Life Sciences 89 (2011) 221–228

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Liver preconditioning induced by iron in a rat model of ischemia/reperfusion

Mónica Galleano [a], Gladys Tapia [b], Susana Puntarulo [a], Patricia Varela [b], Luis A. Videla [b], Virginia Fernández [b,*]

[a] Physical Chemistry-PRALIB, School of Pharmacy and Biochemistry, University of Buenos Aires-CONICET, Buenos Aires, Argentina
[b] Molecular and Clinical Pharmacology Program, Institute of Biomedical Sciences, Faculty of Medicine, University of Chile, Santiago, Chile

ARTICLE INFO

Article history:
Received 24 December 2010
Accepted 26 May 2011

Keywords:
Oxidative stress
Iron administration
Liver preconditioning
Ischemia–reperfusion injury
Nuclear factor-κB
Acute-phase response
Free radicals

ABSTRACT

Aims: Liver preconditioning against ischemia–reperfusion (IR) injury is a major area of experimental research, in which regulation of gene expression with cytoprotective responses due to transient oxidative stress development has been reported. Considering that significant cytoprotection occurs after exposure to low levels of iron (Fe), we tested the hypothesis that sub-chronic administration of Fe to rats underlying transient oxidative stress preconditions the liver against IR injury.
Main methods: Animals received six doses (50 mg Fe–dextran/kg ip) every second day during 10 days, before partial IR (vascular clamping) or sham laparotomy (control). Transient oxidative stress was defined by liver glutathione and protein carbonyl contents (24, 48, and 72 h after Fe treatment). Plasma and liver Fe status and ferritin content (western blot) were assessed in animals not subjected to IR. Liver injury and inflammatory response were evaluated by serum transaminases, liver morphology and serum TNF-α. Fe preconditioning against IR injury was correlated with liver glutathione content and the redox-sensitive NF-κB signaling pathway (EMSA) and western blot analysis of haptoglobin.
Key findings: Significant hepatoprotection against IR injury, underlying transient oxidative stress and enhancement in the total and labile Fe pools, was achieved by Fe administration. Abrogation of IR injury is related to reduced TNF-α response (91%), abolishment of the IR-induced liver glutathione depletion and recovery of the NF-κB signaling pathway (75%), lost during IR.
Significance: Sub-chronic Fe administration protects the liver against IR injury through antioxidant and anti-inflammatory responses, with recovery of NF-κB activation and related acute-phase response signaling.
© 2011 Elsevier Inc. All rights reserved.

Introduction

Reactive oxygen species (ROS) reach relatively low concentrations within the cell, varying from 10^{-16} M for hydroxyl radical to 10^{-7} M for hydrogen peroxide (Boveris et al., 2008), because of their efficient processing by antioxidants. Moderate increases in ROS in a defined period may exert regulation of gene expression with cytoprotective responses (Dröge, 2002), thus protecting organs from detrimental effects of subsequent noxious events (Das and Das, 2008). In this context, induction of a mild oxidative stress in the liver represents a preconditioning strategy against ischemia–reperfusion (IR) injury, as shown for transient ischemia (Peralta et al., 1997; Romanque et al., 2010), hyperthermia (Terajima et al., 2000), hyperbaric oxygen therapy (Yu et al., 2005), ozone (Ajamieh et al., 2004), doxorubicin (Ito et al., 2000), t-butyl hydroperoxide (Rüdiger et al., 2003), and, more recently, thyroid hormone administration (Fernández et al., 2007, 2008). Under these conditions, moderate ROS levels may regulate protein function through reversible sulfhydryl oxidation and/or gene expression by specific kinase, phosphatase, or transcription factor activation (Dröge, 2002; Poli et al., 2004). Conversely, large amounts of ROS trigger pathogenic mechanisms, as shown for IR, with substantial oxidation of biomolecules and dysregulation of signal transduction and gene expression (Dröge, 2002; Poli et al., 2004; Videla, 2009). Consequently, oxidative stress is a redox phenomenon representing a form of hormesis, with biologically beneficial effects in the low-level range and harmful responses at high-level ranges and/or after prolonged ROS exposure (Calabrese, 2008; Hayes, 2008).

Iron (Fe), an essential micronutrient and bio-catalyst of crucial oxidation–reduction reactions, is also a potential toxin. Hormetic responses to Fe exposure are related to its chemistry promoting electron exchange under aerobic conditions, being (i) essential for mitochondrial oxidative phosphorylation and cytoprotective responses at low levels; or (ii) lethal due to uncontrolled ROS generation when Fe-buffering capacity is overcome (Aust et al., 1985; Bacon and Britton, 1990; Pietrangelo, 2003). In agreement, low Fe concentration (20 μM) induced rat cardiomyocyte survival and hypertrophy via an inducible nitric oxide synthase mechanism, whereas cardiomyocyte necrosis is attained at high (80 to 100 μM) concentrations (Muñoz et al., 2010). Heart protection by Fe is further

* Corresponding author at: Molecular and Clinical Pharmacology Program, Institute of Biomedical Sciences, Independencia 1027, Postal Code 8380000, Santiago-7, Chile. Tel.: +56 2 9786256; fax: +56 2 7372783.
E-mail address: vfernand@med.uchile.cl (V. Fernández).

0024-3205/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.lfs.2011.06.005

supported by the findings that (i) ischemic preconditioning triggers a Fe signal leading to ferritin upregulation (Chevion et al., 2008); and (ii) short-term Fe administration up-regulates superoxide dismutase (Metzler et al., 2007). Furthermore, docosahexaenoic acid-dependent Fe accumulation in oligodendroglia cells protects against hydrogen peroxide-induced damage, an effect attributed to enhanced glutathione peroxidase and glutathione reductase activities (Brand et al., 2008), whereas, Fe-induced calcium signals through ROS-mediated ryanodine receptor ERK activation trigger cAMP/calcium response element binding protein-dependent transcription of genes related to synaptic plasticity (Hidalgo and Núñez, 2007).

This work was aimed to test the hypothesis that sub-chronic administration of Fe to rats, involving transient liver oxidative stress, exerts significant protection against IR injury. Optimal conditions for the preconditioning maneuver were studied, followed by assessment of the hepatoprotective effects of Fe administration on subsequent IR injury, tumor necrosis factor-α (TNF-α) levels, NF-κB activation, and the acute-phase response (APR) of the liver.

Materials and methods

Animals

Male Sprague–Dawley rats (Bioterio Central, ICBM, Faculty of Medicine, University of Chile) weighing 140–160 g were housed on a 12-hour light/dark cycle and were provided with rat chow and water ad libitum. All experimental animal protocols and animal procedures complied with the Guide for the Care and Use of Laboratory Animals (National Academy of Sciences, NIH Publication 86-23, revised 1985).

Fe supplementation

At time zero (Fig. 1), animals received 6 intraperitoneal doses of 50 mg of Fe–dextran/kg, dissolved in buffered saline (PBS), or equivalent volumes of saline (controls). Fe–dextran and saline were administered every second day over a 10 day period (Fig. 1).

Induction of liver IR injury

At 72 h after Fe treatment, rats were anesthetized with intraperitoneal (1 mL/kg) zolazepam chlorhydrate (25 mg/mL) and tiletamine chlorhydrate (25 mg/mL) (Zoletil 50; Virbac S/A, Carros, France), and partial IR (1 h ischemia followed by 20 h of reperfusion) was induced by temporarily occluding the blood supply to the left lateral and median lobes of the liver by means of a Schwartz clip (Fine Science Tools Inc., Vancouver, British Columbia, Canada), as previously described (González-Flecha et al., 1993). Control animals were subjected to anesthesia and sham laparotomy. Experimental groups included saline and Fe-treated rats subjected to either sham laparotomy or IR (Fig. 1). Reperfusion was carried out for 20 h, time at which serum GOT and GPT values and liver oxidative stress related parameters (GSH depletion and enhancement in oxidized proteins) are maximal when assessed in the time period of 3–72 h (unpublished data). At the end of the reperfusion period blood samples were obtained by cardiac puncture and centrifuged for serum GPT, GOT, and TNF-α assessments and liver samples, taken from the medial lobes, were frozen in liquid nitrogen and stored at −80 °C (assessments of NF-κB and proteins), or fixed in phosphate-buffered formalin, embedded in paraffin, and stained with hematoxylin–eosin (morphology assessment).

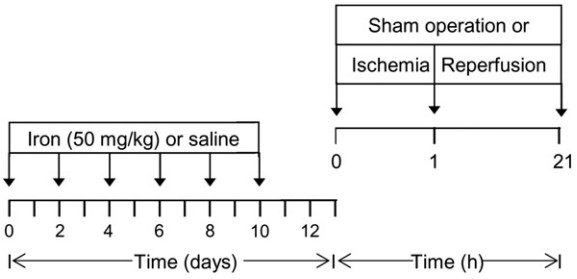

Fig. 1. Experimental protocol for short-term iron preconditioning. Animals were given either saline or daily doses of iron (50 mg/kg) at time 0, 2, 4, 6, 8, and 10 days. Oxidative stress-related parameters were determined at days 11, 12, and 13, and parameters related to iron homeostasis were measured at day 13, experimental time at which groups of control and iron-treated rats were subjected to sham operation or to 1 h ischemia followed by 20 h reperfusion, thus conforming four experimental groups. Blood and liver samples were obtained at the end of the reperfusion period (21 h).

Parameters of oxidative stress

In order to define the time window for development of a transient oxidative stress by Fe, liver samples from animals not subjected to IR or sham laparotomy were immediately processed for glutathione and protein carbonyl contents at 24, 48, and 72 h after Fe treatment, corresponding to days 11, 12, and 13 of the experimental protocol (Fig. 1). For this purpose, livers were perfused in situ, in anesthetized animals, with a cold solution containing 150 mM KCl and 5 mM Tris (pH 7.4) to remove blood. Total reduced glutathione (GSH) equivalents (μmol GSH/g liver) and protein oxidation (nmol carbonyl/mg protein), were assessed as previously described (Tietze, 1969; Reznic and Packer, 1994). Total protein content was calculated from the absorbance at 280 nm. In order to evaluate the effects of Fe supplementation on the IR-induced oxidative stress, total GSH equivalents were also assessed in animals subjected to IR or sham laparotomy.

Plasma and liver Fe status

These studies were carried out in animals not subjected to IR or sham laparotomy after Fe or saline treatments. Plasma Fe and the unsaturated Fe binding capacity were assessed using commercial kits (Wiener Lab., Rosario, Argentina) (Rousseau and Puntarulo, 2009). Plasma Fe plus unsaturated Fe binding capacity gives total Fe binding capacity and the ratio plasma Fe/total Fe binding capacity gives the percentage of transferrin saturation. Total liver Fe content (non-heme plus heme) was assessed by spectrophotometric detection of the Fe–batophenantroline complex, using intact liver samples previously mineralized with HNO_3 plus $HClO_4$ (1:1) (Brumby and Massey, 1967). The labile Fe pool (LIP), redox-active Fe weakly chelated to a variety of ligands, was evaluated by electron paramagnetic resonance (EPR) spectroscopy as paramagnetic complexes in the presence of desferoxamine (Fe^{3+}–DFO) at 77 K (Kozlov et al., 1992). The complex exhibited a signal with g = 4.3 (Fig. 2B, upper panel) and was quantified by double integration using a calibration curve prepared with $Fe(SO_4)_2$ and DFO. EPR spectra were recorded on a Bruker 106 spectrometer using the conditions previously indicated (Rousseau and Puntarulo, 2009).

Parameters of inflammatory response and liver injury

ELISA kits were used for assessment of serum levels (pg/mL) of TNF-α (Biosource International, Camarillo, CA, USA). The severity of liver damage was determined by measuring serum GPT and GOT levels with commercial kits (Valtek Diagnostics, Santiago, Chile) and by performing light microscopy of the liver.

NF-κB electromobility shift assay (EMSA)

Nuclear protein extracts from liver samples were prepared according to Deryckere and Gannon (1994). The samples were subjected to electromobility shift assay for assessment of NF-κB DNA binding using the probe 5′-GAT CTC AGA GGG GAC TTT CCG AG-3′ (Invitrogen Life

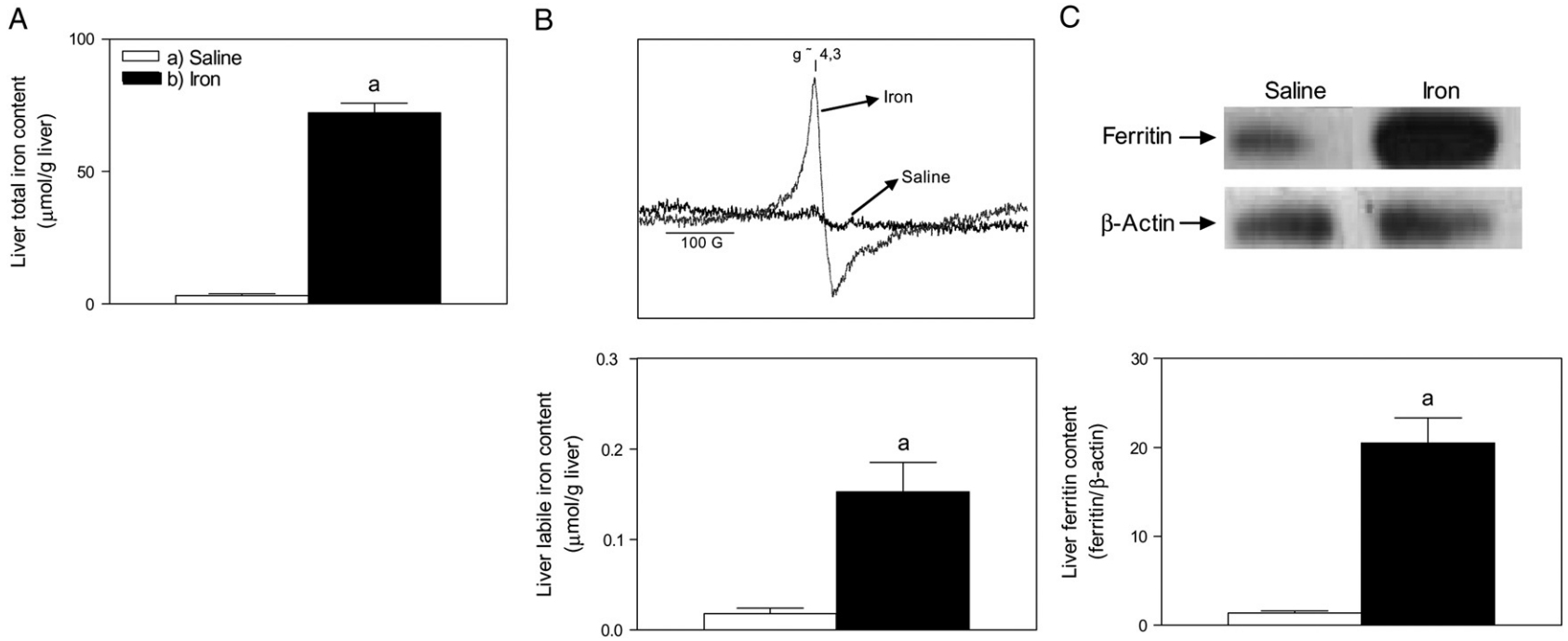

Fig. 2. Content of (A) total iron, (B) labile iron, and (C) ferritin in the livers of saline-control rats and iron-treated animals at day 13 after treatment. Upper panel in B represents EPR signals of Fe^{3+}–DFO complex measured at 77 K, as described in Materials and methods, and the lower panel shows the quantification of labile iron levels expressed as μmol/g of liver. Upper panel in C represents ferritin expression evaluated by Western blotting, using 60 μg of soluble protein from a saline-control rat and an iron-treated animal, and the lower panel shows bar graphs corresponding to densitometric quantification expressed as ferritin/β-actin ratios to compare lane-to-lane equivalency in total protein content. Data are expressed as means ± SEM for 4 animals per group. Significance study (Student's *t*-test for unpaired data), $^{a}P<0.05$ compared to saline-control values.

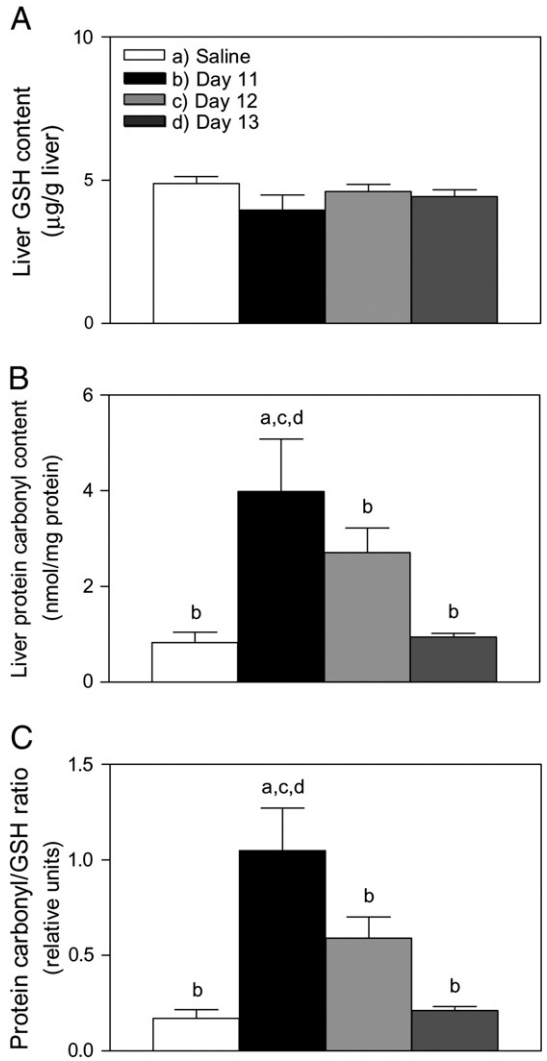

Fig. 3. Effects of iron administration on rat liver (A) reduced glutathione (GSH) content, (B) protein carbonyl content, and (C) the respective protein carbonyl/GSH content ratios at days 11, 12, and 13 after treatment. Data are expressed as means ± SEM for 3 to 8 animals per group. Significance studies ($p<0.05$; one-way ANOVA and the Newman–Keuls' test) are indicated by the letters identifying each experimental group.

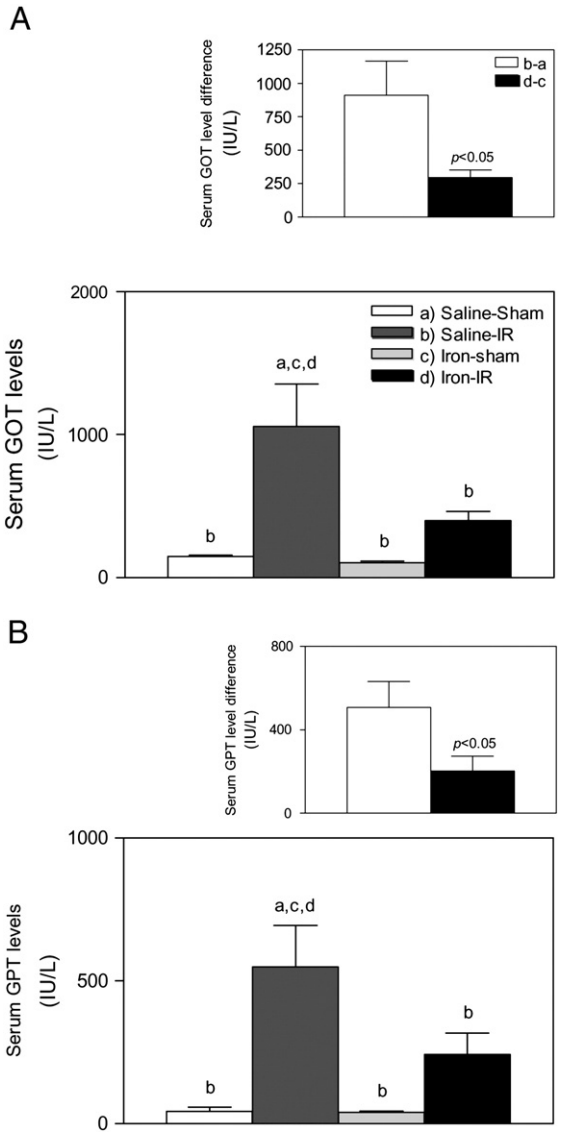

Fig. 4. Serum (A) glutamic oxaloacetate transamine (GOT) and (B) glutamic pyruvate transaminase (GPT) levels after hepatic ischemia–reperfusion (IR) injury in unpreconditioned and iron preconditioned rats. Insets: net changes in serum GOT (A) and GPT (B) induced by IR under condition of no treatment [(b) saline-IR minus (a) saline-sham] and after iron administration [(d) iron-IR minus (c) iron-sham]. Data are expressed as means ± SEM for 4 to 9 animals per group. Significance studies ($P<0.05$; one-way ANOVA and the Newman–Keuls' test) are indicated by the letters identifying each experimental group. Data in the insets were analyzed by Student's *t*-test for unpaired data.

Technologies, Carlsbad, CA), labeled with α-^{32}PdCTP using the Klenow DNA Polymerase Fragment I (Invitrogen Corp., Carlsbad, CA), as described previously (Tapia et al., 2003; Fernández et al., 2005a,b). The specificity of the reaction was determined by a competition assay using 100-fold molar excess of unlabeled DNA probes. Samples were loaded on nondenaturating 6% polyacrylamide gels and run until the free probe reached the end of the gel; NF-κB bands were detected by autoradiography and quantified by densitometry using Scion Image (Scion Corp., Frederick, MD).

Western blot analysis of haptoglobin and ferritin

Liver samples (100–500 mg) frozen in liquid nitrogen were homogenized and suspended in a buffer solution pH 7.9 containing 10 mM HEPES, 1 mM EDTA, 0.6% Nonidet P-40, 150 mM NaCl, and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 mM orthovanadate). Soluble protein fractions (25 μg) were separated on 12% polyacrylamide gels using SDS-PAGE (Towbin et al., 1979) and transferred to nitrocellulose membranes (Laemmli, 1970), which were blocked for 1 h at room temperature with TBS containing 5% nonfat dry milk. The blots were washed with TBS-containing 0.1% Tween 20 and hybridized with rabbit polyclonal antibodies for human haptoglobin and ferritin (Dako Corp., Carpinteria, CA). In all determinations, mouse monoclonal antibody for rat β-actin (ICN Biomedicals, Inc., Aurora, OH) was used as internal control. After extensive washing, the antigen–antibody complexes were detected using horseradish peroxidase labeled goat anti-rabbit IgG or goat antimouse IgG and a SuperSignal West Pico Chemiluminescence Kit Detection System (Pierce, Rockford, IL).

Statistical analyses

Values shown represent the mean ± SEM for the number of separate experiments indicated. Student's*t* test or one-way ANOVA followed by the Newman–Keuls' test assessed the statistical significance of differences between mean values as indicated. A P value of less than 0.05 was considered significant.

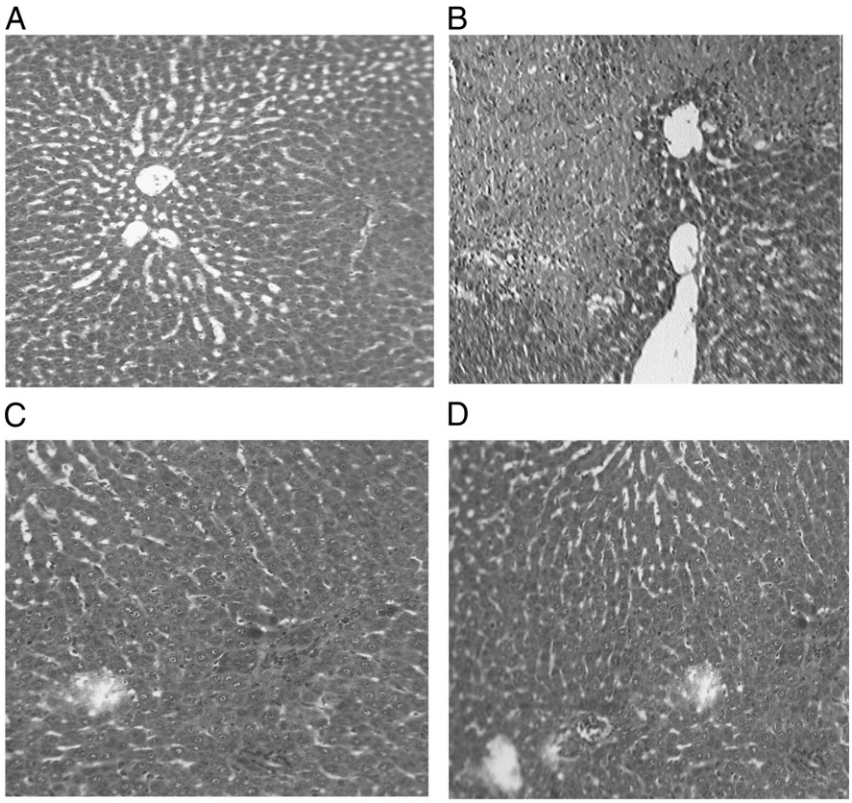

Fig. 5. Liver histology after hepatic ischemia–reperfusion (IR) injury in unpreconditioned and iron preconditioned rats. Representative liver sections from (A) a saline-sham rat, (B) a saline-control animal subjected to 1 h ischemia followed by 20 h reperfusion, (C) an iron-pretreated sham operated rat, and (D) an iron-pretreated animal subjected to IR (hematoxylin–eosin-stained liver sections from a total of 4 to 5 animals per experimental group; original magnification × 20).

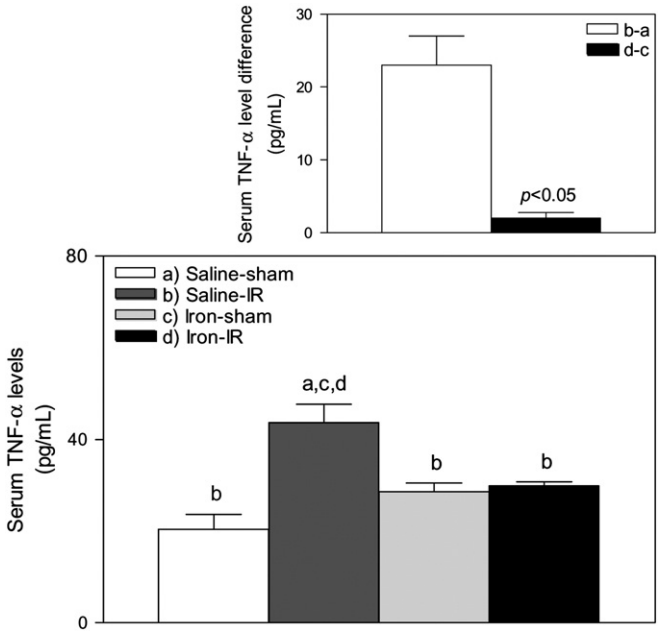

Fig. 6. Serum tumor necrosis factor-α (TNF-α) levels after hepatic ischemia–reperfusion (IR) injury in unpreconditioned and iron preconditioned rats. Inset: net changes in serum TNF-α induced by IR under condition of no treatment [(b) saline-IR minus (a) saline-sham] and after iron administration [(d) iron-IR minus (c) iron-sham]. Data are expressed as means ± SEM for 11 to 13 animals per group. Significance studies ($p<0.05$; one-way ANOVA and the Newman–Keuls' test) are indicated by the letters identifying each experimental group. Data in the inset were analyzed by Student's *t*-test for unpaired data.

Results

Effects of Fe supplementation on the Fe status and induction of reversible oxidative stress in the liver

Assessments of plasma and liver Fe status in animals not subjected to IR or sham laparotomy (day 13th, Fig. 1) showed 22.9-fold (Fig. 2A), 8.3-fold (Fig. 2B), and 15.2-fold (Fig. 2C) increases ($P<0.05$) in the hepatic content of total Fe, LIP, and ferritin, respectively. These modifications occurred without significant changes in the serum levels of transaminases [GOT: controls, 175 ± 28 (n = 5) IU/L; Fe-treated rats, 302 ± 13 (n = 3). GPT: controls, 56 ± 6 (n = 4); Fe-treated rats, 57 ± 6 (n = 3)]. Under these conditions, liver GSH levels were not significantly modified after Fe treatment (Fig. 3A). However, Fe significantly enhanced liver protein carbonyl content (Fig. 3B) and the protein carbonyl/GSH ratio (Fig. 3C) at day 11 (4.8-fold and 6.6-fold increases, respectively; $P<0.05$) over control values, parameters that were normalized at day 13 (Fig. 3B and C), thus establishing a reversible oxidative stress in the liver which may afford protection against subsequent noxious effects induced by IR.

Effects of Fe supplementation on parameters of liver injury

To determine if the changes in Fe and oxidative stress status described above are related to liver protection against IR injury, serum TNF-α levels and transaminase activities were assessed after 1 h of partial ischemia followed by reperfusion for 20 h (Fig. 1). This IR protocol resulted in substantial liver injury, as shown by the 6.1-fold and 12-fold enhancements ($P<0.05$) in the serum levels of GOT (Fig. 4A) and GPT (Fig. 4B), compared to the saline-sham operated control. Rats subjected to Fe administration and sham operation (Fig. 1) exhibited comparable GOT and GPT levels in serum to those in the saline-sham group (Fig. 4). Furthermore, serum GOT and GPT

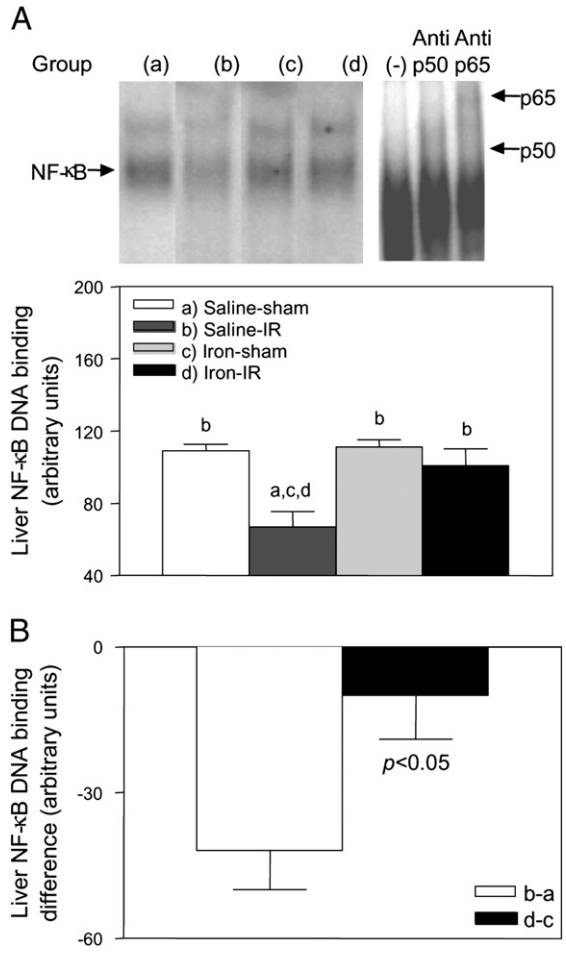

Fig. 7. (A) Liver NF-κB DNA binding on electromobility shift assay after hepatic ischemia–reperfusion (IR) injury in unpreconditioned and iron preconditioned rats. Top autoradiographs represent lanes containing 6.7 μg nuclear protein from each experimental group studied and supershift analysis of nuclear extracts from a saline-sham rat incubated with the labeled probe for NF-κB and with antibodies specific for NF-κB p65 (anti-p65) or p50 (anti-p50). Bar graphs correspond to densitometric quantification of relative NF-κB DNA binding, expressed as means ± SEM for 8 to 12 different animals; significance studies ($P<0.05$; one-way ANOVA and the Newman–Keuls' test) are indicated by the letters identifying each experimental group. (B) Net changes in NF-κB DNA binding induced by IR under condition of no treatment [(b) saline-IR minus (a) saline-sham] and after iron administration [(d) iron-IR minus (c) iron-sham]; significance studies were carried out by Student's *t*-test for unpaired data.

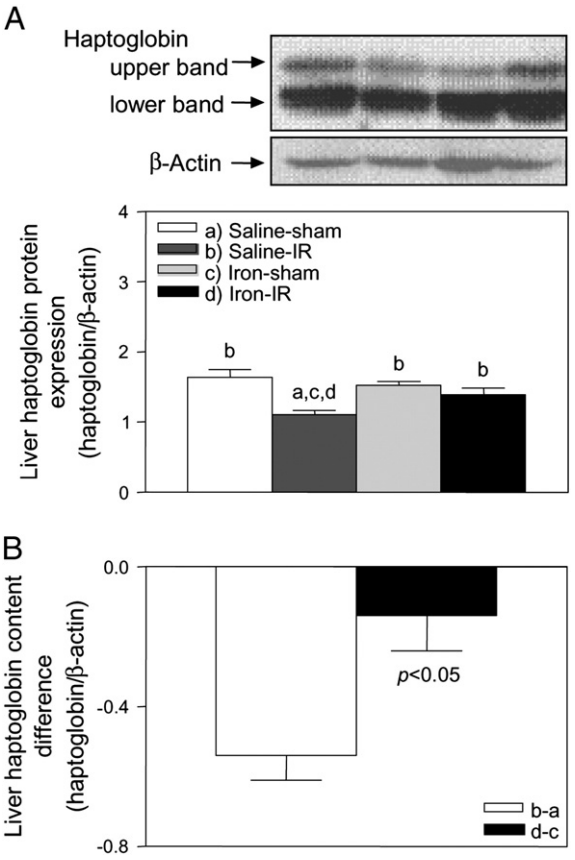

Fig. 8. (A) Liver haptoglobin expression evaluated by Western blotting after hepatic ischemia–reperfusion (IR) injury in unpreconditioned and iron preconditioned rats. Representative blots of haptoglobin and β-actin protein expression are shown, using 25 μg of soluble protein from a different rat of each group studied. Bar graphs correspond to the respective densitometric quantification expressed as haptoglobin/β-actin ratios for both haptoglobin bands, expressed as means ± SEM for 14 different animals; significance studies ($P<0.05$; one-way ANOVA and the Newman–Keuls' test) are indicated by the letters identifying each experimental group. (B) Net changes in haptoglobin expression induced by IR under condition of no treatment [(b) saline-IR minus (a) saline-sham] and after iron administration [(d) iron-IR minus (c) iron-sham]; significance studies were carried out by Student's *t*-test for unpaired data.

levels in Fe preconditioned animals subjected to IR showed 2.8-fold (Fig. 4A) and 5.2-fold (Fig. 4B) increases over those in the Fe-sham operated group, respectively, with net diminutions of 75% (Fig. 4A, inset) and 65% (Fig. 4B, inset) ($P<0.05$), respectively, in relation to IR. These data are in agreement with the morphological characteristics of the liver assessed by light microscopy, showing normal morphology in saline-control (Fig. 5A) and Fe-sham (Fig. 5C) groups, whereas saline-IR animals exhibited distorted architecture with extensive centrolobular and peripheral necrosis and neutrophil infiltration (Fig. 5B); however, normal liver architecture with minimal centrolobular necrosis was achieved by IR in Fe preconditioned rats (Fig. 5D). Hemosiderophages were observed in liver parenchyma from Fe-sham (Fig. 5C) and Fe-IR (Fig. 5D) groups. Furthermore, IR-induced 110% increase in the serum levels of TNF-α over control values in unpreconditioned animals (Fig. 6) ($P<0.05$), with a net 91% diminution being observed in Fe preconditioned rats subjected to IR compared with the respective Fe-sham operated group (Fig. 6, inset).

In addition, IR led to 65% decrease in the hepatic content of GSH ($P<0.05$) over control values in unpreconditioned rats, an effect that was abolished by Fe preconditioning [control-sham, 6.12 ± 0.26 (n = 8) μmoles/g liver; control-IR, 3.98 ± 0.35 (n = 6); Fe-sham, 6.87 ± 0.96 (n = 5); Fe-IR, 6.62 ± 0.26 (n = 6)].

Effects of Fe supplementation on NF-κB signaling activity

Liver NF-κB DNA binding activity in unpreconditioned animals was significantly diminished by IR, in relation to controls (40% diminution; $P<0.05$), an effect that was not observed in the liver of Fe preconditioned animals (Fig. 7A). By comparing liver NF-κB DNA binding difference in unpreconditioned and preconditioned animals, Fe administration elicited a net 76% recovery ($P<0.05$) in the IR-induced loss in hepatic NF-κB activity (Fig. 7B). In agreement, data on the hepatic expression of the NF-κB-controlled acute-phase protein (APP) haptoglobin in unpreconditioned animals indicate a 33% decrease ($P<0.05$) in its protein content induced by IR over control values (Fig. 8A). However, Fe administration prior to IR led to a 9% diminution in haptoglobin content compared to the Fe-sham group, thus eliciting a net 75% recovery (Fig. 8B). Interestingly, liver NF-κB DNA binding and haptoglobin expression were not significantly modified by Fe administration in animals subjected to sham laparotomy compared to controls (Figs. 7 and 8).

Discussion

Transient and moderate enhancement in the oxidative stress status of the liver is considered as a hormetic stimulus leading to preconditioning effects against IR in a variety of experimental conditions (Peralta et al., 1997; Romanque et al., 2010; Terajima et al.; 2000; Yu et al., 2005; Ajamieh et al., 2004; Ito et al., 2000; Rüdiger et al., 2003), including transient ischemia (Romanque et al., 2010), and the in vivo administration of thyroid hormone (Fernández et al., 2007, 2008) or n-3 long-chain polyunsaturated fatty acids (Zúñiga et al., 2010). Although the last three preconditioning strategies have clinical potential, liver preconditioning by thyroid hormone or n-3 long-chain polyunsaturated fatty acids have not been evaluated in man, whereas ischemic preconditioning in human liver resections or in human liver transplantation remains controversial (Videla, 2010). The data presented in this work indicate that Fe administration to rats constitutes an alternate preconditioning strategy that may be of relevance to clinical application, taking into account that repeated injections of 100–125 mg/kg of iron complexes by intramuscular or intravenous routes (100–125 mg/kg, 1–3 times/week for 4–12 weeks) are considered as valid and well tolerated therapeutic strategies in human anemia treatments (Silverstein and Rodgers, 2004; Bayraktar and Bayraktar, 2010). Thus, the dosage protocol used in this work (50 mg iron/kg, 3 times/week for 2 weeks) may constitute an adequate preconditioning strategy with relevance to clinical application, as minor side effects to extrahepatic tissues after iron overload have been documented (Galleano and Puntarulo, 1994; Puntarulo, 2005; Puntarulo and Galleano 2009).

The experimental protocol used for Fe administration to rats resulted in enhancement in the total Fe and LIP of the liver, with consequent up-regulation of ferritin content, when assessed three days after the last dose of Fe administered (day 13th in Fig. 1), leading to a transient increase in the oxidative stress status of the liver. This was evidenced by significant higher hepatic protein carbonyl levels and protein carbonyl/glutathione ratios over control values observed at day one after Fe treatment (day 11th in Fig. 1), parameters that returned to control values at day 13th. Although virtually all the intracellular Fe is tightly bound to proteins, a minor proportion termed LIP is Fe (as Fe^{2+} and Fe^{3+}) loosely bound to low-molecular-mass, low-affinity ligands such as phosphate and citrate, proteins and lipids (Yongmin et al., 2006). This LIP, which can promote cellular ROS generation by different pathways (Galatro and Puntarulo, 2007), could be responsible for the development of the transient oxidative stress condition observed, a condition that is characterized by being a reversible redox imbalance with lack of hepatotoxicity, as shown by the lack of significant changes in the levels of serum GOT and GPT.

Furthermore, data presented demonstrate a significant protection afforded by Fe administration against liver IR injury when given before the IR protocol, as shown by significant diminutions in serum GOT and GPT levels and normal liver architecture in Fe pre-treated animals subjected to IR. Fe preconditioning is related to suppression of the TNF-α and oxidative responses, and reversion of the changes in signal transduction and gene expression that underlie liver injury induced by IR (Dröge, 2002; Fernández et al., 2007; Poli et al., 2004). Our results demonstrate that Fe liver preconditioning is related to recovery of IR-reduced levels of NF-κB activation and haptoglobin to those observed in the liver of control animals. Haptoglobin, an anti-inflammatory and anti-oxidant acute phase protein synthesized by liver cells in response to IL-6, participates in the APR, a major pathophysiological reaction mainly focused on the liver (Samols, et al., 2003; Galicia et al., 2009; Levy et al., 2010). The APR is associated with inflammation and with other not inflammatory conditions, whose main role is to restore homeostasis by contributing to defensive or adaptive capabilities (Samols, et al., 2003; Galicia et al., 2009; Levy et al., 2010). Thus, up-regulation of liver haptoglobin in Fe preconditioning may represent a defense mechanism against the deleterious effects of IR-induced oxidative stress and inflammatory responses, redirecting gene expression into a pattern fulfilling antioxidant, transport, coagulation, and immune functions. The success of the Fe administration protocol used in this study in terms of limiting the damaging consequences of IR, could be due to cellular Fe metabolism over the 72 h time-window (Fig. 1) between Fe administration and the settlement of IR. Under these conditions, expansion of LIP occurs, with the consequent induction of ferritin synthesis (Cairo et al., 1995), a protein that is highly effective in sequestering large amounts of Fe in its central cavity in a soluble, non-toxic bioavailable form (Galatro and Puntarulo, 2007). Thus, the enhancement in liver ferritin content by day 13th may imply that a significant amount of the supplemented Fe is not available for Fe-dependent liver injury, whereas LIP expansion would trigger the transient increase in the oxidative stress status limiting the further pro-oxidant challenge of IR.

Consistent with the data presented in this study, liver ischemia–reperfusion as well as acute or dietary Fe overload, have been reported to significantly enhance the APR through the induction of hepcidin, an antimicrobial peptide which has been postulated to maintain Fe homeostasis by modulating Fe absorption at both the intestinal and macrophage levels, thus regulating Fe recycling and Fe balance (Goss et al., 2005).

Conclusion

The results of this study indicate that transient oxidative stress is induced in the liver following Fe administration, an effect that is related to a regulatory and protective effect of Fe in signaling processes, leading to significant prevention of liver injury associated with IR. Fe preconditioning is related to suppression of the pro-inflammatory and pro-oxidative responses and with the recovery of liver NF-κB DNA binding and APR responses, which are lost during IR. These Fe mediated effects may protect the liver against drastic IR-induced oxidative stress and inflammatory deleterious effects.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

This research was supported by grants 1080039 and 1110006 from FONDECYT, Chile.

References

Ajamieh HH, Menendez S, Martinez-Sanchez G, Candelario-Jalil E, Re L, Giuliani A, et al. Effects of ozone oxidative preconditioning on nitric oxide generation and cellular redox balance in a rat model of hepatic ischemia–reperfusion. Liver Int 2004;24:55–62.

Aust SD, Morehouse LA, Thomes CE. Role of metals in oxygen radical reactions. J Free Radic Biol Med 1985;1:3–15.

Bacon BR, Britton RS. The pathology of hepatic iron overload: a free radical mediated process? Hepatology 1990;11:127–37.

Bayraktar UD, Bayraktar S. Treatment of iron deficiency anemia associated with gastrointestinal tract diseases. World J Gastroenterol 2010;16:2720–5.

Boveris A, Repetto MG, Bustamante J, Boveris AD, Valdez LB. The concept of oxidative stress in pathology. In: Alvarez S, Evelson P., editors. Free radical pathophysiology. India: Transword Research Network Kerala; 2008. p. 1–17.

Brand A, Schonfeld E, Isharel I, Yavin E. Docosahexaenoic acid-dependent iron accumulation in oligodendroglia cells protects from hydrogen peroxide-induced damage. J Neurochem 2008;105:1325–35.

Brumby PE, Massey V. Determination of non-heme iron, total iron, and copper. Methods Enzymol 1967;10:463–72.

Cairo G, Tacchini L, Pogliaghi G, Anzon E, Tomasi A, Bernelli-Zazzera A. Induction of ferritin synthesis by oxidative stress. Transcriptional and post-transcriptional regulation by expansion of the "free" iron pool. J Biol Chem 1995;270:700–13.

Calabrese EJ. Converging concepts: adaptive response, preconditioning, and the Yerkes–Dobson Law are manifestations of hormesis. Ageing Res Rev 2008;7:8–20.

Chevion M, Leibowitz S, Aye NN, Novogrodsky O, Singer A, Avizemer O, et al. Heart protection by ischemic preconditioning: a novel pathway initiated by iron and mediated by ferritin. J Mol Cell Cardiol 2008;45:839–45.

Das M, Das DK. Molecular mechanism of preconditioning. IUBMB Life 2008;60:199–203.

Deryckere F, Gannon F. A one-hour minipreparation technique for extraction of DNA-binding proteins from animal tissues. Biotechniques 1994;16:405.
Dröge W. Free radicals in the physiological control of cell function. Physiol Rev 2002;82:47–95.
Fernández V, Tapia G, Varela P, Videla LA. Redox regulation of thyroid hormone-induced Kupffer cell-dependent IκB-α phosphorylation in relation to inducible nitric oxide synthase expression. Free Radic Res 2005a;39:411–8.
Fernández V, Tapia G, Varela P, Castillo I, Mora C, Moya F, et al. Redox up-regulated expression of rat liver manganese superoxide dismutase and Bcl-2 by thyroid hormone is associated with inhibitor of IκB-α phosphorylation and nuclear factor-κB activation. J Endocrinol 2005b;186:539–47.
Fernández V, Castillo I, Tapia G, Romanque P, Uribe-Echevarría S, Uribe M, et al. Thyroid hormone preconditioning: protection against ischemia–reperfusion liver injury in the rat. Hepatology 2007;45:170–7.
Fernández V, Tapia G, Varela P, Gaete L, Vera G, Mora C, et al. Causal role of oxidative stress in liver preconditioning by thyroid hormone in rats. Free Radic Biol Med 2008;44:1724–31.
Galatro A, Puntarulo S. Mitochondrial ferritin in animals and plants. Front Biosci 2007;12:1063–71.
Galleano M, Puntarulo S. Effect of mild iron overload on liver and kidney lipid peroxidation. Braz J Med Biol Res 1994;27:2349–58.
Galicia G, Maes W, Verbinnen B, Kasran A, Bullens D, Arredouani M, et al. Haptoglobin deficiency facilitates the development of autoimmune inflammation. Eur J Immunol 2009;39:3404–12.
González-Flecha B, Cutrín JC, Boveris A. Time course and mechanism of oxidative stress and tissue damage in rat liver subjected to in vivo ischemia–reperfusion. J Clin Invest 1993;91:456–64.
Goss JA, Seu P, Gao FQ, Wyllie S. Ischemia–reperfusion of rat liver modulates hepcidin in vivo expression. Liver Transpl 2005;11:800–6.
Hayes DP. Adverse effects of nutritional inadequacy and excess: a hormetic model. Am J Clin Nutr 2008;88:578S–81S.
Hidalgo C, Núñez MT. Calcium, iron and neuronal function. IUBMB Life 2007;59:280–5.
Ito K, Ozasa H, Sanada K, Horikawa S. Doxorubicin preconditioning: a protection against rat hepatic ischemia–reperfusion injury. Hepatology 2000;31:416–9.
Kozlov AV, Yegorov DY, Vladimirov YA, Azizova OA. Intracellular free iron in liver tissue and liver homogenate: studies with electron paramagnetic resonance on the formation of paramagnetic complexes with desferal and nitric oxide. Free Radic Biol Med 1992;13:9–16.
Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–5.
Levy AP, Asleh R, Blum S, Levy NS, Miller-Lotan R, Kalet-Litman S, et al. Haptoglobin: basic and clinical aspects. Antioxid Redox Signal 2010;12:293–304.
Metzler B, Jehle J, Theurl I, Ludwiczek S, Obrist P, Pachinger O, et al. Short term protective effects of iron in a murine model if ischemia/reperfusion. Biometals 2007;20:205–15.
Muñoz JP, Chiong M, García L, Troncoso R, Toro B, Pedrozo A, et al. Iron induces protection and necrosis in cultured cardiomyocytes: role of reactive oxygen species and nitric oxide. Free Radic Biol Med 2010;48:526–34.
Peralta C, Hotter G, Closa D, Gelpi E, Bulbena O, Roselló-Catafau J. Protective effects of preconditioning on the injury associated to ischemia–reperfusion in the rat: role of nitric oxide and adenosine. Hepatology 1997;25:934–7.
Pietrangelo A. Iron-induced oxidant stress in alcoholic liver fibrogenesis. Alcohol 2003;30:121–9.
Poli G, Leonarduzzi G, Biasi F, Chiarpotto E. Oxidative stress and cell signaling. Curr Med Chem 2004;11:1163–82.
Puntarulo S. Iron, oxidative stress and human health. Mol Aspects Med 2005;26:299–312.
Puntarulo S, Galleano M. Forms of iron binding in the cells and the chemical features of chelation therapy. Mini Rev Med Chem 2009;9:1136–46.
Reznic AZ, Packer L. Oxidative damage to proteins: spectrophotometric method for carbonyl assay. Methods Enzymol 1994;233:357–63.
Romanque P, Díaz A, Tapia G, Uribe-Echevarría S, Videla LA, Fernández V. Delayed ischemic preconditioning protects against liver ischemia–reperfusion injury in vivo. Transplant Proc 2010;42:1569–75.
Rousseau I, Puntarulo S. Ferritin-dependent radical generation in rat liver homogenates. Toxicology 2009;264:155–61.
Rüdiger HA, Graf R, Clavien PA. Sublethal oxidative stress triggers the protective effects of ischemic preconditioning in the mouse liver. J Hepatol 2003;39:972–7.
Samols D, Agrawal A, Krushner I. Acute phase proteins. In: Oppenheim JJ, Feldman M, editors. Cytokine reference on-line. London: Academic Press; 2003.
Silverstein SB, Rodgers GM. Parenteral iron therapy options. Am J Hematol 2004;76:74–8.
Tapia G, Fernández V, Varela P, Cornejo P, Guerrero J, Videla LA. Thyroid hormone-induced oxidative stress triggers nuclear factor-κB and cytokine gene expression in rat liver. Free Radic Biol Med 2003;35:257–65.
Terajima H, Enders G, Thiaener A, Hammer C, Kondo T, Thiery J, et al. Impact of hyperthermic preconditioning on postischemic hepatic microcirculatory disturbances in an isolated perfusion model of the rat liver. Hepatology 2000;31:407–15.
Tietze F. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. Anal Biochem 1969;27:287–90.
Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A 1979;76:4350–4.
Videla LA. Oxidative stress signaling underlying liver disease and hepatoprotective mechanisms. World J Hepatol 2009;1:73–9.
Videla LA. Hormetic responses of thyroid hormone calorigenesis in the liver: association with oxidative stress. IUBMB Life 2010;62:460–6.
Yongmin MA, de Groot H, Liu Z, Hider RC, Petrat F. Chelation and determination of labile iron in primary hepatocytes by piridinone fluorescent probes. Biochem J 2006;395:49–55.
Yu SY, Chiu JH, Yang SD, Yu HY, Hsieh CC, Chen PJ, et al. Preconditioned hyperbaric oxygenation protects the liver against ischemia–reperfusion injury in rats. J Surg Res 2005;128:28–36.
Zúñiga J, Venegas F, Villarreal M, Núñez D, Chandía M, Valenzuela R, et al. Protection against in vivo liver ischemia reperfusion injury by n-3 long-chain polyunsaturated fatty acids in the rat. Free Radic Res 2010;44:854–63.