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# Combination of iron overload plus ethanol and ischemia alone give rise to the same endogenous free iron pool

Odile Sergent[1], Aldo Tomasi[2], Daniela Ceccarelli[2], Alberto Masini[2], Hans Nohl[3], Pierre Cillard[1], Josiane Cillard[1], Yuri A. Vladimirov[4] & Andrey V. Kozlov[5,*]
[1]*Laboratoire de Biologie Cellulaire et Vegetale, UPRES 3891, UFR des Sciences Pharmaceutiques et Biologiques, University of Rennes 1, 2 AVE du Pr. Léon Bernard, CS, 34317 35043, Rennes Cedex, France;* [2]*Biomedical Science Department, University of Modena, Via Campi, 287, 41100, Modena, Italy;* [3]*Research Institute for Biochemical Pharmacology and Toxicology, Veterinary University of Vienna, Veterinaerplatz 1, A-1210, Vienna, Austria;* [4]*Department of Biophysics, Russian State Medical University, Ostrovitjanova Str. 1, 117997, Moscow, Russia;* [5]*Ludwig Boltzmann Institute for Experimental and Clinical Traumatology, Donaueschinger Str. 13, A-1200, Vienna, Austria; *Author for correspondence (E-mail: Andrey.Kozlov@vu-wien.ac.at)*

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**Abstract**

Iron overload aggravates tissue damage caused by ischemia and ethanol intoxication. The underlying mechanisms of this phenomenon are not yet clear. To clarify these mechanisms we followed free iron ("loosely" bound redox-active iron) concentration in livers from rats subjected to experimental iron overload, acute ethanol intoxication, and *ex vivo* warm ischemia. The levels of free iron in non-homogenized liver tissues, liver homogenates, and hepatocyte cultures were analyzed by means of EPR spectroscopy. Ischemia gradually increased the levels of endogenous free iron in liver tissues and in liver homogenates. The increase was accompanied by the accumulation of lipid peroxidation products. Iron overload alone, known to increase significantly the total tissue iron, did not affect either free iron levels or lipid peroxidation. Homogenization of iron-loaded livers, however, resulted in the release of a significant portion of free iron from endogenous depositories. Acute ethanol intoxication increased free iron levels in liver tissue and diminished the portion of free iron releasing during homogenization. Similarly to liver tissue, the primary hepatocyte culture loaded with iron *in vitro* released significantly more free iron during homogenization compared to non iron-loaded hepatocyte culture. Analyzing three possible sources of free iron release under these experimental conditions in liver cells, namely ferritin, intracellular transferrin-receptor complex and heme oxygenase, we suggest that redox active free iron is released from ferritin under ischemic conditions whereas ethanol and homogenization facilitate the release of iron from endosomes containing transferrin-receptor complexes.

## Introduction

Iron is not only an essential element for normal cellular function and survival but it may paradoxically play also a fatal role under certain conditions. A number of human pathological conditions including ischemia-reperfusion injury, hemochromatosis, hemorrhagic shock, and certain neurodegenerative diseases are associated with anomalies in iron metabolism. In addition it has been shown that serious symptoms occurring in these diseases can be reversed in animals (Babbs 1985; Richardson & Ponka 1998) and in patients (Dragsten *et al.* 2000) by the administration of iron chelators. There are clinical, biochemical, and morphological evidences, indicating that iron

overload creates a background amplifying tissue damage caused by ischemia-reperfusion (Pucheu *et al.* 1993; Wu and Paller 1994; Turoczi *et al.* 2003; Mehta *et al.* 2004) and ethanol intoxication (Wisniewska-Knypl & Wronska-Nofer 1994; Minotti *et al.* 1995; Stal *et al.* 1996).

In contrast, results of epidemiological studies that relate iron stores to the risk of coronary heart disease have been inconsistent (Ascherio *et al.* 2001). Therefore, it has been hypothesized that iron markers common in epidemiologic studies, such as serum ferritin, transferrin saturation, total iron, or iron-binding capacity, are inappropriate to investigate harmful effects related to iron overload, since oxygen free radicals involved in iron mediated tissue damage are produced only by free iron, a small portion of intracellular iron which has a catalytic activity similar to that of ferrous ions (Lee & Jacobs 2004). It is already known from *in vitro* studies that ferrous ions quickly react with lipids inducing lipid peroxidation and give rise to hydroxyl radicals, which are able to damage all important biomolecules, namely lipids, proteins, and DNA (Aust *et al.* 1985; Arora & Gores 1996; Abalea *et al.* 1998; Abalea *et al.* 1999).

Therefore, free iron concentration is the reliable parameter of iron toxicity. Three main technical approaches have been adopted to detect endogenous free iron pool. One of them is based on the detection of low molecular iron complexes by HPLC (Ferrali *et al.* 1990), the second one applies a fluorescence probe (Epsztejn *et al.* 1997), and the last one is an electron paramagnetic resonance spectroscopy (EPR) assay (Kozlov *et al.* 1992). The HPLC method determines free iron levels in a low molecular weight chemical form in tissue and cell homogenates (Ferrali *et al.* 1990); the fluorescent assay can be used to determine free iron in non-homogenized cell cultures (Epsztejn *et al.* 1997), and the assay based on EPR spectroscopy detects free iron in cell homogenates and in whole tissues (Kozlov *et al.* 1992). The latter is based on the detection of nitrosyl non-heme iron complexes in the presence of an excess of nitrite and has been shown to detect the same iron pool as desferrioxamine, an iron chelator binding selectively endogenous free iron in the body (Kozlov *et al.* 1992; Kozlov *et al.* 1996).

The aim of this work is to clarify the mechanisms by which iron overload amplifies the tissue damage caused by ischemia-reperfusion and ethanol intoxication using advantages of EPR spectroscopy.

## Experimental procedures

### *Animal models*

Female Wistar albino rats (100–120 g body weight) were subjected to experimental siderosis by feeding a standard diet purchased from Piccioni (Brescia, Italy) supplemented with 2.5% (w/w) carbonyl iron (Fluka). Carbonyl iron is an extremely pure form of elemental iron physically shaped as microscopic spheres less than 5 $\mu$ in size. These animals were used as a model of iron overload. After 20, 40, and 60 days the animals were sacrificed by decapitation and the livers were extracted. *Ex vivo* model of warm liver ischemia was made in the same strain of rats. The rats were decapitated, then a midline laparotomy was performed, a clip was used to close the arterial and the portal venous vessels supplying blood to the left lobe of the liver; liver was extracted and stored in Ringer solution at 37 °C. Acute alcohol intoxication was induced by giving a single intraperitoneal injection of 3.5 g ethanol/kg of body weight as a 20% (w/w) solution in saline to control rats and rats kept on iron diet for 60 days.

A group of rats was i.p. injected with saline and used as controls. The animals were sacrificed by decapitation 6 h after injections. All experiments were performed under the conditions described in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (publication NIH 86–23; revised 1985).

### *Hepatocyte preparation*

Hepatocytes were isolated by perfusion of the liver as previously described (Guguen-Guillouzo *et al.* 1983) except that a liberase (Roche, France) solution (23.3 $\mu$g/ml) was used for dissociation of liver parenchymal cells instead of a collagenase solution. Following dissociation, cell viability was estimated by the trypan blue exclusion test and was above 90%. Cells were seeded at densities of $20 \times 10^6$ cells in 175 $cm^2$ Falcon® flasks with Eagle's minimum essential medium and 25% medium 199 with Hanks salts and containing: streptomycin (50 $\mu$g), penicillin (50 $\mu$g), bovine insulin (5 $\mu$g),

bovine serum albumin (1 mg) and $NaHCO_3$ (2.2 mg). After 3–4 h, the medium was changed and renewed with the same medium as above but deprived of serum and supplemented with $10^{-7}$ M dexamethasone. Sets of cultures were treated for 24 h with 100 $\mu$M ferric nitrilotriacetate (iron-NTA). Another set of cultures was pretreated with 10 mM $NH_4Cl$ for 4 h before the addition of 50 mM ethanol for 1.5 h. Simultaneously, control cultures were performed without $NH_4Cl$, ethanol or both.

*Free iron detection*

Free iron concentration in liver tissue was measured by EPR spectroscopy as described in our previous publication (Kozlov *et al.* 1992) using a nitrite-assay: sodium nitrite (0.05 ml, 8.7 M) was added to 0.8–0.9 g of liver tissue and incubated at 20 °C for 20 min. Nitric oxide (NO) produced from the reduction of sodium nitrite, gives rise to a dinitrosyl iron complex with endogenous free iron characterized by an EPR absorption at $g = 2.04$ (Vanin & Chetverikov 1968). To measure free iron content in liver homogenates or hepatocyte suspensions sodium nitrite (0.05 ml, 8.7 M) was added to 0.5 ml of homogenate or hepatocyte suspension. The mixture was incubated at 20 °C for 10 min and the subsequent procedure was the same as for whole liver. Free iron concentration was calculated on a calibration plot obtained by adding incremental volumes of $FeSO_{4*}7H_2O$ to tissue homogenate. The concentration of ferrous ions in the stock solution was measured using the o-phenanthroline assay. The homogenate was then treated as described above and EPR spectra were collected. Since no difference in the shape of the spectra was observed, the calibration curve was built using the magnitude of the EPR signals and the concentration of added iron. For experiments performed with hepatocyte cultures, free iron was chelated by deferiprone instead of nitrites.

*TBARS detection*

Thiobarbituric acid reactive substances (TBARS) were determined as previously described (Mihara *et al.* 1980) with minor modifications (Kozlov *et al.* 1991): 0.25 ml of the tissue homogenate was mixed with 3.0 ml of 1.5% phosphoric acid and 1.0 ml of 0.5% TBA. To prevent iron mediated oxidation 2 mM diethylenetriaminepentaacetic acid (DETAPAC) was added to each sample. Samples were incubated at 100 °C for 45 min then cooled at room temperature and extracted in 4.0 ml n-butanol; the mixture was stirred and centrifuged at 1800 g for 10 min. The upper butanol fraction was collected and absorbtion spectra were recorded in the region of 514–554 nm. The intensity of the 534 nm peak was measured with two base points at 514 and at 554 nm.

*Presentation of results and statistical methods*

The data are expressed as mean ± standard error of mean, and the statistical significance of differences was estimated by ANOVA. The significance level was set at 95%.

## Results

The livers of animals subjected to acute ischemia (1, 2, and 3 h, respectively) and to chronic experimental iron overload (20, 40, and 60 days, respectively) were tested at different time points of iron overload and ischemia in order to obtain comparable free iron levels in both models. Since it has been already documented that free iron levels in liver homogenates were increased under iron overload conditions (Ferrali *et al.* 1997), we used EPR spectroscopy to confirm Ferrali's result with an independent method. As expected, the typical spectra of non-heme nitrosyl complexes were observed in liver homogenates (Figure 1). It appeared that both ischemia and iron overload resulted in an increase of free iron levels in liver homogenates. The time necessary to reach comparable free iron levels was quite different being in a range of hours for ischemia and in a range of weeks for iron overload. Figure 2a shows free iron levels determined in non-homogenized liver and in liver homogenate. During the time course of ischemia and iron overload the levels of free iron in liver homogenates became approx. 5 fold greater compared to control tissues. Free iron levels in ischemic liver tissue were similar to those found in liver homogenates. On the contrary in iron-loaded livers, free iron levels in non-homogenized livers were lower than those detected in homogenates from the same livers. This is a clear indication that

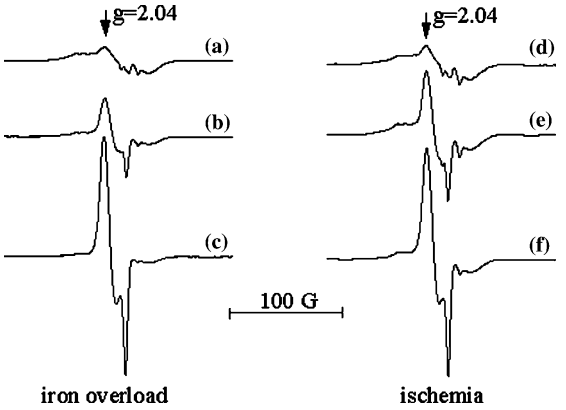


*Figure 1.* Low temperature EPR spectra of nitrosyl complexes observed in liver homogenate under ischemia and iron overload. The liver homogenate (1:2 w/v) was incubated 10 min with 7 M $NaN0_2$, placed in a Teflon cylindrical form and frozen in liquid nitrogen. "a" and "d" represent untreated liver in iron overload and ischemia experiments, respectively.; "b" and "c" are the typical EPR spectra observed after 40 and 60 days of iron overload, respectively; "e" and "f" are the typical EPR spectra observed after 2 and 3 h of ischemia, respectively.

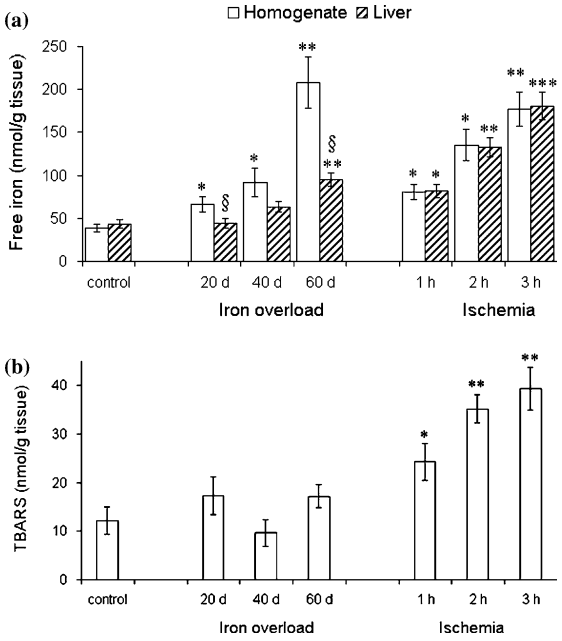


*Figure 2.* (a). The time course of free iron levels in liver homogenate and in non-homogenized liver during experimental iron overload and warm liver ischemia. The time axes indicate the time of liver ischemia and the time used to feed animals with iron-enriched diet for ischemia and iron overload models, respectively. *; **; *** – significantly different to control (* – $P<0.05$; ** – $P<0.005$; *** – $P<0.0005$). § – significantly different to homogenate ($P<0.05$). (b). The time course of TBARS in liver homogenate during experimental ischemia and iron overload. The time axes indicate the time of liver ischemia and the time used to feed animals with iron-enriched diet for ischemia and iron overload models, respectively. *; ** – significantly different to control (* – $P<0.05$; ** – $P<0.005$).

significant portion of free iron has been released during the mechanical homogenization procedure.

The measurement of lipid peroxidation products (TBARS) in liver homogenates (in the presence of an excess of iron chelator) revealed an accumulation of TBARS in ischemic livers whereas no significant changes of TBARS levels resulted in livers from rats subjected to iron overload (Figure 2b). This result suggests that the pool of iron accumulating in the liver during overload remains silent, in terms of catalytic activity, until the tissue is homogenized. During homogenization this iron is released from intracellular or extracellular compartments due to the mechanical disruption of biomembranes.

To ascertain whether the source of free iron released during homogenization is located inside the cells we have performed experiments with primary hepatocyte cultures, which obviously do not contain iron stores other than intracellular. Figure 3 shows that control hepatocytes did not release a significant portion of iron during homogenization. In contrast, a significant release of free iron was observed in iron loaded (100 $\mu$M iron-NTA) hepatocytes after homogenization, similarly to that in iron loaded hepatic tissue. This experiment demonstrates that free iron released during homogenization originates from intracellular compartments. It should be noted that no changes could be found, when hepatocytes were treated only with NTA (data not shown).

Since it is widely accepted that ethanol and its derivatives are able to modify the physical state of the cell membranes (Chin & Goldstein 1977; Polokoff *et al.* 1985), we intended to test whether ethanol had an effect on free iron content in iron loaded liver similar to that of homogenization. Figure 4 shows that ethanol facilitates the release of a small portion of iron in control liver and that this portion is drastically increased if ethanol intoxication is combined with iron overload.

Since a small portion of iron is released in liver of control animals treated with ethanol, we tried to reproduce this effect in hepatocyte culture. Hepatocytes were incubated 1.5 h with 50 mM ethanol with or without $NH_4Cl$, an inhibitor of iron release from endosomes (Paterson & Morgan 1980; Morgan 1981). We observed a significant

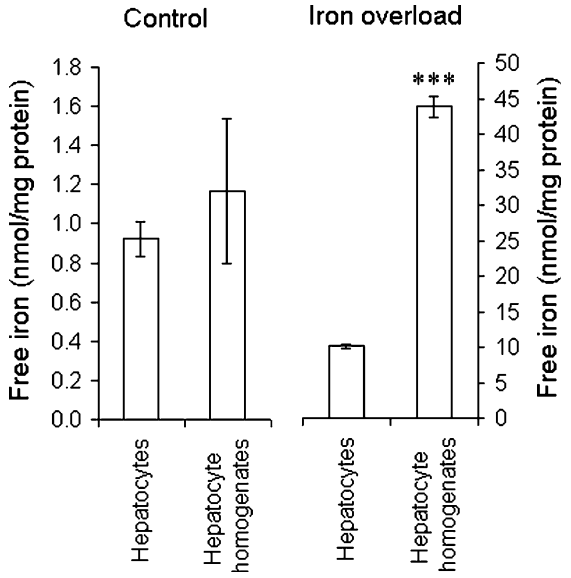


*Figure 3*. Effect of homogenization on the levels of endogenous free iron contents in control hepatocytes and hepatocytes loaded with iron. *** – significantly different to control ($P < 0.0005$).

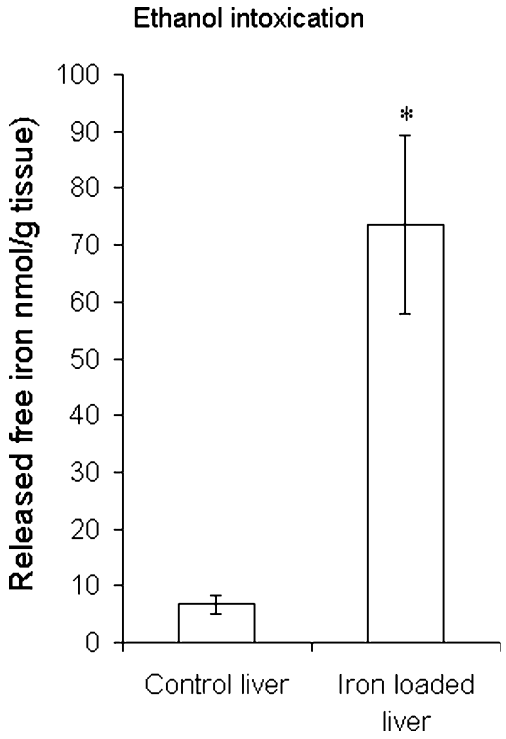


*Figure 4*. Effect of iron overload on free iron amount releasing during homogenation in liver obtained from control and iron loaded rats (60 days on) without further treatment and subjected to acute ethanol intoxication. * – significantly different to control ($P < 0.05$).

increase in the levels of endogenous free iron in ethanol treated cells (Figure 5). Addition of $NH_4Cl$ did not influence iron levels in control hepatocytes but it abolished the effect of ethanol. This confirms that ethanol facilitates the release of iron from endosomes.

## Discussion

Our results suggest that there are two different mechanisms elevating the levels of endogenous free iron in the liver during experimental iron overload and ischemia, respectively. In case of ischemia similar concentrations of free iron were detectable both in homogenized and not homogenized liver. In contrast, during iron overload the levels of free iron detectable in liver homogenate were significantly higher than those in non-homogenized liver. Since homogenization affects first of all membrane compartments we propose that this iron pool was originally kept in membrane compartments and released while these compartments were destroyed during homogenization. Another interesting phenomenon observed in this study is that iron overload combined with ethanol results in an increase of free iron levels already in non-homogenized liver, similarly to the effect of homogenization.

A prooxidant action of ethanol *in vivo* has already been documented. In biological systems ethanol has been shown to stimulate free radical reactions, resulting in the generation of hydroxyl radicals as the initial products (Albano *et al.* 1988; Reinke *et al.* 1991; Knecht *et al.* 1995; Rao *et al.* 1996; Sun *et al.* 2002). It is well known that hydroxyl radicals are produced in the reaction between iron and hydrogen peroxide (Fenton reaction). Therefore it is expected that ethanol should elevate either hydrogen peroxide production or catalytically active iron levels or both. It has been already shown that ethanol stimulates superoxide radical production in microsomes (Albano *et al.* 1988). This can be the mechanism elevating the levels of hydrogen peroxide, which is formed via disproportionation of superoxide radicals. Here we show that there is an additional mechanism amplifying ethanol toxicity, ethanol mediated release of an additional portion of iron from intracellular membrane compartments. We have shown that iron accumulates during iron overload in a store, which looses the iron during

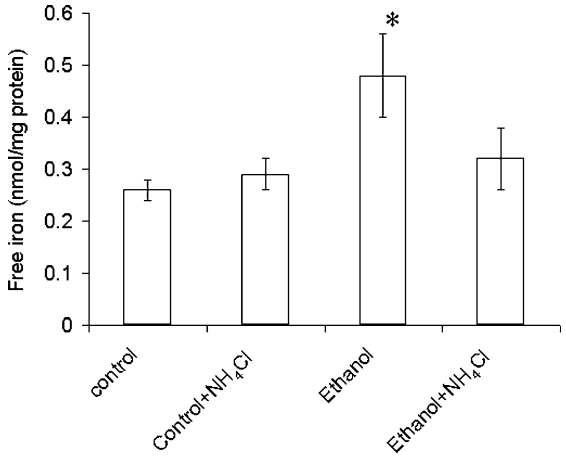


*Figure 5*. Effect of ethanol and $NH_4Cl$ on the levels of free iron in hepatocyte culture. The hepatocytes were incubated for 90 min without (control) and with 50 mM ethanol (ethanol). 10 mM of $NH_4Cl$ was added to both control (control + $NH_4Cl$) and ethanol (ethanol + $NH_4Cl$) groups. * – Significantly different to all other groups. $P < 0.05$ to all other groups.

homogenization. Similar results were obtained with primary hepatocyte culture indicating that this is an intracellular pool located in hepatocytes.

There are three sources that can elevate intracellular free iron levels, namely ferritin, an endogenous store of iron, transferrin, a protein transporting iron through the blood and into the cells, and heme oxygenase (HO) releasing iron from heme-proteins. HO requires oxygen and can not operate under ischemic conditions. It is known that the levels of CYP2E1, a cytochrome P450 monooxygenase, are elevated after acute and chronic alcohol treatment (Kessova & Cederbaum 2003). In turn, high activity of CYP2E1 can induce HO expression (Gong *et al*. 2003), but this process takes weeks and cannot be the case in acute ethanol intoxication. Therefore, HO is not likely an iron source either during ischemia or in acute ethanol intoxication. Since iron overload is accompanied by iron transport via transferrin from the blood into the cells this is an important source of intracellular iron (Aisen *et al*. 2001), but not for ischemic tissue. There is no or low blood flow through ischemic organs, consequently free iron source(s) should have intracellular location. This fact suggests that ferritin is the most probable source of free iron under ischemic conditions.

Previously published and presented here data allow us to suggest the following mechanism of free iron metabolism during iron overload combined with acute ethanol intoxication and ischemia (Figure 5). The uptake of iron via transferrin receptors involves two phases. The first is the accumulation of transferrin iron complexes in endosomes, and the second is the reductive release of iron from transferrin (Thorstensen & Romslo 1990; Scheiber & Goldenberg 1993), requiring reducing agents such as NAD(P)H (Scheiber & Goldenberg 1993) and re-cycling of transferrin receptors back to the cell surface. Since the levels of reducing agents is reduced under iron overload conditions (Pietrangelo *et al*. 1995) one can expect that iron overload leads to the accumulation of transferrin-iron complexes in endosomes. These complexes are dissociated during homogenization due to availability of reducing agents from other subcellular compartments. The transferrin mediated mechanism of iron absorption is regulated by the number of transferrin receptors expressed on the cell surface (Young & Aisen 1981). These receptors, however, are down-regulated during iron overload (Lu *et al*. 1989; Richardson *et al*. 1999). An alternative mechanism occurring in iron overload is the uptake of non-transferrin bound iron (Brissot *et al*. 1985). These complexes have been shown to occur in iron overload related diseases (Hershko *et al*. 1978; Batey *et al*. 1980).

In contrast to iron overload, free iron released during ischemia becomes catalytically active immediately after release, catalyzing lipid peroxidation. We suggest that ferritin is a possible source of this iron (Figure 6). It is well known that ischemia rapidly causes an increase in cellular NADH levels (Obi-Tabot *et al*. 1993; Thorniley *et al*. 1996;

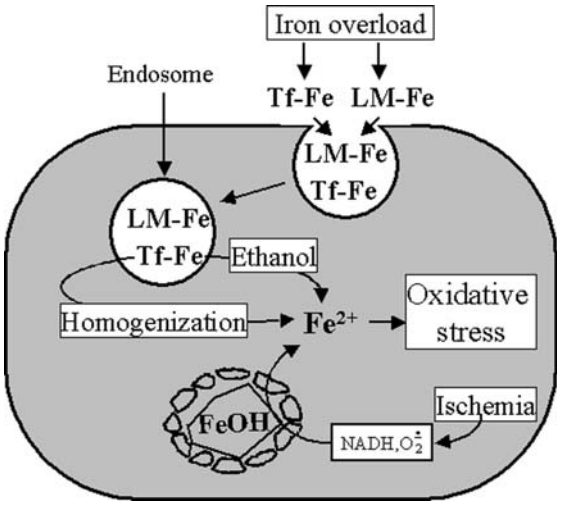


*Figure 6*. Two pathways of iron metabolism induced by iron overload and by ischemia respectively. LM-Fe – low molecular iron complexes; Tf-Fe – transferrin iron complexes.

Fellman & Raivio 1997) and that NADH or NADPH can serve as reducing co-factors for the enzyme-mediated reduction of ferritin iron (Topham *et al*. 1989; Jaeschke *et al*. 1992). Flavin nucleotide has been suggested to shuttle the electrons to the ferritin iron (Topham *et al*. 1989). The release of iron from ferritin can be due to direct reduction of iron by NADH/NADPH, or can be mediated by superoxide radical generated by NADH/NADPH dependent enzymes (Bando & Aki 1990).

We did not detect significant increase in TBARS in iron-loaded liver, but there was a strong trend at least at 20 and 60 days of treatment (Figure 2). Prolonged exposure of intracellular organelles to oxidative stress reactions could have increased the fragility of intracellular membranes, which in turn would have facilitated the release of storage iron from membrane compartments (Figure 4). As we show here, acute ethanol intoxication combined with iron overload facilitates the release of iron. It is well known that alcohol-induced hepatotoxicity is primarily due to membrane damage induced by the direct solvent properties of the alcohols or its derivatives (Strubelt *et al*. 1999). This seems to be one of the mechanisms by which ethanol induces the release of iron accumulated during iron overload (Figure 6). An alternative mechanism could be ethanol-induced release of iron from transferrin–transferrin receptor complex. To prove whether ethanol facilitates the release of iron from endosomes, we performed a series of experiments using $NH_4Cl$, an inhibitor of iron release from endosomes. From the Figure 4, one can see that $NH_4Cl$ abolishes ethanol mediated iron release. This confirms the ''endosomal'' nature of iron released during ethanol intoxication. We propose that the release of iron from intracellular storage is the mechanism that amplifies liver disease if ethanol intoxication is combined with iron overload, a phenomenon documented in biochemical and clinical studies (Pietrangelo 2003). Some evidences presented in this study can be done only in hepatocyte culture. Therefore, the validation of the mechanisms suggested here for *in vivo* situations requires further studies.

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