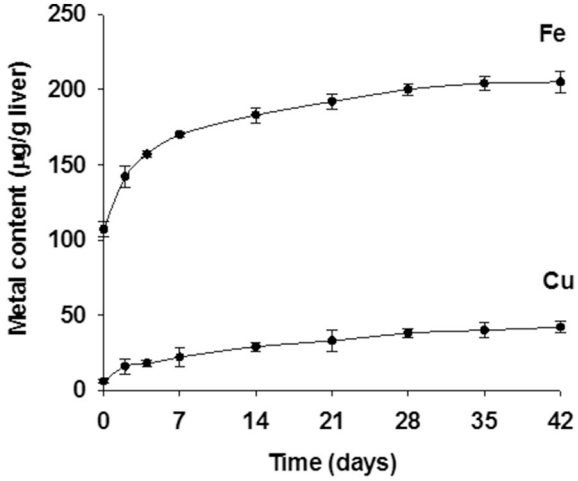

Fig. 2. Liver contents of Fe and Cu during the chronic administration of either Fe(II) or Cu(II) in the drinking water for 42 days.

3.4. Liver oxidative stress and damage

The situation of oxidative stress and oxidative damage in rat liver was established by the enhancements of liver chemiluminescence, phospholipid peroxidation, and protein oxidation (Figs. 3–5) [18,19] and by the decreased GSH content and GSH/GSSG ratio (see below).

3.4.1. Liver chemiluminescence

In vivo liver surface chemiluminescence in Fe- and Cu-overloads showed significant increases of organ light emission from the initial value of 12 ± 1 cps/cm^2; Fe-induced increment was of 108% and Cu-induced increment was of 91%. A plateau in liver chemiluminescence was reached at day 7 both for Fe and Cu (Fig. 3). The determinations of liver metal contents and of chemiluminescence (Figs. 2 and 3) provided robust data with similar kinetic behavior during the treatments with both metals.

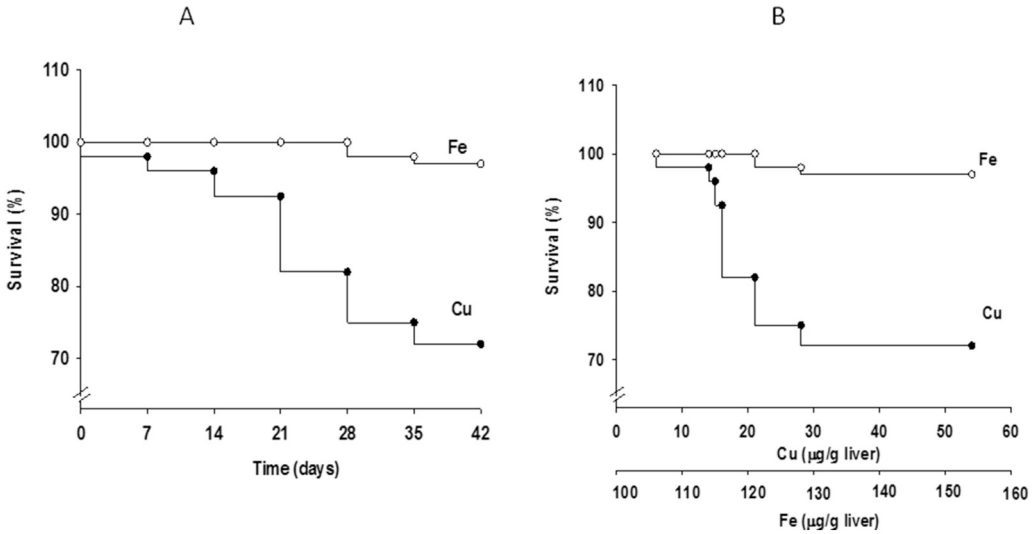

Fig. 1. Rat survival during the chronic administration of either $FeCl_2$ or $CuSO_4$, expressed as function of the time of treatment (A) and in relation to the liver Fe and Cu contents (B).

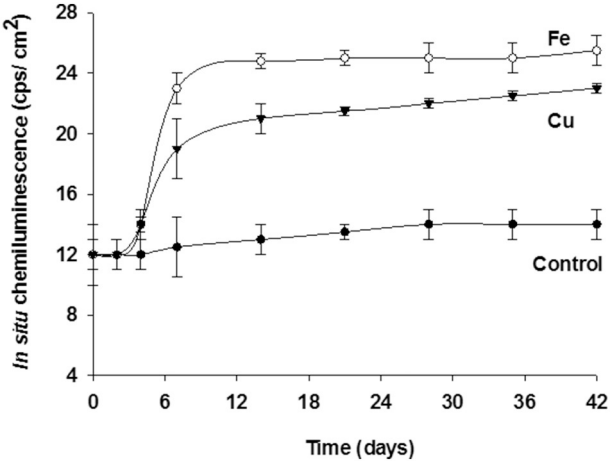

Fig. 3. Surface chemiluminescence of rat liver in the chronic overloads with Fe (II) and Cu(II).

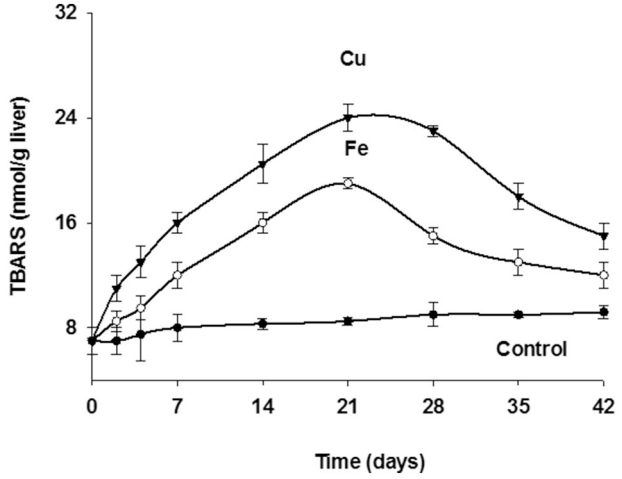

Fig. 4. The process of phospholipid peroxidation determined as TBARS in the chronic overloads with either Fe or Cu.

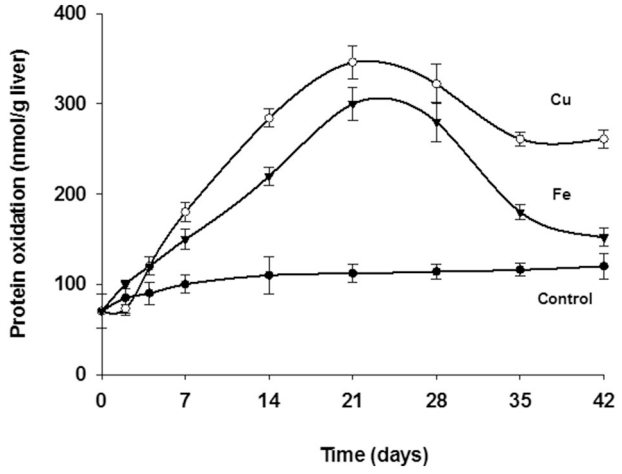

Fig. 5. Liver protein oxidation measured as carbonyl groups (> C=O) in the chronic overload with either Fe or Cu.

3.4.2. Liver phospholipid peroxidation

TBARS, the products of phospholipid peroxidation were significantly increased in the chronic overloads with Fe and Cu. The whole increment was biphasic, reaching maximal levels at day 21 (Fig. 4), with maximal increases of 132% for Fe overload and of 286% for Cu overload (both, $p < 0.01$). The increases of phospholipid peroxidation products in liver homogenates correlated with liver chemiluminescence until day 21. After that, phospholipid peroxidation products decreased to nearly control levels (7 ± 1 nmol TBARS/g liver), likely by metabolic utilization of the phospholipid peroxidation products or by synthesis of new phospholipids.

3.4.3. Liver protein oxidation

A biphasic process was also observed in the chronic overloads with Fe and Cu with a maximal content of carbonyl groups at day 21, with a kinetics that resembles the one of phospholipid peroxidation. Both determinations indicate that the level of oxidative free radicals in the organ had a burst about day 21. The initial level of oxidized proteins was increased at the points of maximal effect by 178% after Fe overload and by 374% after Cu overload (Fig. 5).

3.4.4. Oxidative damage index in liver homogenates

The index of oxidative damage was calculated by combining two indicators: TBARS and protein carbonyls [15,16]. Oxidative damage became evident after the second day to the end of treatment, showing a maximum at day 21 and was greater in Cu overload than in Fe supplementation (Fig. 6).

3.5. Antioxidant protection. GSH content and the GSH/GSSG ratio

3.5.1. Liver GSH content

The chronic treatment with Fe led to a marked decrease in liver GSH content to 30% at days 7–14 from the initial value of 6.3 ± 0.2 mM GSH ($p < 0.01$) with a recovery to nearly normal levels at days 14–28. Interestingly, during Cu overload only a small decrease of 17% without functional significance was observed in GSH content (Fig. 7).

3.5.2. Liver GSH/GSSG ratio

The GSH/GSSG ratio was a more sensitive indicator of oxidative stress and damage than GSH content. In both metal overloads this parameter was deeply decreased, reaching a plateau at days 2–7. In both cases of metal intoxication a marked decrease of about 40% of the initial value was observed. The GSH/GSSG ratio remained below control values (49 ± 1) during the 42 days of the treatment with both metals (Fig. 8).

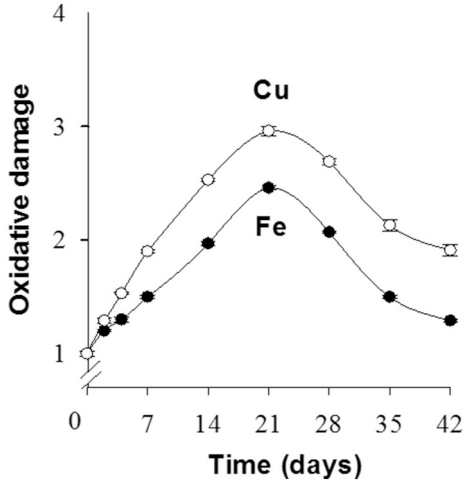

Fig. 6. Oxidative damage index in rat liver in the chronic treatments with either Fe or Cu overloads.

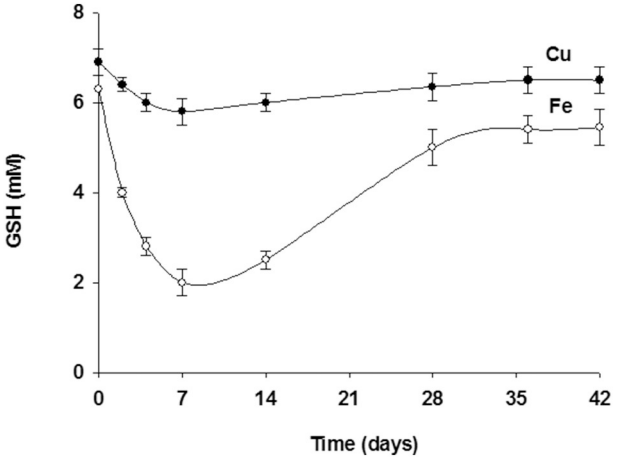

Fig. 7. Rat liver GSH concentration in the chronic overloads with either Fe or Cu.

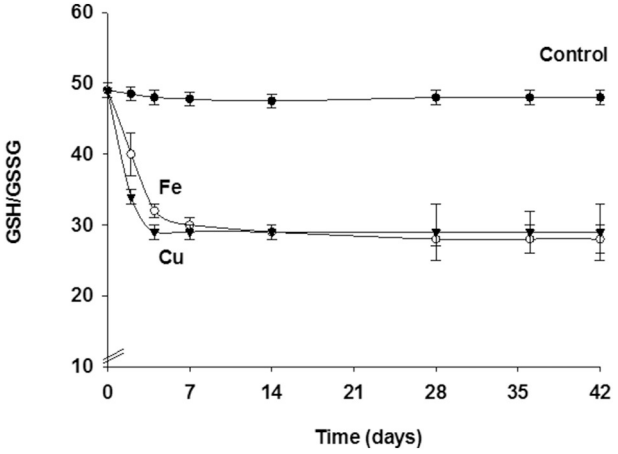

Fig. 8. GSH/GSSG ratio in rat liver during the chronic overloads with either Fe or Cu.

3.6. Kinetic analysis of the initial changes in the chronic overloads with Fe or Cu

A kinetic analysis of liver biochemical changes during the initial 10 days of the chronic overloads with Fe or Cu was performed (Fig. 9). This analysis was done by ordering the $t_{½}$ values, the time required to achieve one half of the maximal change for each determination from left to right, with the faster processes on the left.

The considered biochemical indicators were: liver surface chemiluminescence, phospholipid peroxidation, protein oxidation, GSH content and the GSH/GSSG ratio. The consequence of Fe and Cu overloads is well described as a shift towards a more oxidized cellular redox state. GSH content ($t_{½}$ of 2 days for both Fe and Cu) and the GSH/GSSG ratio ($t_{½}$ of 3–2 days for Fe and Cu, respectively) were the most sensitive and earliest indicators of the impaired redox homeostasis. This consideration highlights the defensive involvement of GSH in the metal overloads.

Liver chemiluminescence increases showed a $t_{½}$ of 5.3 days for both metals. The destructive process of phospholipid peroxidation showed $t_{½}$ of 9.5 and 7.0 days for Fe and Cu treatments, respectively. Protein oxidation, the second destructive process, showed $t_{½}$ of 10 and 8.8 days for Fe and Cu intoxications. The kinetics of the changes clearly indicates that GSH oxidation, *i.e.* the decrease of the main cellular antioxidant, occurs previously to the oxidative damage to phospholipids and proteins. In other words, biomolecule oxidation takes place after the impairment of the GSH-dependent antioxidant defense. However, in the chronic treatment with Cu, GSH content remained fairly constant. Decreased liver GSH level occurs with downstream effects on cellular metabolism and signaling, that are interpreted as regulatory actions that constitute a hepatocyte attempt to reestablish the normal composition of membranes and proteins.

4. Discussion

In this study, young rats were treated with either Fe(II) or Cu(II) ions in the drinking water for 42 days, a period that is roughly equivalent to 6 years of human life, considering that one week of rat life corresponds to about one year of human life.

The chronic treatment of rats with overloads of either Fe or Cu reproduced the liver oxidative phenomena that were observed in the acute (one single dose) overload with the same metals [18,19]. The similarity entangled both the qualitative (increase/decrease) and the quantitative aspects of the changes. Both, acute and chronic treatments significantly increased liver Fe and Cu contents, which obviously are the initial cause of metal toxicity. The chronic treatment produced increased Fe and Cu liver contents by 87% and 450%, respectively (Fig. 2). In the acute intoxication, observed 16 h after the overloads, Fe and Cu contents were increased by 40% and 750%, respectively. Although the quantitative changes in metal organ contents were slightly different in both treatments, they were remarkably similar in their biochemical effects. The situations of acute and chronic Fe or Cu overloads produced marked and significant increases in oxidative

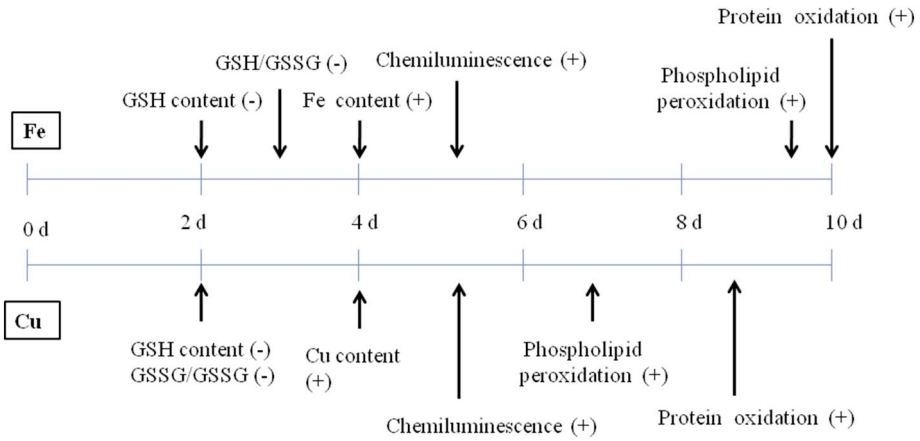

Fig. 9. Kinetic analysis of the liver biochemical changes during the initial 10 days of the chronic overloads with Fe or Cu. The arrows indicate the $t_{½}$, the time to reach one half of the changes in reactions and processes. Increases (+) and decreases (−) are indicated.

Table 1
Maximal percentage of change of intracellular redox indicators in rat liver in acute and chronic Fe and Cu overloads.

Redox indicator	Fe acute	Fe chronic	Cu acute	Cu chronic
Chemiluminescence	+208	+108	+102	+91
Phospholipid peroxidation	+192	+132	+108	+286
Protein oxidation	+60	+178	+40	+375
GSH content	–58	–70	–80	–17
GSH/GSSG ratio	–50	–60	–50	–44

processes as liver chemiluminescence, phospholipid peroxidation and protein oxidation and marked and significant decreases in GSH content and in the GSH/GSSG ratio. Altogether, the increases in oxidative processes constitute a fair indication of oxidative stress in Fe and Cu overloads where liver cells are unable to counteract the enhanced production of oxidant molecular species.

A comparison of the increased liver oxidative processes in acute [18,19] and chronic Fe and Cu overloads is given in Table 1.

According to the classic definition by Sies of oxidative stress, an increase in oxidative processes or a decrease in the antioxidant defense establish the condition of oxidative stress and damage [20]. In the present study both conditions are indeed met in rat liver that shows increased oxidative processes (Figs. 3, 4, and 5) and decreased antioxidant defense (Figs. 7 and 8) in a clear situation of liver oxidative stress and damage (Fig. 6).

Liver is the main organ involved in the physiology and metabolism of both metals. After metal absorption in the upper intestine, liver partially stores and partially eliminates the metals. The immediate liver response is to secrete the metals into the bile; however, in metal overloads this physiological mechanism does not totally prevents the increased liver content of the metals (Fig. 2).

The concept that oxidative free radical reactions afford the molecular mechanism of both Fe and Cu chronic liver toxicity is well supported by the observations of increased *in vivo* liver chemiluminescence (Fig. 3) and the *ex vivo* determined increments of phospholipid peroxidation and of protein oxidation (Figs. 4, 5 and 6) as well as by the decreased GSH content and GSH/GSSG ratio (Figs. 7 and 8).

Organ chemiluminescence is a physiological phenomenon with non-zero values (10–30 cps/cm^2, depending on the counter settings) that is a consequence of aerobic metabolism and endogenous phospholipid peroxidation [7–9]. Organ chemiluminescence has been determined *in vivo* in rat liver, brain, skeletal muscle and heart, and in perfused lung. Marked increments of 200–400% were observed in the organs of animals subjected to oxidative stress and damage. In the chronic overloads with Fe and Cu, liver chemiluminescence was significantly increased by 108% and by 91%, respectively (Fig. 3 and Table 1).

The accepted mechanism of organ photoemission is that it is mainly given by the red dimol emission of singlet oxygen ($^1O_2$) at 643 nm and at 714 nm, accompanied by a smaller blue emission at about 450 nm of excited carbonyl groups ($> CO_*$). The molecular mechanism is that two peroxyl radicals (ROO·) from the phospholipid peroxidation process produce the transient and unstable lipid peroxide, ROOOOR, intermediate that immediately cleaves yielding mainly $^1O_2$ and in a smaller proportion > CO*. The electronically excited states quickly returns to the basal state with the emission of a photon. The number of photons emitted depends on the steady-state concentrations of $^1O_2$, and of > CO* (the light emitting chemical species) and of the predecessor ROO· molecules formed in phospholipid peroxidation [7–9]. The reactions take place in the lipid phase of the phospholipid bilayer of intracellular cell membranes.

GSH is present at the high concentration of 4–8 mM in liver cells [21] and is considered the most important intracellular antioxidant in mammalian organs where it also participates in a vast series of defense mechanisms. The ratio GSH/GSSG, *i.e.* the reduced/oxidized ratio, establishes the cellular redox potential. Cellular GSH is an effective protection against oxidative stress, situation that is consequence of aerobic life. GSH has other important physiological functions as well, such as the detoxification of xenobiotics and the regulation of redox sensitive proteins [22]. The marked fall in liver GSH concentration, to about one fourth, in chronic Fe overload (Fig. 7) is quantitatively similar to the one observed in the acute Fe treatments [18]. Moreover, liver GSH is able to fully react with Cu, Se, Cr and Zn that are present in submicromolar concentrations by electrovalent bonding and in non-enzymatic reactions.

Increased liver contents of Fe and Cu are characteristic and diagnostic criteria for two human diseases: hemochromatosis and Wilson's disease. Concerning Fe, increases of about 100% in the hepatic content of Fe were reported in liver biopsies of patients with hemochromatosis [23–25]. Concerning Cu, the Cu liver content of biopsies is the diagnostic criterion for Wilson's disease. It has been established that values > 250 μg Cu/g liver dry weight (an increase of 4.5 times respect to the normal liver Cu content of 55 μg Cu/g liver dry weight) indicate the existence of Wilson's disease [26,27].

Animal studies, as the present one on the chronic overloads of Fe and Cu, could be useful in the understanding of the biochemical mechanisms of the toxicity of both metals. The observations and interpretations could be extrapolated to human patients in order to develop therapeutic actions in hemochromatosis and in Wilson's disease, cases in which liver accumulation of either Fe or Cu in human liver leads to situations of oxidative stress and damage. For instance, it is likely that the therapeutic intervention of supplementation with exogenous antioxidants, as vitamin E and ascorbic acid, would improve patient health.

5. Conclusion

The chronic overload of rats with either Fe(II) or Cu(II) increases the liver content of the metals, a fact that establishes a situation of oxidative stress and damage with augmented oxidative free radical reactions. There are marked increases in the *in vivo* liver chemiluminescence and in the *ex-vivo* measured products of phospholipid peroxidation and of protein oxidation. Simultaneously, there are marked decreases of liver GSH content and of the GSH/GSSG ratio. The hepatic contents of Fe and Cu found in rats in chronic experiments are quantitatively similar to the ones found in human hemochromatosis and in Wilson's disease, respectively.

Authors' statement

All authors have made a significant contribution to the paper: Rosario Musacco Sebio, Nidia Ferrarotti and Christian Saporito Magriñá have performed the determinations of chemiluminescence, phospholipid and protein oxidation, have participated in the acquisition of data, performed oxidative damage and protein content determinations and in the final approval of the version to be submitted. Julián Fuda and Horacio Torti have performed metal determinations in liver by atomic absorption method and in the final approval of the version to be submitted. Alberto Boveris, Fabiana Lairión and Marisa G. Repetto have participated in the conception and design of the study, analysis and interpretation of data, drafting the article and leading it to the final version.

Abbreviations

> CO	carbonyl groups
$> CO_*$	excited carbonyl groups
DTNB	5,5′-dithio-bis-(2-nitrobenzoic acid) (Ellman's Reagent)
GSH	reduced glutathione
GSSG	oxidized glutathione
H_2O_2	hydrogen peroxide
NADPH	nicotinamide adenine dinucleotide phosphate reduced form
OH·	hydroxyl radical

$^1O_2$	singlet oxygen, the excited state of O_2
ROOH	organic peroxide
ROO·	peroxyl radical
ROOOOR	lipid peroxide
TBARS	thiobarbituric acid reactive substances
TNB	2-nitro-5-thiobenzoate

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The authors declare that they do not have conflict of interests.

References

[1] D. Waggoner, T. Bartnikas, J. Gitlin, Neurobiol. Dis. 6 (1999) 221–230.
[2] H. Kozlowski, A. Janicka-Klos, J. Brasun, E. Gaggelli, D. Valensin, G. Valensin, Chem. Rev. 253 (2009) 2665–2685.
[3] R.C. Hider, X. Kong, Iron: effect of overload and deficiency, in: A. Sigel, H. Sigel, R.K.O. Sigel (Eds.), Metal Ions in Life Sci, de Gruyter, Berlin, 2013, pp. 229–294.
[4] D. Skoog, D. West, F. Holler, Fundamentos de Química Analítica, McGraw-Hill, Mexico, 2014.
[5] J. Callaghan, S. Gutman, Clin. Chem. 52 (2006) 1256–1257.
[6] G. Krumschnabel, C. Manzl, C. Berger, B. Hofer, Toxicol. Appl. Pharmacol. 209 (2005) 62–73.
[7] A. Boveris, E. Cadenas, R. Reiter, M. Filipkowski, Y. Nakase, B. Chance, Proc. Natl. Acad. Sci. U. S. A. 177 (1980) 347–351.
[8] E. Cadenas, H. Sies, Methods Enzymol. 105 (1984) 211–230.
[9] J.C. Cutrin, A. Boveris, B. Zingaro, G. Corvetti, G. Poli, Hepatology 31 (2000) 622–632.
[10] B. González Flecha, S. Llesuy, A. Boveris, Free Radic. Biol. Med. 10 (1991) 93–100.
[11] A. Boveris, N. Oshino, B. Chance, Biochem. J. 128 (1972) 617–627.
[12] M.G. Repetto, G. Ossani, A.J. Monserrat, A. Boveris, Exp. Mol. Pathol. 88 (2010) 143–149.
[13] C. Fraga, B. Leibovitz, A.L. Tappel, Free Radic. Biol. Med. 4 (1988) 155–161.
[14] A. Reznick, L. Packer, Methods Enzymol. 233 (1994) 357–363.
[15] A. Navarro, A. Boveris, Am. J. Phys. Cell Phys. 92 (2007) C670–C686.
[16] A. Boveris, R. Musacco-Sebio, N. Ferrarotti, C. Saporito-Magriñá, H. Torti, F. Massot, M.G. Repetto, J. Inorg. Biochem. 116 (2012) 63–69.
[17] T.P. Akerboom, H. Sies, Methods Enzymol. 77 (1981) 373–382.
[18] R. Musacco-Sebio, C. Saporito-Magriñá, J. Semprine, H. Torti, N. Ferrarotti, M. Castro-Parodi, A. Damiano, A. Boveris, M.G. Repetto, J. Inorg. Biochem. 137 (2014) 94–100.
[19] R. Musacco-Sebio, C. Saporito-Magriñá, J.M. Acosta, A. Boveris, M.G. Repetto, Liver Res. Open J. 2 (2017) 9–13.
[20] H. Sies, Am. J. Med. 91 (1991) 31S–38S.
[21] H. Forman, H. Zhang, A. Rinna, Mol. Asp. Med. 30 (2009) 1–12.
[22] N. Ballatori, S. Krance, S. Notenboom, S. Shi, K. Tieu, C. Hammond, Biol. Chem. 390 (2009) 191–214.
[23] K.L. Nuttall, J. Palaty, G. Lockitch, J, Ann. Clin. Lab. Sci. 33 (2003) 443–450.
[24] P.C. Adams, C. Bradley, A.R. Henderson, J. Lab. Clin. Med. 130 (1997) 509–514.
[25] B.R. Bacon, P.C. Adams, K.V. Kowdley, L.W. Powell, A.S. Tavill, Diagnosis and Management of Hemochromatosis: 2011 Practice Guideline, American, Association for the Study of Liver Diseases, Alexandria, Va, 2011.
[26] A. Ala, A.P. Walker, K. Ashkan, J.S. Dooley, M.L. Schilsky, Lancet 369 (2007) 397–408.
[27] K.I. Rodriguez-Castro, F.J. Hevia-Urrutia, G. Sturniolo, World J. Hepatol. 7 (2015) 2859–2870.