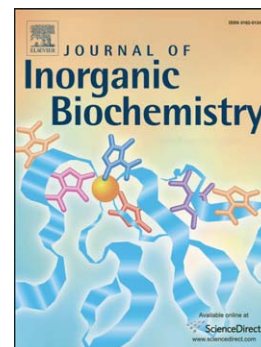# Accepted Manuscript

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RAT LIVER ANTIOXIDANT RESPONSE TO IRON AND COPPER OVERLOADS

Rosario Musacco-Sebio[a], Christian Saporito-Magriñá[a], Jimena Semprine[a], Horacio Torti[b], Nidia Ferrarotti[a,c], Mauricio Castro-Parodi[d], Alicia Damiano[d], Alberto Boveris[a] and Marisa G. Repetto[a*]

[a]Department of General and Inorganic Chemistry, [b]Department of Physics, [c]Laboratory of Clinical Immunology, Department of Clinical Biochemistry and [d]Department of Molecular Biology, School of Pharmacy and Biochemistry, University of Buenos Aires, C1113AAD Buenos Aires, Argentina.

Running title: Fe and Cu overloads and liver antioxidant response.

*Corresponding Author:

Prof. Dr. Marisa G. Repetto

Química General e Inorgánica

Facultad de Farmacia y Bioquímica

Junin 956

C1113AAD Buenos Aires, Argentina

Phone: 54-11-4964-8249

Fax: 54-11-4508-3648

E-mail: mrepetto@ffyb.uba.ar

Abstract

The rat liver antioxidant response to Fe and Cu overloads (0-60 mg/kg) was studied. Dose- and time-responses were determined and summarized by $t_{1/2}$ and C_{50}, the time and the liver metal content for half maximal oxidative responses. Liver GSH (reduced glutathione) and GSSG (glutathione disulfide) were determined. The GSH content and the GSH/GSSG ratio markedly decreased after Fe (58-66%) and Cu (79-81%) loads, with $t_{1/2}$ of 4.0 and 2.0 h. The C_{50} were in a similar range for all the indicators (110-124 μg Fe/g and 40-50 μg Cu/g) and suggest a unique free-radical mediated process. Hydrophilic antioxidants markedly decreased after Fe and Cu (60-75%; $t_{1/2}$: 4.5 and 4.0 h). Lipophilic antioxidants were also decreased (30-92%; $t_{1/2}$: 7.0 and 5.5 h after Fe and Cu. Superoxide dismutase (SOD) activities (Cu,Zn-SOD and Mn-SOD) and protein expression were adaptively increased after metal overloads (Cu,Zn-SOD: $t_{1/2}$: 8-8.5 h and Mn-SOD: $t_{1/2}$: 8.5-8.0 h. Catalase activity was increased after Fe (65%; $t_{1/2}$: 8.5 h) and decreased after Cu (26%; $t_{1/2}$: 8.0 h), whereas catalase expression was increased after Fe and decreased after Cu overloads. Glutathione peroxidase activity decreased after metal loads by 22-39% with a $t_{1/2}$ of 4.5 h and with unchanged protein expression. GSH is the main and fastest responder antioxidant in Fe and Cu overloads. The results indicate that thiol (-SH) content and antioxidant enzyme activities are central to the antioxidant defence in the oxidative stress and damage after Fe and Cu overloads.

1. Introduction

Oxidative stress was originally described with the concept of an unbalance between the production of oxidants and the antioxidant defenses in biological systems, such as cells, tissues or organisms [1]. Reactive oxygen species (ROS, which include the species of the partial reduction of O_2: $O_2^{\bullet-}$, H_2O_2 and $HO^{\bullet}$) are physiologically produced *in vivo* as by-products of oxidative metabolism and an increase in their rate of production defines the oxidative stress condition, which is frequently associated with pathological situations [1,2,3]. The key importance of superoxide anion ($O_2^{\bullet-}$) in free-radical mediated oxidations relays on its dismutation to H_2O_2 and on its ability to reduce sequestered ions Fe^{3+} and Cu^{2+} to Fe^{2+} and Cu^{+}, that are released. The reduced forms of both metals catalyze the homolysis of H_2O_2 and ROOH yielding $HO^{\bullet}$ and $RO^{\bullet}$ [4,5,6]. Production of $HO^{\bullet}$ from H_2O_2 and Fe^{2+} by the Fenton reaction has been considered for a long time as the likely rate-limiting step for physiological lipid peroxidation [2,5,6].

The "redox hypothesis" extends the classical concept of oxidative stress and proposes that oxidative stress occurs as a result of oxidative phenomena leading to disruption of redox signaling and control and to molecular damage. The steady state redox state of thiol groups (GSH (reduced glutathione) and other low molecular weight thiol proteins) are involved in cell signaling and regulation [7]. Reactive oxygen species (O_2^{-}, H_2O_2 and $HO^{\bullet}$) and the products of the oxidation of lipids or proteins (peroxides, aldehydes, quinones) would be responsible for the oxidation of thiol groups [7].

The two transition metals Fe and Cu are absolutely required for mammalian life. Humans have an RDI (Reference Daily Intake) of 10-15 mg Fe/day and 1-3 mg Cu/day. Higher intakes, such as > 25 mg Fe/day and > 10 mg Cu/day are toxic for humans [8]. Fe and Cu are accumulated in liver up to 50% and 10 times of their normal contents,

respectively [8]. The cell toxicity produced by Fe and Cu overloads encompasses multiple simultaneous chemical pathways, such as lipid peroxidation, protein oxidation, macromolecule inactivation and thiol pool oxidation. Fe^{3+}- and Cu^{2+}-promoted lipid peroxidation in the model system of phospholipid liposomes supplemented with H_2O_2 [6] and in rat liver [8], likely through $HO^{\bullet}$ and $RO^{\bullet}$ generation by homolysis of H_2O_2 and of endogenous organic hydroperoxides.

. Antioxidants are normal cell constituents whose function is to decrease the level of oxidative chemical species. Cellular antioxidants include the classic antioxidant enzymes: superoxide dismutases (Cu,Zn-SOD and Mn-SOD), catalase, glutathione peroxidase [2], and the thioredoxin system (thioredoxin reductase and thioredoxin) [9] and small molecules that are able to trap free-radicals and excited species, such as GSH, α-tocopherol and β-carotene. Small antioxidant molecules in the low micromolar range, reduce the extent of membrane phospholipid peroxidation and protein oxidation.

The aim of this work is to analyze the time course of the antioxidant response in rat liver after Fe and Cu overloads, a process that produces oxidative stress and oxidative damage.

2. Experimental methods

2.1. Experimental animal model

Sprague-Dawley male rats (50 days, weighting 200 ± 8 g) received ferrous chloride ($FeCl_2$, 0-60 mg/kg, intraperitoneal, n = 24) and cupric sulphate ($CuSO_4$, 0-30 mg/kg, ip, n = 24) with control rats receiving 0.9% NaCl and were assayed after 16 h of metal administration with a maximal and stable metal level in the liver [8]. In separate

experiments, to determine the time course of the assayed indicators, rats received a single dose of 30 mg $FeCl_2$ (n = 24) or 10 mg $CuSO_4$ (n = 18) and were sacrificed after 2 to 48 h. Rats were anesthetized with 15 % (w/v) urethane at 1.5 g/kg (ip). 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. 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 liver homogenate [10] that were frozen and thawed to disrupt mitochondrial membranes and to release mitochondrial contents.

2.3. In situ liver chemiluminescence

The whole procedure followed a previously used protocol [8,11]. 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 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) and photoemission was expressed as counts per second (cps/cm^2) of exposed liver surface.

2.4. Antioxidant content

2.4.1. Glutathione, glutathione disulfide and the glutathione ratio (GSH/GSSG)

Homogenate samples were treated with 2 M perchloric acid and the supernatant was neutralized with 2 M K_3PO_4. The reaction medium consists in 100 mM phosphate buffer (pH 7.20). GSH was determined by its reaction with 70 µM 5,5'-dithio-bis(2-nitrobenzoic acid (ε_{412}=13.5 $mM^{-1}cm^{-1}$), and GSSG was determined using 0.2 mM NADPH and 0.2 U/mL glutathione reductase as reductants [12].

2.4.2. Content of hydrophilic antioxidants

The homogenate hydrophilic antioxidant potential of liver homogenates was measured in a reaction medium with 20 mM 2,2- azobis (2- amidinopropane) (ABAP), 100 mM phosphate buffer (pH 7.40) and 40 µM luminol. The assay was made with 10 µL of homogenate that prevented light emission for a period proportional to the amount of antioxidants in the sample [13]. The system was calibrated with Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid), the hydrophilic analogue of vitamin E. Hydrophilic antioxidants are expressed in µmol Trolox/g of liver, or mM Trolox [13].

2.4.3. Content of lipophilic antioxidants

The *tert*-butylhydroperoxide (tBOOH) initiated chemiluminescence of tissue homogenates assays the tissue content of lipophylic antioxidants [10]. The level of chemiluminescence reflects, with an inverse relationship, the level of non-enzymatic lipophilic antioxidants (α−tocopherol and carotenoids). tBOOH-initiated chemiluminescence was determined in a Packard Tri-carb model 3355 liquid scintillation counter in the out-of-coincidence mode at 30 °C, in 120 mM KCl, 30 mM phosphate buffer (pH 7.40), 3 mM tert-butyl hydroperoxide and 0.1-0.2 mg protein/mL of liver homogenate. A maximal level of emission was reached after 15-20 min. The results obtained in cpm/g liver are expressed as α-tocopherol content, considering that 0.2 μM α-tocopherol inhibits chemiluminescence by 50% [10].

2.4.4. Antioxidant enzyme activity

SOD activity: Cu,Zn-SOD (SOD1) and Mn-SOD (SOD2) were determined by the inhibition of the autocatalytic adrenochrome formation at 480 nm [14]. Catalase activity was determined by the decrease in H_2O_2 absorption at 240 nm [15]. Glutathione peroxidase (GPx) activity (GPx2 and GPx4) was assayed by measuring the glutathione disulfide reduction mediated by NADPH oxidation at 340 nm [16].

2.4.5. Western Blot analysis for protein expression

Liver was homogenized in 10 mM triethanolamine, 250 mM sucrose, pH 7.60, with proteinase inhibitors (0.2 mM PMSF, 25 mg/mL p-aminobenzamidine, 20 mg/mL aprotinin, 10 mg/mL, leupeptin, 10 mg/mL pepstatin). Homogenate (0.1 mg protein) was loaded in 12.5% polyacrylamide gel using a discontinuous system and electrotransferred onto nitrocellulose membranes (Hybond ECL, Amersham Pharmacia Biotech). Samples were incubated overnight with the primary antibodies anti-SOD1, anti-SOD2 (1:1000), anti-catalase (1:1000) and anti-GPX2 and anti-GPX4 (1:1000) (from Sigma-Aldrich), and after that with a goat anti-rabbit immunoglobulin G (Jackson Immuno Research Laboratories, 1:20,000) conjugated to peroxidase and detected using the enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotech. Samples were incubated overnight with a monoclonal anti-β-actin antibody (Alpha Diagnostic International, 1:2000) and developed. The densitometry of the bands was quantified by the ImageJ 1.45s® software package [17]. Immunoblot analysis were run in triplicates and expressed by SOD1/β-actin, SOD2/β-actin, catalase/β-actin, GPX2/β-actin and GPX4/β-actin ratios.

2.5. Liver metal content

Liver metal content was measured in an atomic absorption spectrometer (Buck model 200 A, East Norwalk, CN) after 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 [8]. Results are expressed in μg metal/g liver.

2.6. Chemicals

tert-Butyl hydroperoxide was obtained from Aldrich Chemical Co (Milwaukee,WI), and other chemicals were purchased from Sigma-Aldrich Chemical Co (St Louis, MO).

2.7. Statistical Analysis

Data were analyzed using the Tukey-Kramer multiple comparison test, and significance was indicated by $p < 0.05$. Results indicate the mean value $\pm$ standard error (SEM).

3. Results

3.1. Liver antioxidants

3.1.1. Glutathione and the ratio GSH/GSSG

The liver GSH content decreased 58% after Fe load, with a $t_{1/2}$ of 4.0 h and with a C_{50} of 116 µg Fe/g liver, and 79% after Cu load, with a $t_{1/2}$ of 4.0 h and a C_{50} of 40 µg Cu/g liver (Fig. 1A). The ratio GSH/GSSG was the most sensitive indicator of oxidative stress with a $t_{1/2}$ of 2.0 h. Due to its mathematical nature, a change from the normal 97% GSH and 3 % GSSG (GSH/GSSG = 32.3) to 94% GSH and 6% GSSG (GSH/GSSG = 15.6) implies a 50% decrease in the ratio (Fig. 1A and Table 1).

3.1.2. Hydrophilic antioxidants

The pool of hydrophilic antioxidants (GSH, uric acid, ascorbic acid) showed marked decreases after Fe and Cu overloads, 60% and 75%, respectively (Fig. 1B) with a $t_{1/2}$ of 4.5 h for Fe and of 4.0 h for Cu, and with C_{50} of 118 µg Fe/g and 42 µg Cu/g (Table 1).

3.1.3. Lipophilic antioxidants

The pool of liver lipophilic antioxidants were decreased, 30% and 92%, after Fe and Cu; the $t_{1/2}$ were 7.0 h (Fe) and 5.5 h (Cu) and with C_{50} of 124 µg Fe/g liver and 50 µg Cu/g liver (Fig. 1C and Table 1). Metal accumulation correlated with lipophilic antioxidant consumption (r = 0.81 and 0.90 for Fe and Cu; p < 0.01).

3.1.4. Liver chemiluminescence and GSSG

In situ and *in vivo* liver chemiluminescence is the photon emission of the de-excitation of the electronically excited species derived from lipid peroxidation, that are singlet oxygen and excited carbonyl groups. Spectral analysis shows that singlet oxygen is responsible of the main part (> 90 %) of the normal liver photoemission [18,19]. The process is markedly increased after Fe and Cu overloads [8]. Significant correlations were found between liver chemiluminescence and GSSG content (r = 0.96 (Fe) and 0.97 (Cu)), same with GSH content (r = -0.88 (Fe); -0.64 (Cu)), and with the ratio GSH/GSSG (r = -0.99 (Fe); -0.86 (Cu), and with liver lipophilic antioxidant content (r = -0.99 (Fe) and -0.94 (Cu) Indicators of the same oxidative process are naturally correlated but this does not imply that, for instance, GSSG has any chemical role in photon emission.

3.2.1. *Antioxidant enzyme activities*

Cytosolic superoxide dismutase (Cu,Zn-SOD or SOD1) activity in liver increased, understood as an adaptive response, 57% and 127% after Fe and Cu overloads with $t_{1/2}$ of 8.0 h (Fe) and 8.5 h (Cu) and with C_{50} of 114 µg Fe/g liver and 42 µg Cu/g liver. Mitochondrial superoxide dismutase (Mn-SOD or SOD2) activity increased 45% and 125% after Fe and Cu overloads with $t_{1/2}$ of 8.5 h (Fe) and 8.0 h (Cu), and with a C_{50} of 118 µg Fe/g liver and 40 µg Cu/g liver (Fig. 2A and Table 1).

After Fe overload, catalase activity increased by 65% with a $t_{1/2}$ of 8.5 h and a C_{50} of 110 µg Fe/g liver. Catalase activity decreased 26% after Cu overload with a $t_{1/2}$ of 8.0 h and a C_{50} of 44 µg Cu/g liver (Fig. 2B and Table 1).

Liver glutathione peroxidase activity decreased 39% and 22% after Fe and Cu overloads with a $t_{1/2}$ of 4.5 h for both metals and C_{50} of 120 µg Fe/g liver and 48 µg Cu/g liver (Fig. 2C and Table 1).

3.2.2. Antioxidant enzyme expression

Immunoblots show the bands of 16 kDa and 25 kDa corresponding to Cu,Zn-SOD (SOD1) and Mn-SOD (SOD2) (Fig. 3A), the band of 60 kDa corresponding to catalase (Fig. 3B) and the bands of 22 kDa and 29 kDa corresponding to GPx2 and GPx4 (Fig. 3C). Densitometry of the immunoblots were plotted as SOD1/β-actin, SOD2/β-actin, catalase/β-actin and GPx2/β-actin, GPx4/β-actin ratios.

SOD1 protein expression increased 30% after 6 h of Fe overload. However, Cu treatment had no effect on SOD1 expression. Regarding SOD2, the protein expression increased 70% and 40% after 6 h of Fe and Cu overloads (Fig. 3A). Regarding catalase,

protein expression increased 30% and 60% after 6 h and 16 h of Fe overload. Nevertheless, the catalase expression decreased 70% after 6 h and 140% after 16 h of Cu overload (Fig. 3B). GPx2 protein expression did not change after Fe and Cu overloads. In the case of GPx4, the protein expression slightly increased 30% after Fe load while it was not modified by Cu load (Fig. 3C).

4. Discussion

Liver accumulation of Fe and Cu is an effective process, considering the linear part of the accumulation/dose ratio that is 7% and 20% for Fe and Cu [8]. It is well established that Fe and Cu overloads have toxic effects in mammalian organs [4,5,20]. A recent study [8] on Fe and Cu acute liver toxicity used a kinetic approach by determining $t_{1/2}$, the time required for half maximal oxidative response, and C_{50}, the metal tissue content for half maximal oxidative response. Both, Fe and Cu overloads show similar time courses with a $t_{1/2}$ of about 4.0-4.5 h for metal accumulation, *in situ* liver chemiluminescence, and homogenate phosphoplipid and protein oxidations, indicating simultaneous biochemical processes [8]. The same kinetic approach was used to show that spontaneous liver chemiluminescence precedes necrosis in vitamin E-deficient rats [21] and that lipoperoxidation occurs before kidney necrosis [22]; in other words, that biochemistry comes first than histology.

The same experimental model and the same kinetic approach that was used before to characterize the oxidative process is used in this study to describe the liver antioxidant response. The free-radical mediated oxidative process initiated by Fe and Cu includes various simultaneous intracellular biochemical pathways, which are instantaneous,

continuous and homogeneous. In the case of Fe and Cu overloads, the indicators (*in vivo*

liver chemiluminescence and homogenate phospholipid and protein oxidation products)

show similar C_{50} for Fe (110-118 μg/g) and Cu (42-54 μg/g), what indicates a single

process [8]. Liver GSH is rapidly oxidized responding to relatively small increases in Fe

and Cu organ contents (Fig. 1 and Table 1). The $t_{1/2}$ of the processes show that GSH

oxidation is the fastest initial event in the liver toxicity of both metals and that the ratio

GSH/GSSG is indeed the most sensitive indicator of the oxidative challenge after metal

overloads (Fig.4). Ratios GSH/GSSG of 30, 15 to 6, and less than 4 correspond to

physiological, oxidative stress and oxidative damage conditions [23].

Both redox forms determine the potential of the couple GSH/GSSG, according to E'

$= E°´ + RT \ln [GSH]^2/GSSG$ with $E°´= -220$ mV. Cellular GSH levels are physiologically

maintained by constant reduction of GSSG by glutathione reductase and NADPH reducing

equivalents [24]. However, in oxidative stress and damage, GSH is oxidized, deeply

changing the ratio GSH/GSSG, which is frequently considered as the redox potential of the

whole cell [25]. The calculated cellular redox potential for normal conditions is -340 mV,

and for maximal Fe and Cu effects, -170 mV, and -64 mV, respectively. The protective

roles of GSH against oxidative stress and damage are multiple: it scavenges $HO^•$, $RO^•$ in

the hydrophilic domain, is a cofactor of glutathione peroxidase and participates in the

regeneration of the reduced form of protein thiols [26-29].

Hydrophilic antioxidants is a concept based in the use of the ABAP assay to

determine tissue antioxidants [13], integrating in a functional basis the contents of GSH,

ascorbic acid, uric acids, and other water soluble antioxidants. Hydrophilic antioxidants

decreased in Fe and Cu loads (Fig. 4) in parallel to GSH, which is easily understood

because GSH largely accounts for hydrophilic antioxidants [13]. Similarly, hydrophobic antioxidants is another concept based in the use of tBOOH-initiated chemiluminescence [10]. The assay integrates the antioxidant function of α-tocopherol, β-carotene and other lipid soluble antioxidants. A marked decrease in hydrophobic antioxidants is observed after Fe and Cu overloads which is a direct consequence of the increased lipoperoxidation process. It is accepted that cellular antioxidants do not act independently and that they function in a co-operative way in the form of network or cascade, as initially indicated by Sen and Packer [30] and later by Crichton et al. [31]. A synergic effect of GSH and α-tocopherol in Fe-induced hepatotoxicity has been reported [26]. It has been also claimed that reduction of Fe^{3+} to Fe^{2+} and of Cu^{2+} to Cu^+ by GSH are major initiating events in liver metal-catalyzed oxidations [29].

It is apparent, considering the whole data, that both transition metals enhance the endogenous rate of free radical reactions through the generation of $HO^{\bullet}$ and $RO^{\bullet}$ by a Fenton-like homolytic scission of H_2O_2 and ROOH, that operates as the rate-limiting step of the process [2,5,27,28]. Considering the O-O bond homolysis, there are two points of view. The classical one is that the free ions Fe^{2+} and Cu^+ catalyze the reaction. The second ones, is that the binding of positively charged species Fe^{2+} and Cu^+ to the negatively-charged head groups of phospholipids or to a specific peptide or a protein site, favor the reaction with H_2O_2 generating $HO^{\bullet}$ that immediately oxidizes neighbor amino acids with cross-linking, fragmentation and denaturation [32,33].

There are two simultaneous responses of the antioxidant enzymes in liver after metal overloads. The first one, almost immediate, involves enzyme protein damage with loss of enzymatic activity, similar to non-enzymatic antioxidant consumption. The second one,

delayed, is the adaptive increase in the activity of antioxidant enzymes. The increase in SOD activities (Cu,Zn-SOD and Mn-SOD) is understood as an adaptive response, with increase of protein expression. In rat liver, about 85% of total SOD activity is Cu,Zn-SOD activity and the remaining 15% corresponds to Mn-SOD. Both enzymatic activities were similarly enhanced after Fe and Cu overloads. This adaptive response of SOD to an acute oxidative stress in a mammalian organ is to be remarked, but it is not infrequent. Indeed, liver and plasma increased SOD activity have been reported after Cu-mediated oxidative stress [34]. Acute Pb^{2+} intoxication enhance SOD activity in human blood cells [35]. In animals, increased SOD activities were reported in serum after Zn^{2+} load [36], after Cd^{2+} intoxication in rat liver [37-38] and after Zn^{2+} [39] and Mn^{2+} [40] loads in rat brain. Similarly, loads of Hg^{2+} in mouse brain [41], and of Pb^{2+} in rat aorta [42] increased SOD activity. Concerning the decrease, in the range of 25 to 70 %, of catalase after Cu overload and with glutathione peroxidase after both metals, it is understood that they are part of the general free-radical mediated process of protein inactivation.

Increased activity of the SOD in cytosol and mitochondria decreases the steady-states of $O_2^{\bullet-}$ and its reaction rates with nitric oxide (NO) to yield peroxynitrite ($ONOO^-$) and increases intracellular H_2O_2 steady state concentrations that are potentiated by the decreases in glutathione peroxidase activity and GSH content [43]. Increased cytosolic levels of Fe^{2+}, of Cu^+ and of H_2O_2 are central to the hypothesis that Fe and Cu toxicities are mediated by increased rates of $HO^\bullet$ and $RO^\bullet$ formation.

5. Conclusions

Increments in Fe and Cu liver contents enhance the rate of physiological free-radical reactions leading to oxidative stress and damage. The chemical mechanisms are similar for

Fe and Cu through formation of HO$^{\bullet}$ and RO$^{\bullet}$ by scission of H_2O_2 and ROOH. Liver antioxidant protection, mainly given by GSH, is highly effective in preventing organ oxidative damage. The observed results support the concept that thiol redox state is essential for cell homeostasis, involving antioxidant enzyme activity and expression. Reduced thiols prevent the oxidative stress and damage induced by Fe and Cu overloads. A pharmacological treatment for oxidative damage in Fe and Cu toxicity is possible using the GSH precursor N-acetylcysteine.

6. Abbreviations

ABAP = 2,2- azobis (2-amidinopropane)

PMSF = phenylmethylsulfonyl fluoride

Aknowledgements

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

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Table 1. Indicators of antioxidant content and of oxidative stress in rat liver after acute Fe

and Cu loads. $t_{1/2}$ and C_{50}, are the time and the metal content for half maximal responses.

Type/Indicator	$t_{1/2}$ - Fe (h)	C_{50} - Fe ($\mu g/g$)	$t_{1/2}$ - Cu (h)	C_{50} -Cu ($\mu g/g$)
Non enzymatic antioxidants				
GSH content	4.0	116	4.0	40
GSH/GSSG ratio	2.0	108	2.0	30
Hydrophilic antioxidants	4.5	118	4.0	42
Lipophilic antioxidants	7.0	124	5.5	50
Antioxidant enzyme activities				
Cu,Zn-SOD	8.0	114	8.5	42
Mn-SOD	8.5	118	8.0	40
Catalase	8.5	110	8.0	44
Glutathione peroxidase	4.5	120	4.5	48
Oxidative process				
Liver chemiluminescence	4.0	114	4.0	42

Legends to the figures

Fig.1. A. Glutathione (GSH). A1. Time course of liver GSH content after Fe and Cu overloads. Insert A2. Time course of the ratio GSH/GSSG after metal treatment. A3. C_{50} of liver GSH after Fe and Cu loads. Insert: A4. C_{50} of liver GSH/GSSG ratio in Fe and Cu loads. **B. Hydrophilic antioxidants.** B1. Time course of liver hydrophilic antioxidant content after Fe and Cu overloads. B2. Liver hydrophilic antioxidant content after Fe and Cu accumulation. **C. Lipophilic antioxidants.** C1. Time course of liver lipophilic antioxidant content after Fe and Cu loads. C2. Liver lipophilic antioxidant content after Fe and Cu accumulation.

Fig.2. A. Superoxide dismutase activity. A1. Time course of liver Cu,Zn-SOD and Mn-SOD activities after Fe and Cu loads. A2. Liver Cu,Zn-SOD and Mn-SOD activities in relation to Fe and Cu accumulation after acute metal loads. **B. Catalase activity.** B1. Time course of liver catalase after Fe and Cu overloads. B2. Liver catalase in relation to Fe and Cu accumulation. **C. Glutathione peroxidase activity**. C1. Time course of liver glutathione peroxidase activity after Fe and Cu loads. C2. Liver glutathione peroxidase activity in relation to Fe and Cu accumulation.

Fig. 3. Protein expression in rat liver after Fe and Cu overloads. A. Superoxide dismutase Cu,Zn-SOD (SOD1) and Mn-SOD (SOD2). B. Catalase. C. Glutathione peroxidase (GPx2 and GPx4).

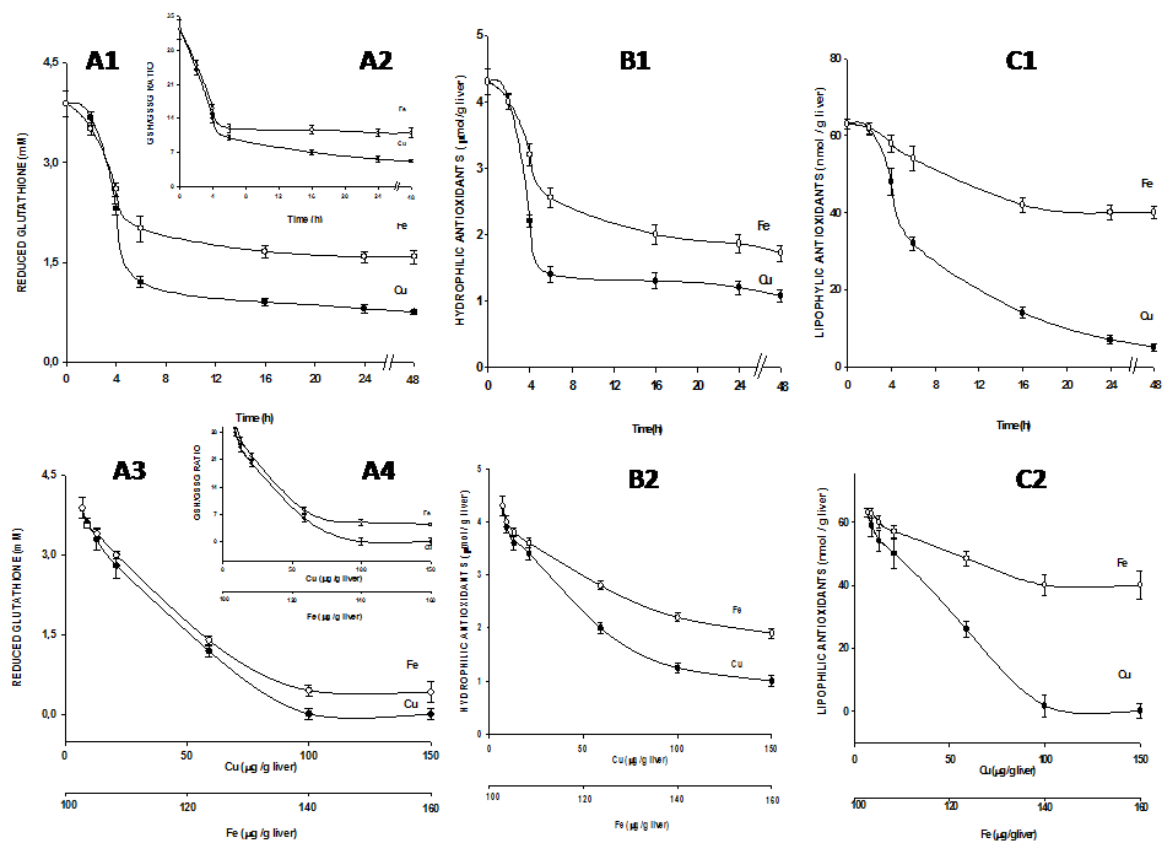**Fig.4.** Scheme of the time course of the rat liver antioxidant response to the oxidative challenge of Fe and Cu overloads. (-) and (+) indicate metal decreased or increased effect.
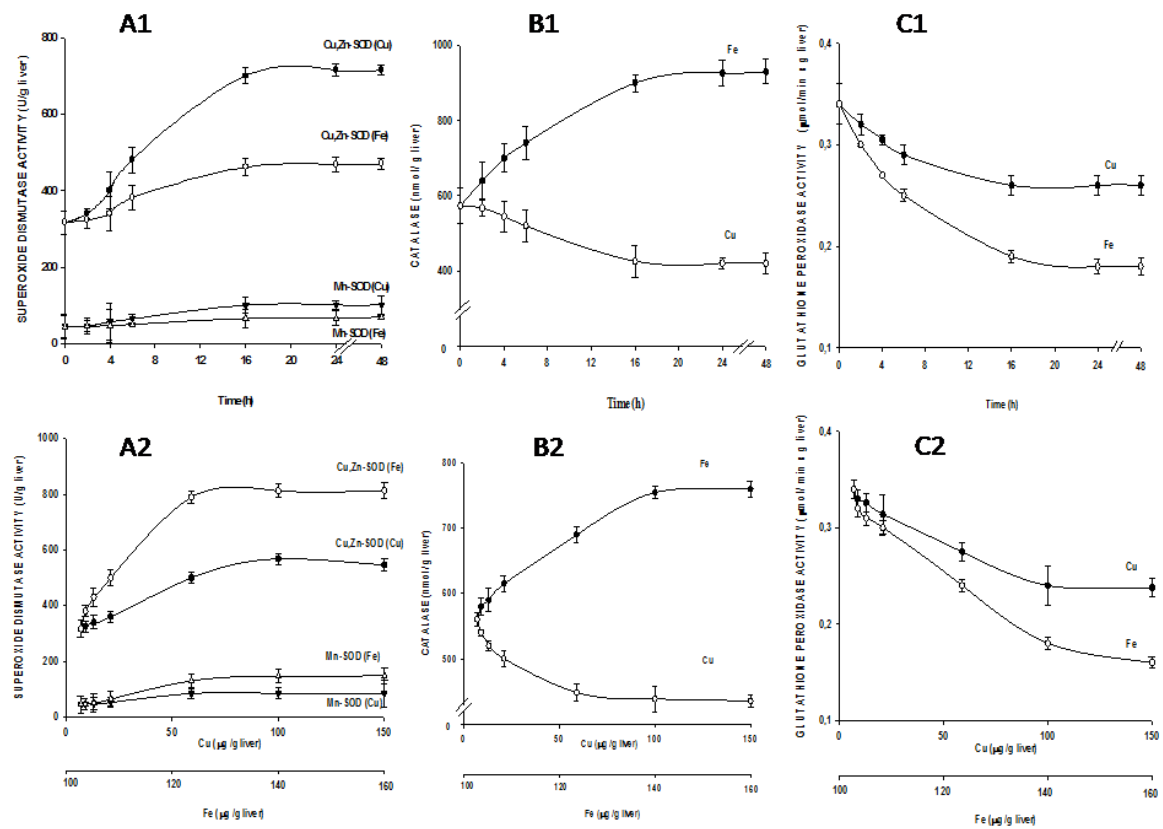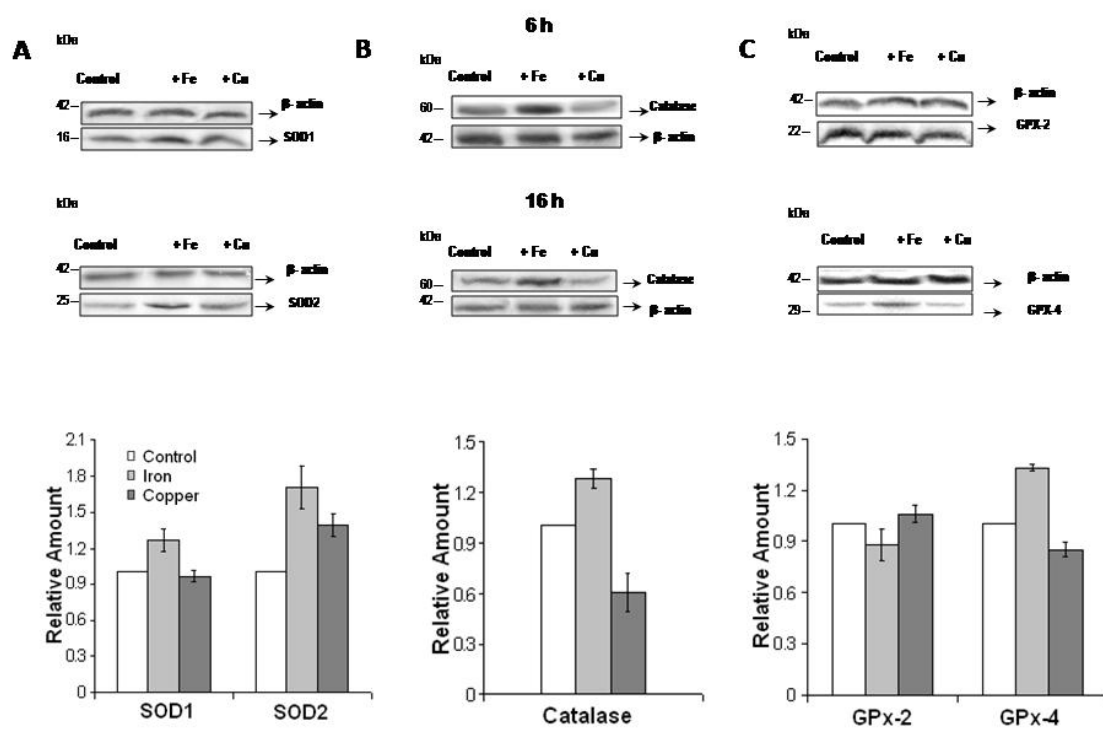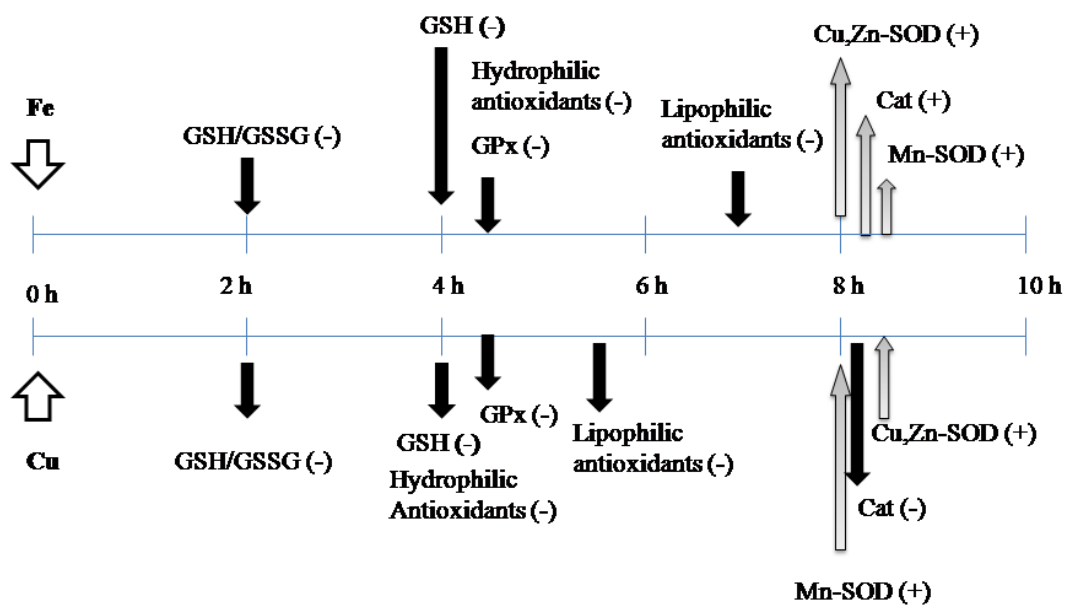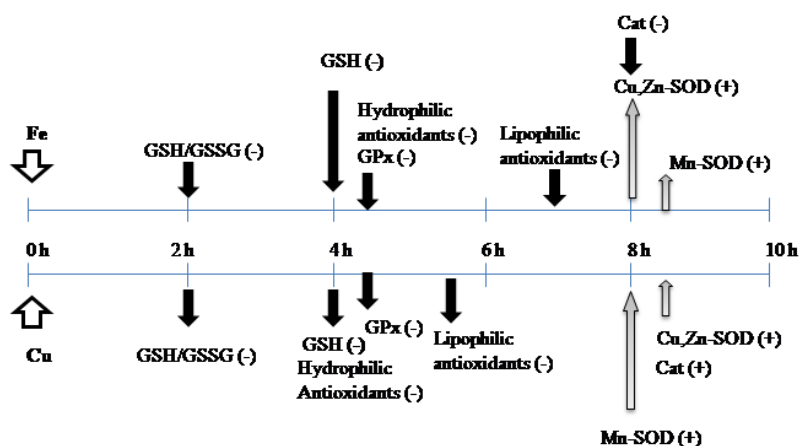
Figure 1

Figure 2

Figure 3

Figure 4

Graphical Abstract

The antioxidant protection in liver is highly affected after Fe and Cu acute overloads. GSH is the main and fastest-responder antioxidant. An adaptive response of increased expression and activity of SOD1, SOD2 and catalase follows to Fe and Cu overloads. Increased cytosolic levels of Fe^{2+} and Cu^+ and of H_2O_2 are central to the hypothesis that Fe and Cu toxicities are mediated by increased rates of $HO^{\bullet}$ and $RO^{\bullet}$ formation.

Highlights

- Liver antioxidant protection is highly affected in acute Fe and Cu overloads.
- Liver accumulation of 7% Fe and 20% Cu generate non enzymatic antioxidant consumption.
- GSH is the main and fastest-responder antioxidant.
- Fe and Cu trigger increased activities and protein expression of SOD and catalase.
- Increased levels of Fe^{2+}, Cu^+ and of H_2O_2 generate increased rates of $HO^{\bullet}$ formation.