This is an Accepted Manuscript, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this Accepted Manuscript with the edited and formatted Advance Article as soon as it is available.

You can find more information about Accepted Manuscripts in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal’s standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this Accepted Manuscript or any consequences arising from the use of any information it contains.
Graphical Abstract

There are two responses of the brain antioxidant system to the oxidative stress that follows to increased cytosolic levels of Fe and Cu. The first one is the consumption of cellular antioxidants (GSH and hydrophilic and lipophilic antioxidants) where GSH is the main and fastest-responder (left side of the figure). The second one is an adaptive increase in antioxidant enzyme activities with increased protein expression (right side of the figure).
Brain antioxidant response to iron and copper acute intoxications in rats

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

1 Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX
DOI: 10.1039/c0xx00000x

Dose- and time-dependent antioxidant responses to Fe (0-60 mg/kg) and Cu overloads (0-30 mg/kg) in rat brain are described by C50 and t1/2, the brain metal concentration and the time for half maximal oxidative responses. Brain GSH and the GSH/GSSG ratio markedly decreased after Fe and Cu treatments (50-80%) with a t1/2 of 9-10 h for GSH and of 4 h for GSH/GSSG for both metals. The GSH/GSSG ratio was the most sensitive indicator of brain oxidative stress. The decrease in GSH and the increase of in vivo chemiluminescence had similar time courses. The C50 for brain chemiluminescence, GSH and for hydrophilic and lipophilic antioxidants were in similar ranges (32-36 µg Fe/g brain and 10-18 µg Cu/g brain), which indicates a unique free-radical mediated process for each metal. The brain concentration of hydrophilic and lipophilic antioxidants decreased after Fe and Cu loads; hydrophilic antioxidants by 46-68% with a t1/2 of 10-11 h and lipophilic antioxidants by 75-45% with a t1/2 of 12-10 h. Cu,Zn-SOD and CAT activities and protein expression were adaptively increased (100-90%, after Fe and Cu loads), with t1/2 of 8-12 h. GPx-4 activity decreased after both metal loads by 73-27% with a t1/2 of 8-4 h with decreased protein expression.

Keywords: Iron; copper; oxidative stress and damage; brain chemiluminescence; glutathione; antioxidants;

Introduction

The transition metals iron (Fe) and copper (Cu) are absolutely required for mammalian aerobic brain metabolism. Fe and Cu are clearly hormetic1,2,3; humans have an RDI (Reference Daily Intake) of 10-15 mg Fe/day and 1-3 mg Cu/day, but higher intakes, > 25 mg Fe/day and > 10 mg Cu/day are definitively toxic4. In metal loads, Fe and Cu accumulate mainly in liver and secondarily in brain: 7% and 20% of the dose in the liver and 3.4% and 3.7% of the dose in the brain, for Fe and Cu, respectively4,5. The brain toxicity produced by Fe and Cu overloads encompasses multiple simultaneous chemical pathways involving oxidative reactions, most of them free-radical mediated reactions. Recent studies2-4 described rat liver and brain oxidative damages after an acute toxic load of Fe or Cu in kinetic terms considering the time (t1/2) and the organ metal concentration (C50) that produced half maximal oxidative effects. The following indicators were considering for the oxidative process: animal survival, in vivo brain and liver spontaneous chemiluminescence, and phospholipid and protein oxidation products in organ homogenates. The same kinetic approach, by determining t1/2 and C50, was used to analyze the liver antioxidant response of glutathione (GSH), and hydrophilic and lipophilic antioxidants in liver4 and is used here to determine the antioxidant response in rat brain.

The original and classical concept of oxidative stress was the idea of an unbalance between the production of oxidants and the antioxidant defenses in cells and tissues5. The concept was extremely successful and is currently applied to cells, tissues and organisms. The recognition of the role of highly reduced cellular thiols (–SH) in keeping normal metabolism extended the classical concept of oxidative stress to the disruption of the redox signaling and regulation due to a redox shift of ~SH to disulfide (~SS) groups6. The redox state of the ~SH groups in GSH, thioredoxin and other low molecular weight ~SH equilibrate with ~SH/~SS ratio in proteins and regulatory factors and is consequently deeply involved in cell signaling and regulation7. The current interpretation of cellular oxidative stress is that the condition implies an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and control8. It has been observed in rat liver that the increase in oxidants, free radicals and related species, and the shift in the ~SH/~SS redox couple occur simultaneously and have synergic effects8. The same process is described here for the brain, situation that if sustained leads to molecular and cellular damage and eventually to neuronal death.

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 (CAT), glutathione peroxidase9, and the thioredoxin system (NADPH, thioredoxin reductase and thioredoxin10). Small molecules, such as GSH, α-tocopherol and β-carotene11, are also constitutive part of the cellular antioxidant defense. These
antioxidant molecules in the mM level, as GSH, and in the µM range, as α-tocopherol and β-carotene, are able to reduce the rate of free radical mediated reactions and the extent of membrane phospholipid peroxidation and protein oxidation in vivo, in brain\textsuperscript{4} and heart\textsuperscript{10} homogenates.

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

1 Experimental animal model

The experimental animal model of Fe and Cu overloads was previously utilized\textsuperscript{2-4}. Male Sprague-Dawley rats (200 g) were purchased from the Central Animal House, School of Pharmacy and Biochemistry, University of Buenos Aires, and acclimatized under laboratory condition for 7 days before starting the experiment. Rats were provided with standard diet and water and maintained under standard conditions of temperature (23 °C - 25 °C) and humidity (50%) with an alternation of 12 h light/dark cycle. Rats received i.p. single doses of 30 mg FeCl\textsubscript{3} (n = 24) or 10 mg CuSO\textsubscript{4} (n = 18), 6 rats died during the experiment and were sacrificed at various times, 0 to 48 h, to determine the time course and sequence of the metal effects\textsuperscript{1}. In a separate experiment, to establish the relationship between metal concentration and effects, rats received i.p. different doses of FeCl\textsubscript{3} (0.03, 0.3, 3, 10, 30 and 60 mg Fe/kg, n = 24) or CuSO\textsubscript{4} (0.5, 1, 3, 7.5, 10 and 30 mg Cu/kg, n = 24) and were sacrificed after 16 h. Control rats (n = 24) received i.p. the same volumes of 0.9 % NaCl. Before sacrifice, 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 Brain metal concentrations

Brain metal concentrations were measured in an atomic absorption spectrometer (AAS method; Buck model 200 A, East Norwalk, CN) after samples were incinerated for 4 h in a graphite muffle at 500 °C. Calibration was made using standard solutions of 0.1 to 3 mg/L of Fe and Cu\textsuperscript{1}, obtained from Merck Company. The conditions for Fe determinations were: wavelength, 248.3 nm; slit, 0.2 nm; current: 1.5 mA, with acetylene-air flame (rich). For Cu determinations the conditions were: wavelength, 324.8 nm; slit 0.7 nm; Current: 1.0 mA with acetylene-air flame (lean). Samples were weighed on a Shimadzu balance AY220 with a sensibility of 0.1 mg and daily controlled with a Shimadzu standard weight of 100 g. Micropipettes used were weekly controlled by gravimetry. Laboratory analysis and standard solutions were used according to the ISO 17025 standards. The limits of detection for AAS metal determinations (DL) for Fe and Cu were 0.03 mg Fe/L and 0.025 mg Cu/L, and the coefficient of variations (CV) were 2% for both metals.

3 In vivo and in situ brain chemiluminescence

Brain chemiluminescence was recorded to follow simultaneously the oxidative process and the antioxidant response, considering that organ spontaneous chemiluminescence reports in real time the physiological level of \textsuperscript{5}O\textsubscript{2} and the rate of the whole lipid peroxidation and free-radical mediated process\textsuperscript{5}. Brain photoemission was determined 15 min after anesthesia with stable readings, usually after 5 min, following a previously used protocol\textsuperscript{1,11}. The head skin of the anesthetized rats was removed, parietal bones were cut out with a curved scissor, and the exposed brain was washed with 0.9 % NaCl. Brain was exposed and the animal was covered with aluminum foil, in which an about 2.0 cm\textsuperscript{2} window allowed brain cortex exposure. Brain 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 of exposed brain surface (cps/cm\textsuperscript{2}).

4 Brain homogenates

Brain homogenates were used in this study to follow the process of oxidative stress and damage in the tissue, based on the simultaneity of lipid peroxidation in all brain organelles and membranes\textsuperscript{2-4}9. After determination of brain chemiluminescence, the whole brain was rapidly excised, weighed and homogenized at a ratio of 1 g brain/9 mL of medium in 120 mM KCl, 30 mM phosphate buffer, pH 7.40, at 0 °C. The homogenates were centrifuged at 600 g for 10 min to discard nuclei and cell debris. The supernatant, a suspension of subcellular fractions, vesicles, organelles and endoplasmic reticulum membranes in diluted cytosol, was used as brain homogenate. The homogenates were frozen and thawed to disrupt mitochondrial membranes and to release mitochondrial components\textsuperscript{3,12}. Antioxidant concentrations and activities are expressed per g of wet brain to approach the physiological situation.

5 Antioxidant concentrations and enzymatic activities

5.1 GSH and the GSH/GSSG ratio

Brain homogenate samples were treated with 2 M perchloric acid and the supernatant was neutralized with 2 M K\textsubscript{2}PO\textsubscript{4}. The sample was diluted in 100 mM phosphate buffer (pH 7.20). GSH and oxidized glutathione (GSSG) were determined in a model U-2000 Milli-Q water, ultrapure water quality was obtained from a Osmon reverse osmosis system, according with the standard specifications for ASTM D-1193, grade water I (resistivity of 18.2 MOhms.cm at 25 °C). The chemicals used were of the highest quality and were obtained from Merck Company. The certified and determined values of Fe and Cu (Certified Reference Materials, CRM) were: for Fe, a standard solution of 0.5 M Fe(NO\textsubscript{3})\textsubscript{3} in nitric acid (HNO\textsubscript{3}) Certipur\textsuperscript{®} Merck (998 ± 2 mg/L of Fe, lot OC495679), and for Cu, a standard solution of 0.5 M Cu(NO\textsubscript{3})\textsubscript{2} in HNO\textsubscript{3} Certipur\textsuperscript{®} Merck (1001 ± 2 mg/L of Cu, lot OC467334). Hydrochloric acid was from Cicarelli Pro-analysis (ACS), lot 59916. Results are expressed in µg metal/g wet brain.
Hitachi UV-visible spectrophotometer, GSH concentration by its reaction with 70 μM 5,5'-dithio-bis(2-nitrobenzoic acid (ε412=13.5 mM⁻¹ cm⁻¹) and GSSG, using 0.2 mM NADPH and 0.2 U/mL glutathione reductase as reductants.  

5.2 Hydrophilic antioxidants
The total endogenous hydrophilic antioxidant potential of brain homogenates was determined in a Packard Tri-carb model 3355 liquid scintillation counter in the out-of-coincidence mode at 30 °C, by the ABAP assay with luminol as indicator of the reaction end. The reaction medium was 20 mM 2,2'-azobis (2-amidinopropane) (ABAP), 100 mM phosphate buffer (pH 7.40), 40 μM luminol and 10 μL of brain homogenate. In the assay, the endogenous hydrophilic and low-molecular weight antioxidants prevented the burst of luminol light emission for a time proportional to the amount of antioxidants in the sample. The system was calibrated with Trolox, the hydrophobic vitamin E analogue, and results are expressed as equivalents to µmol Trolox/g of brain or mM Trolox considering 1 g of brain as 1 mL of water.

5.3 Lipophilic antioxidants
The chemiluminescence of tissue homogenates was used to determine the tissue concentration of lipophilic antioxidants. The level of chemiluminescence in the assay reflects, with an inverse relationship, the concentration of non-enzymatic lipophilic antioxidants, mainly α-tocopherol and β-carotene, in the sample. 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), and 0.1-0.2 mg protein/mL of brain homogenate. Counting was followed until a maximal level of emission, usually after 15-20 min, was reached. The results of light emission inhibition are expressed as equivalent to α-tocopherol concentration in nmol/g brain, considering that 0.2 nmol α-tocopherol/g brain inhibits chemiluminescence by 50%.

5.4 Antioxidant enzyme activities
Antioxidant enzyme activities were determined with a Hitachi UV-visible spectrophotometer, model U-2000: SOD activity (Cu,Zn-SOD, SOD1) was determined by the inhibition of the autocatalytic adrenochrome formation at 480 nm; CAT activity was determined by the decrease in H₂O₂ absorption at 240 nm; and glutathione peroxidase activity (GPx-4) was measured at 340 nm by the NADPH-mediated glutathione disulfide reduction.

5.5 Western blot analysis for the protein expression of antioxidant enzymes
The Western blot analysis of brain homogenates for the protein expression of antioxidant enzymes was done as described before.

6 Chemicals

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

7 Statistical analysis
Data was analyzed using the Tukey-Kramer multiple comparison test. A p-value of less than 0.05 was considered to be statistically significant. Results indicate mean values ± standard errors (SEM). Graph Pad InStat software program was employed to statistically analyze the data.

Results

1 Brain antioxidants
1.1 GSH and the ratio GSH/GSSG. The brain concentration of GSH decreased 65% after Fe, with a t₁/₂ of 9 h and a C₅₀ of 33 μg Fe/g brain. After Cu, the decrease in brain GSH was 50% with a t₁/₂ of 10 h and a C₅₀ of 15 μg Cu/g brain (Fig. 1, Table 1). The same Fig. 1 includes the GSH/GSSG ratio, that markedly decreased 73% and 89% after Fe and Cu overloads, with a t₁/₂ of 4 h for both metals and a C₅₀ of 30 μg Fe/g brain and 5 μg Cu/g brain (Fig. 1 and Table 1).

Figure 1. A. Time course of brain GSH concentration after Fe and Cu overloads (Fe: ●, Cu: ▼), and time course of the ratio GSH/GSSG after metal treatment (Fe: △, Cu: ▽). B. Brain GSH concentration after Fe and Cu loads (Fe: ●, Cu: ●), and brain GSH/GSSG ratio in Fe and Cu loads (Fe: △, Cu: ▽).

The ratio GSH/GSSG was the most sensitive indicator of brain oxidative stress; it drastically decreased after metal loads, with t₁/₂ of 4 h (Fig. 1). Due to its mathematical nature, a slight change from the normal brain concentrations of 95-96% GSH and 4.5% GSSG (GSH/GSSG = 21.2) to 90% GSH and 10% GSSG (GSH/GSSG = 9) implies a 57% decrease in the ratio (Fig. 1 and Table 1).

1.2 Hydrophilic antioxidants in relation to the oxidative process reported by in vivo brain chemiluminescence. Brain hydrophilic antioxidants were determined as a pool, which in the assay conditions is constituted by GSH and ascorbic acid, and were 0.48 µmol Trolox equivalents/g brain, which considering 0.3 GSH = 1 Trolox²⁰, accounts for 1.6 μM GSH/g brain. The hydrophilic antioxidant concentration was severely decreased after Fe and Cu loads, 46% (Fe) and 68% (Cu), with t₁/₂ of 10 h.
for Fe and of 11 h for Cu, and with \( C_{50} \) of 35 \( \mu g \) Fe/g brain and 10 \( \mu g \) Cu/g brain (Fig. 2 and Table 1).

### Table 1. Rat brain antioxidant response to acute Fe and Cu overloads described by the \( t_{1/2} \) and \( C_{50} \), the time and the metal concentration for half maximal oxidative response.

<table>
<thead>
<tr>
<th>Type/Indicator</th>
<th>( t_{1/2} ) - Fe</th>
<th>( C_{50} ) - Fe</th>
<th>( t_{1/2} ) - Cu</th>
<th>( C_{50} ) - Cu</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemiluminescence</td>
<td>11</td>
<td>36</td>
<td>14</td>
<td>18</td>
</tr>
<tr>
<td>Non enzymatic antioxidants</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSH</td>
<td>9</td>
<td>33</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>GSH/GSSG ratio</td>
<td>4</td>
<td>30</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Lipophilic antioxidants</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-tocopherol</td>
<td>10</td>
<td>35</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>Lipophilic antioxidants</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-tocopherol</td>
<td>10</td>
<td>32</td>
<td>12</td>
<td>17</td>
</tr>
<tr>
<td>Antioxidant enzyme activities</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cu,ZnHSOD</td>
<td>10</td>
<td>49</td>
<td>12</td>
<td>20</td>
</tr>
<tr>
<td>CAT</td>
<td>8</td>
<td>40</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>GPx-4</td>
<td>8</td>
<td>30</td>
<td>4</td>
<td>15</td>
</tr>
<tr>
<td>The values of the Table 1 were calculated from mean values of the data in Figs. 1 to 6.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The oxidative process reported by in vivo brain chemiluminescence in relation to GSH and to the ratio GSH/GSSG. Fig. 2 includes the simultaneous in vivo brain chemiluminescence, which markedly increased (4 and 3 times) after Fe and Cu administration. In vivo brain chemiluminescence detects the formation of electronically excited species derived from phospholipid peroxidation, \(^{1} O_2 \) and excited \(^{1} CO^* \) groups, with \(^{1} O_2 \) responsible of the main part (> 90 %) of emission. The increase in in vivo brain chemiluminescence and the decrease in hydrophilic homogenate antioxidants, mainly GSH, showed similar \( t_{1/2} \) for both oxidative responses \((10 \pm 1 \) h). The \( C_{50} \) for the increase in brain chemiluminescence and the decrease in homogenate GSH were similar for both metals (30-36 \( \mu g \) Fe/g brain, and 10-18 \( \mu g \) Cu/g brain; Table 1). Significant correlations were found between brain chemiluminescence and the GSH concentration \((r = - 0.88, Fe; - 0.64, Cu)\); and the ratio GSH/GSSG \((r = - 0.99, Fe; - 0.86, Cu)\). The indicators of the same oxidative process are naturally correlated.

### 1.4 Lipophilic antioxidants. Brain lipophilic antioxidants were determined in a pool that in the assay conditions includes mainly \( \alpha \)-tocopherol and \( \beta \)-carotene. This pool of antioxidants decreased 75 % and 45 %, after Fe and Cu, with \( t_{1/2} \) of 10 h (Fe) and of 12 h (Cu) and with \( C_{50} \) of 32 \( \mu g \) Fe/g brain and 17 \( \mu g \) Cu/g brain (Fig. 3 and Table 1).

Figure 2. Hydrophilic antioxidants. A. Time course of brain hydrophilic antioxidant concentration after Fe and Cu loads (Fe: ●, Cu: ◀), and in vivo brain chemiluminescence after Fe and Cu treatment (Fe: △, Cu: ▽). B. Brain hydrophilic antioxidant concentration after Fe and Cu loads.

Figure 3. Lipophilic antioxidants. A. Time course of brain lipophilic antioxidant concentration after Fe and Cu loads. B. Brain lipophilic antioxidant concentration after Fe and Cu accumulation. Brain metal concentration and lipophilic antioxidant consumption showed correlations of \( r = 0.91 \) and 0.99 for Fe and Cu \((p < 0.01)\).

### 2 Antioxidant enzymes: superoxide dismutase, catalase and glutathione peroxidase

#### 2.1 Superoxide dismutase. Brain cytosolic superoxide dismutase (Cu,Zn-SOD) activity in increased 4 times and 90 % after Fe and Cu loads with \( t_{1/2} \) of 10 and 12 h for Fe and Cu, respectively (Fig. 4) and with \( C_{50} \) of 49 \( \mu g \) Fe/g brain and 20 \( \mu g \) Cu/g brain.

Figure 4. Superoxide dismutase activity. A. Time course of brain Cu,Zn-SOD activity after Fe and Cu loads. B. Brain Cu,Zn-SOD activity in relation to Fe and Cu accumulation after acute metal loads.
2.2 Catalase. Catalase activity (CAT) increased 2.7 times after Fe administration with a $t_{1/2}$ of 8 h and a $C_{50}$ of 40 µg Fe/g brain. Similarly, after Cu load, CAT activity increased by 90 % with a $t_{1/2}$ of 10 h and a $C_{50}$ of 10 µg Cu/g brain (Fig. 5 and Table 1).

![Figure 5. Catalase activity.](image)

A. Time course of brain CAT activity after Fe and Cu overloads. B. Brain CAT activities in relation to Fe and Cu concentrations.

2.3 Glutathione peroxidase. Brain glutathione peroxidase (GPxH4) activity decreased 73 % and 27 % after Fe and Cu, with $t_{1/2}$ of 8 h for Fe and 15 h for Cu and $C_{50}$ of 30 µg Fe/g brain and 15 µg Cu/g brain (Fig. 6 and Table 1).

![Figure 6. Glutathione peroxidase activity.](image)

A. Time course of brain glutathione peroxidase activity after Fe and Cu loads. B. Brain glutathione peroxidase activity in relation to Fe and Cu accumulation.

2.4 Antioxidant enzyme expression. Immunoblots show the bands of 16 kDa corresponding to Cu,Zn-SOD (SOD1) (Fig. 7A), the band of 60 kDa corresponding to CAT (Fig. 7B) and the band of 29 kDa corresponding to GPx-4 (Fig. 3C). The densitometry of the immunoblots was plotted as SOD1/β-actin, CAT/β-actin and GPx-4/β-actin ratios. SOD1 protein expression increased 100% after 16 h of Fe overload and 62% after 16 h of Cu treatment (Fig. 7A). Regarding CAT, protein expression increased 67% and 52% after 16 h of Fe and Cu loads, respectively (Fig. 7B). In the case of GPx-4, the protein expression decreased 62% after Fe load and 42% after Cu load (Fig. 7C).

![Figure 7. Protein expression in rat brain after Fe and Cu overloads.](image)

A. Cu,Zn-SOD (SOD1) superoxide dismutase. B. Catalase (CAT). C. Glutathione peroxidase (GPX-4). * Statistically highly significant compared to control; p < 0.01); ** Statistically significant compared to control; with p < 0.05.

Discussion

It is well established that Fe and Cu overloads have toxic effects in mammalian organs, especially in liver and brain where metal accumulation is higher. Recent studies on Fe and Cu acute liver and brain toxicity used the kinetic approach in order to define the number of oxidative processes and their sequence. This was done by determining $C_{50}$, the metal brain concentration for half maximal oxidative response, and $t_{1/2}$, the time required for half maximal oxidative response. The $C_{50}$ identified two single biochemical processes for the cases of Fe and Cu loads: The $t_{1/2}$, of the indicators after Fe and Cu, in each case identified a simultaneous process of oxidative reactions and of antioxidant consumption. This kinetic approach has been used to identify that in vivo liver chemiluminescence precedes necrosis in vitamin E-deficient rats and that phospholipid peroxidation occurs before kidney necrosis; in other words, that biochemistry precedes histology in time. The same experimental model used for Fe and Cu intoxication and the same kinetic approach utilized before is used here to describe the brain antioxidant response. The free-radical mediated oxidative-processes initiated by Fe and Cu start immediately after Fe and Cu increase their concentrations in the brain. The brain GSH concentration markedly decreased after Fe and Cu loads and revealed a marked brain oxidative stress process. In vivo brain chemiluminescence increased by 2-3 times after metal administration and organ in vivo photoemission and GSH homogenate oxidation showed similar $t_{1/2}$ (10 ± 1 h). Brain GSH is rapidly oxidized responding to relatively small increases in Fe and Cu organ concentrations. The ratio GSH/GSSG was the
most sensitive indicator of brain oxidative stress, with similar $t_1/2$ (4 h) for Fe and Cu. Ratios GSH/GSSG of 30 to 25, 15 to 6, and less than 4 have been assigned to physiological, oxidative stress and oxidative damage conditions\(^{25}\). The $C_{50}$ for the increase in brain chemiluminescence and for the decrease in homogenate GSH were also similar, 36-33 µg Fe/g brain and 18-15 µg Cu/g brain (Table 1).

The redox potential of the couple GSH/GSSG is determined by the concentration of both chemical species, according to $E^\circ = E^\circ + RT \ln [\text{GSH}] / [\text{GSSG}]$ with $E^\circ = -220$ mV. Cellular GSH levels are physiologically maintained by constant reduction of GSSG by NADPH and glutathione reductase\(^{26}\). However, in oxidative stress and damage, GSH is oxidized, deeply changing the ratio GSH/GSSG, which is frequently considered as the redox potential or the $\text{SH}/\text{SS}$-potential of the whole cell\(^{27}\). 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\(^2\) and RO\(^•\) in the hydrophilic domain, it is a cofactor of GPxs, it is part of the glutaredoxin system and participates in the regeneration of the reduced form of protein thiols\(^{28-30}\).

Hydrophilic antioxidants is a concept based in the use of the ABAP assay to determine tissue antioxidants\(^{31,32}\) that integrates in a functional antioxidant basis the concentration of GSH, ascorbic acids and other water soluble antioxidants. Hydrophilic antioxidants decreased in Fe and Cu intoxication in parallel to GSH, which is easily understood because GSH largely accounts for the whole hydrophilic antioxidants.

Similarly, lipophilic antioxidant is a concept based in the use of chemiluminescence assay\(^{33}\). The test integrates the antioxidant function of α-tocopherol, β-carotene and other lipid soluble antioxidants. A marked decrease in lipophilic antioxidants is observed after Fe and Cu overloads as a consequence of the increased phospholipid peroxidation process occurring in the phospholipid bilayer of the hydrophobic domain of neuron membranes.

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\(^{34}\) and later by Crichton et al.\(^{32}\).

It is apparent, considering the whole data and the literature, that both transition metals, Fe and Cu, enhance the intracellular rate of generation of HO\(^•\) and RO\(^•\) by a Haber-Weiss type homolytic scission of H\(_2\)O\(_2\) and ROOH\(^{26,27,29}\). These clearly defined initiation reactions operate as the rate-limiting step of the whole free-radical mediated process that leads to the cellular situation of oxidative stress.

Reactive oxygen species are physiologically produced in vivo in oxidative metabolism and an increase in their rate of production defines the oxidative stress condition, which is frequently associated with pathological situations\(^{35,26,29}\). The key importance of superoxide anion (O\(_2^{-}\)) in free-radical mediated oxidations is based in two of its properties, the dismutation to H\(_2\)O\(_2\) and in the ability to reduce the cytosolic transition metals, Fe\(^{3+}\) and Cu\(^{2+}\), to Fe\(^{2+}\) and Cu\(^{+}\). These reduced species catalyze the homolysis of H\(_2\)O\(_2\) and ROOH yielding HO\(^•\) and RO\(^•\)\(^{25,32,34}\). The production of HO\(^•\) and RO\(^•\) by the Fenton/Haber-Weiss reactions has been considered for a long time as the rate-limiting step for physiological phospholipid peroxidation\(^{36,32,33}\). Considering the O-O bond homolysis, there are two points of view. The classical one is that free ions Fe\(^{3+}\) and Cu\(^{+}\) catalyze the reaction. The second ones, is that binding of the positively-charged Fe\(^{3+}\) and Cu\(^{+}\) to negatively-charged head groups of phospholipids\(^{35}\) or peptides or protein sites, are the catalytically competent reaction centers for H\(_2\)O\(_2\) homolysis\(^{36}\).

Two different responses were recognized in the antioxidant enzyme expression and activity in brain homogenates after Fe and Cu overloads. The first type of response, observed with GPx-4, is a decrease in activity and in protein level due to free-radical mediated protein damage with loss of enzymatic activity, in agreement with the increased concentration of protein carbonyl groups. The second type of response, observed with Cu,Zn-SOD and with CAT, is an adaptive increase in enzyme activity and protein expression. Interestingly, this adaptive response of antioxidant enzymes to oxidative stress in mammalian organs is to be remarked, but it is not infrequently found. Rat liver increased Cu,Zn-SOD and Mn-SOD activity after Fe and Cu overloads\(^4\) and after Cu\(^{35}\) and Cd-produced oxidative stress\(^{38,40}\). Rat brain show increased Cu,Zn-SOD activity after Zn(II)\(^{41}\) and Mn(II)\(^{42}\) treatments. Increased SOD activity were also reported in rat serum and brain after Zn(II) load\(^{43}\) and after Hg(II) in mouse brain\(^{44}\). The brain of hyperthyroid rats shows oxidative stress and increased activities of the dismutases, catalase and glutathione peroxidases\(^{45}\).

Conclusions

The results of this study support that concept that a decrease in the $\text{SH}$ concentration is involved in the cell injury that follows to the oxidative stress produced by Fe and Cu loads. The oxidative process of oxidant generation and phospholipid peroxidation determined by in vivo brain chemiluminescence shows a similar time course than antioxidant (GSH and hydrophilic and lipophilic antioxidants) consumption. A pharmacological treatment for the oxidative damage in acute Fe and Cu toxicity seems possible by using N-acetylcysteine which is a GSH precursor.

Abbreviations

109 ABAP = 2,2-azobis (2-amidinopropane)
110 GSH = glutathione, reduced glutathione
111 GSSG = glutathione disulfide
112 SOD1 = Cu,Zn-SOD or Cu,Zn-superoxide dismutase

Aknowledgements

105 This study was supported by grants from the University of Buenos Aires (B056 and 200201000369), CONICET, and ANPCYT (PICT 1138-2008 and 0964-2012).
105 The authors declare that they have no conflict of interest.
Notes and references

7. A. Holmgren, Antioxid Redox Signal, 1 2007, 9, 25;
17. A.C. Maehly and B. Chance, B, Meth Biochem Anal, 1954, 1, 357;
26. B. Chance, H. Sies and A. Boveris, Physiol Rev, 1979, 59, 527;
33. D. Carter, Environ Health Perspect, 1995, 103, 17;
34. B. Hallwell and J. Gutteridge, Biochem J, 1984, 219, 1;
35. M.G. Repetto, N.F. Ferrarotti and A. Boveris, Arch Toxicol, 2010, 84, 255;