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Iron-Induced Oxidant Stress Leads to Irreversible Mitochondrial Dysfunctions and Fibrosis in the Liver of Chronic Iron-Dosed Gerbils. The Effect of Silybin[1]

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Hepatic iron toxicity because of iron overload seems to be mediated by lipid peroxidation of biological membranes and the associated organelle dysfunctions. However, the basic mechanisms underlying this process *in vivo* are still little understood. Gerbils were dosed with weekly injections of iron–dextran alone or in combination with sylibin, a well–known antioxidant, by gavage for 8 weeks. A strict correlation was found between lipid peroxidation and the level of desferrioxamine chelatable iron pool. A consequent derangement in the mitochondrial energy-transducing capability, resulting from a reduction in the respiratory chain enzyme activities, occurred. These irreversible oxidative anomalies brought about a dramatic drop in tissue ATP level. The mitochondrial oxidative derangement was associated with the development of fibrosis in the hepatic tissue. Silybin administration significantly reduced both functional anomalies and the fibrotic process by chelating desferrioxamine chelatable iron.

KEY WORDS: Iron; oxidant stress; liver mitochondria (gerbil).

INTRODUCTION

Chronic iron overload in humans is associated with hepatocellular damage and fibrosis, ultimately leading to cirrhosis (Bassett *et. al.*, 1984; Friedman, 1993). Although toxicity of excess iron has been well-established clinically (Bothwell and Isaacson, 1962; Barry *et al.*, 1974; Bassett *et al.*, 1986), the specific cytopathological mechanisms whereby hepatic cells are injured remain to be fully elucidated (Powell, 1985). A proposed mechanism by which cellular toxicity may occur in response to excess iron involves oxidant stress, including lipid peroxidation of biological membranes, resulting in functional impairment of hepatic subcellular organelles (Hanstein *et al.*, 1981; Bacon *et al.*, 1983, 1986, 1989; Masini *et al.*, 1984, 1989; Galleano and Puntarulo, 1992; Pietrangelo *et al.*, 1995). However, some report does not support this view (Tangeras, 1983). Furthermore, a correlation between lipid peroxidation and total iron level does not appear to exist from all these studies. The form of intracellular iron responsible for the induction of oxidant stress is still unknown. The proposal has been put forward that an increase in the intracellular transit pool of iron may account for the induction of lipid peroxidation (Thomas *et al.*, 1985; Britton *et al.*, 1990). In fact, this pool of low-molecular weight iron chelatable by desferrioxamine (DFO-chelatable iron) is catalytically active in initiating free-radical reactions and lipid peroxidation (Halliwell and Gutteridge, 1986).

[1] Key to abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; ALT, alanine aminotransferase; BHT, butylated hydroxytoluene; DFO, desferrioxamine, $\Delta\Psi$, transmembrane electrical potential; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone; MDA, malondialdehyde; ROS, reactive oxygen species; TPP^+, tetraphenylphosphonium chloride.

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An aspect which merits consideration is that functional and structural alterations so far reported have been obtained in an experimental model of chronic dietary iron overload in the rat after a maximal period of treatment of 3 months. It is noteworthy that this species requires the feeding of a diet incorporated carbonyl iron for at least 12 months before hepatic fibrosis is produced (Park *et al.*, 1987). More recently, a new experimental model has been presented where the parenteral administration of iron dextran to gerbils brings about the development of hepatic fibrosis in 8 weeks (Carthew *et al.*, 1991).

In the present research, we have measured the total and DFO-chelatable iron concentration in the hepatic tissue and in the mitochondrial fraction in an experimental model of an iron-dosed gerbil, to identify the form of iron responsible for the induction of oxidant stress. We have then investigated the effect of oxidant stress on the mitochondrial functions and the progression of fibrosis. To better clarify these aspects, we have also performed these measurements on iron-dosed gerbils contemporary administered silybin, a well-known antioxidant with possibly iron-chelating activity (Pietrangelo *et al.*, 1995).

MATERIALS AND METHODS

Animals

Male gerbils (6 weeks of age) were housed in stainless steel cages and divided into four groups; group A: gerbils were given an initial subcutaneous injection of iron-dextran (1 mg/gm body wt) followed at weekly intervals by seven successive doses of half the amount given initially ($n = 12$); group B: gerbils subcutaneously dosed with dextran alone; group C: gerbils subcutaneously dosed with iron-dextran and treated by gavage with 100 mg/kg body wt/day silybin (740 mg silybin-β-cyclodestrin complex/kg body wt/day) suspended in 10% acacia solution for 8 weeks; group D: gerbils subcutaneously dosed with dextran alone and treated with silybin. A further group of animals was gavaged with the vehicle used to administer silybin (i.e., 10% acacia solution). Treated and control animals fasting overnight were killed after 8 weeks by cervical dislocation and the livers rapidly removed and used for morphological, biochemical, and functional studies.

Mitochondrial Studies

Preparation of Mitochondrial Fraction

A portion of the liver was used for preparation of mitochondrial fraction in 0.25 M sucrose according to a standard procedure (Masini *et al.*, 1983). The cytosolic contamination in the mitochondrial preparation obtained from either control or siderotic gerbils did not exceed 2% of the total protein content, as assessed by recovery of marker enzymes [i.e., glutamate dehydrogenase and cytocrome *c* oxidase for mitochondria and acid phosphatase and *N*-acetylglucosaminidase (NAGA) for lysosomal contamination (Botti *et al.*, 1989; Gianetto and deDuve, 1955)]. Protein concentration was determined by the biuret method with bovine serum albumin as a standard.

Lipid Peroxidation

Thiobarbituric acid-reactive substances (TBARS) in the mitochondrial fraction, as index of malondialdehyde (MDA) accumulation, were measured by the thiobarbituric acid method in the presence of 0.1% (w/v) butylated hydroxytoluene and 0.5 mM $FeCl_3$ added to the sample immediately before the addition of the thiobarbituric acid mixture (Tangeras, 1983).

Mitochondrial Transmembrane Electrical Potential

The standard incubation medium had the following composition: 100 mM NaCl, 5 mM sodium-potassium phosphate buffer (pH 7.4), 10 mM Tris-HCl buffer (pH 7.4), and 10 mM $MgCl_2$. The transmembrane electrical potential ($\Delta\psi$) was measured at 25°C, in a final volume of 1.5 ml of incubation medium containing 20 μM tetraphenylphosphonium chloride (TPP^+), by monitoring with a TPP^+ selective electrode, the movements of TPP^+ across the membrane according to Kamo *et al.* (1979). The respiratory states were those defined by Chance and Williams (1956) on the basis of the factors limiting the respiration.

Mitochondrial Desferrioxamine (DFO) Chelatable Iron

Mitochondrial DFO-chelatable iron concentration was measured as desferrioxamine–iron complex (FO) as previously described (Ferrali *et al.*, 1989) Briefly, mitochondrial samples (60–90 mg protein ml) containing 25 μM desferrioxamine were lysed by freezing and thawing. Mitochondria were centrifuged at 100,000 × *g* for 30 min. The supernatant was ultrafiltered by membrane cones (Centriflo CF 25, Amicon). The DFO–iron content of the aproteic ultrafiltrates was measured by an HPLC method (Kruck *et al.*, 1985).

Total Iron Determination

Iron content in samples from liver tissue and mitochondrial fraction was analyzed by atomic absorption spectroscopy, as previously reported (Cairo *et al.*, 1989).

Enzyme Assays

Succinate dehydrogenase and cytochrome oxidase activity was measured polarographically in freeze-thawed mitochondria as described in Moreno and Madeira (1991) at pH 7.4 and 25°C. Unless otherwise stated, 0.3 mg of lubrol/mg protein were present in the incubation medium (Muscatello and Carafoli, 1969). Succinate: cytochrome *c* reductase and rotenone-sensitive NADH-cytochrome *c* reductase activities were determined spectrophotometrically, 550-nm freeze-thawed mitochondria (pH 7.4, 25°C) as described in Tisdale (1967) and Hatefy and Stiggal (1987). The rotenone-sensitive NADH-cytochrome *c* reductase rate was calculated by subtracting the rotenone-insensitive rate, *i.e.*, in the presence of 2.5 μM rotenone, from the overall rate.

Measurement of Hepatic Adenosine Triphosphate. Hepatic adenosine triphosphate concentration was measured in neutral extract using the high-performance liquid chromatography method of Stocchi *et al.* (1985) with some modifications as described in Pietrangelo *et al.* (1995) in a Hewlett-Packard 1090 liquid chromatography equipped with diode-array detector (Hewlett-Packard, Palo Alto, CA).

Hepatocellular Integrity. Liver damage was assessed by measuring gerbil serum alanine aminotransferase (ALT) activity by a GPO–PAP high-performance reagent set purchased from Boehringer Mannheim (Milano, Italy). The method was adapted to a clinical chemistry automated analyzer Hitachi 737 (Hitachi Ltd., Tokyo, Japan).

Light Microscopy Studies. Thin liver slices were cut, fixed in a 4% solution of paraformaldehyde in 0.1 M phosphate buffer (PBS), pH 7.4, and embedded in paraffin. Sections 5μm were prepared and stained with hematoxylin and eosin and Mallory and Perls' Prussian blue.

Reagents. Iron-dextran was purchased from Sigma Chemical Co., St. Louis, MO. All chemicals and reagents were of the highest available purity.

Statistical Analyses. All data are expressed as mean ± SD. Results were analyzed using a two-way analysis of variance to determine differences between the various treatments (control, iron, silybin, and iron plus silybin) with a level of significance of $p < .05$.

RESULTS

The hepatic total iron content of chronic iron treated gerbils over an 8-week period was largely increased compared to control (Table I). No significant reduction in the total iron was observed when silybin was contemporary administered with iron. The body and the liver weight were not appreciably affected by iron treatment, nor was hepatic cell viability.

The following sections report the results of biochemical and functional analyses. Data of the group treated with the 10% acacia solution alone were not significantly different from those of control groups B or D and were not reported.

Iron and Lipid Peroxidation

A major role in generation of noxious reactive oxygen species (ROS) in the cell is now ascribed to the so-called "chelatable iron-pool" (Rothman *et al.*, 1992; Ferrali *et al.*, 1990; Kozlov *et al.*, 1992; Breuer

Table I. Liver Total Iron, Weight Parameters, and Serum ALT Activities in Various Experimental Groups[a]

Experimental condition	Total iron (μg/gm wet wt)	Body weight (gm)	Liver weight (gm)	ALT (U/L)
Control	312 ± 46	63 ± 7	1.3 ± 0.2	78 ± 16
Iron	9672 ± 280[b]	56 ± 4	1.3 ± 0.1	93 ± 20
Iron + silybin	9637 ± 301[b]	58 ± 5	1.4 ± 0.2	77 ± 15

[a] Total iron and ALT analyses were performed as described in the section on Materials and Methods. Data are expressed as mean ± SD of three experiments performed on a pool of four animals.
[b] $p < 0.001$ in comparison to control.

et al., 1995). Yet, mitochondria are essential in the handling of iron and represent an important source of ROS in physiological and pathophysiological states (Fisher, 1987). For these reasons, we studied the effect of hepatic iron overload alone or in combination with silybin treatment on the level of this critical iron pool, on lipid peroxidation, and energy functions in the mitochondrial fraction. The content of total iron as well as of DFO-chelatable iron in the hepatic mitochondrial fraction largely increased following iron overload. The *in vivo* administration of silybin did not significantly modify the level of total iron (Table II). By contrast, silybin was able to significantly reduce the amount of DFO-chelatable iron. The same table shows that MDA content of mitochondrial membranes from siderotic gerbils was largely higher than that of controls. It can also be observed that the combined treatment of silybin and iron significantly reduced MDA level, although not up to the control value.

Mitochondrial Oxidative Metabolism

Mitochondria play a pivotal role in the energy production by oxidative phosphorylation process within the cell. Therefore, we studied the effect of hepatic iron overload alone or in combination with silybin on mitochondrial functional efficiency by measuring the mitochondrial transmembrane electrical potential ($\Delta\psi$), a parameter that gives direct indication of the energy-transducing capability of the inner membrane. Figure 1A shows that liver mitochondria from control gerbils on addition of an oxidizable substrate, such as succinate, developed a $\Delta\psi$ of about 197 mV (State 4). The addition of ADP, which induced the metabolic transition to State 3, caused an immediate decrease to 154 mV, which corresponds to the energy used for ATP synthesis. When the phosphorylation cycle was completed (approximately 3 min), membrane potential returned to the pre-ADP level. At this point, addition of an uncoupler, such as FCCP immediately caused $\Delta\psi$ to collapse. By contrast, mitochondria from iron-treated gerbils (Fig. 1B), in the presence of succinate (State 4), developed a maximum $\Delta\psi$ of 164 mV, a value substantially lower than that of control. Furthermore, the membrane potential, after the drop to 149 mV caused by ADP, remained at this decreased level. The subsequent addition of oligomycin, a well-known inhibitor of respiration tightly coupled to phosphorylation (Masini *et al.*, 1983), made the $\Delta\psi$ increase up to 170 mV. On the contrary, the addition of either an antioxidant, such as BHT, or of an iron chelator,

Table II. Total Iron, DFO-Chelatable Iron, and TBA-RS in the Hepatic Mitochondrial Fraction from Iron-Treated Gerbils[a]

Experimental condition	Total iron (nmol/mg protein)	DFO-chelatable iron (pmol/mg protein)	TBA-RS (nmol/mg protein)
Control	33 ± 5	273 ± 38	0.187 ± 0.015
Iron	456 ± 42[b]	2482 ± 220[b]	1.208 ± 0.11[a]
Iron + silybin	467 ± 36[b]	1588 ± 160[b,c]	0.711 ± 0.11[b,c]

[a] Total iron, DFO-chelatable iron, and TBA-RS analyses were performed as described in the section on Materials and Methods. Data are expressed as mean ± SD of three experiments performed on a pool of four animals.
[b] $p < 0.01$ in comparison to control.
[c] $p < 0.05$ in comparison to iron.

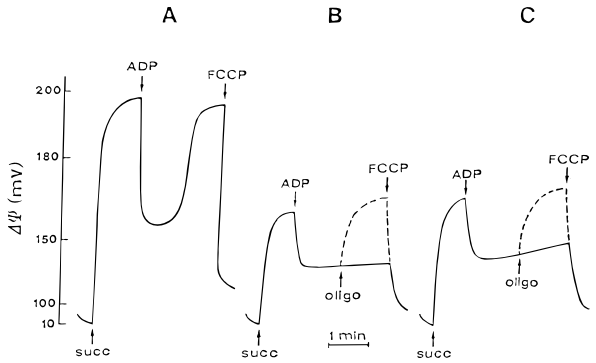

Fig. 1. Membrane potential during a complete cycle of phosphorylation in liver mitochondria from iron-dosed gerbils. Mitochondria (2 mg/ml) were incubated in the standard incubatium medium containing 5 μM rotenone for 2 min and then energized by the addition of 2.5 mM succinate (succ.). The arrows indicate the following addition: 0.25 mM adenosine diphosphate (ADP), 0.2 μM carbonyl cyanide-*p*-trifluoromethoxyphenylydrazone (FCCP), 2 μg/mg protein oligomycin (oligo). (A) control mitochondria; (B) mitochondria from iron-treated gerbils; (C) mitochondria from iron-treated and silybin-adminestered gerbils. The mitochondrial membrane potential was measured by a tetraphenylphosphonium selective electrode as described in the section on Materials and Methods. The traces shown are representative of at least three different experiments performed on a pool of four animals.

such as DFO, did not modify the $\Delta\psi$ trace (not shown). At this point, addition of FCCP promptly collapsed $\Delta\psi$. It is noteworthy, that the State 4 respiratory rate presented by these mitochondria was significantly lower than that of control mitochondria, as well as the increase in oxygen consumption following the addition of either ADP or FCCP (not shown). The contemporary administration of silybin to iron-treated gerbils slightly ameliorated membrane potential anomalies during a complete phosphorylation cycle (Fig. 1C).

A generalized reduction in the respiratory chain enzyme activities, *i.e.*, NADH-cytochrome *c* reductase (complex I + III), succinate: cytochrome *c* reductase (complex II + III), cytochrome *c* oxidase (complex IV), and succinate dehydrogenase (complex II), appeared to be associated with iron treatment (Table III). The contemporary administration of silybin partially prevented this decrease.

The functional integrity of cytochrome *c* oxidase activity depends on the presence of intact phospholipids, especially cardiolipin (Tzagoloff and MacLennan, 1965). The presence or the absence in the standard reaction medium of lubrol, a nonionic detergent, which facilitates the substrate accessibility, may give insight into the structural integrity of the inner mitochondrial membrane (Muscatello and Carafoli, 1969). Figure 2 shows that chronic iron administration to gerbils largely reduced the extent of lubrol activation of cytochrome *c* oxidase activity in freeze-thawed liver mitochondria in comparison to control (7 and 62%, respectively). The contemporary administration of silybin significantly increased the lubrol activation effect in comparison to iron alone. Similar results were obtained for succinate dehydrogenase activity (not shown).

Hepatic Adenosine Triphosphate Content

Associated with the mitochondrial functional derangement due to iron treatment, a dramatic decrease in the hepatic ATP content, *i.e.*, 0.39 ± 0.05 μmol/gm wet wt in comparison to 1.98 ± 0.11 μmol/gm wet wt of control, was detected in intoxicated gerbils (Fig. 3). Silybin administration partially prevented the ATP decrease.

Liver Fibrogenesis

In gerbils, chronic iron overload caused a dramatic proliferation of preexisting hemorragic necrotic foci and led to a dramatic accumulation of collagen with appearance of typical nodularity (Fig. 4B). Interestingly, silybin treatment significantly prevented collagen accumulation, although it was unable to restore a normal architecture (Fig. 4C).

DISCUSSION

In the present experimental model of heavy iron overload, the enhancement of lipid peroxidation in mitochondrial membranes appears to be correlated with the expansion of the catalytically active DFO-chelatable iron pool in gerbil liver. The observation that the protective action of silybin against iron-induced oxidative injury strictly depends on its capability to counteract the rise of mitochondrial chelatable iron level, in spite of unchanged total liver iron burden, gives experimental support to the above conclusion. Lipid peroxidation may lead to modifications of the mitochondrial inner membrane functional integrity. The evaluation of transmembrane potential clearly reveals an irreversible derangement in the energy-transducing capability of the inner membrane. The low polarization of the inner membrane after the addition

Table III. Enzyme Activities of the Respiratory Chain in the Hepatic Mitochondrial Fraction from Iron-Treated Gerbils[a]

	Control	Iron	Iron + silybin
NADH-cytochrome *c* reductase (nmol-min^{-1}-mg^{-1} protein)	139 ± 10	52 ± 7[b]	79 ± 9[b,c]
Succinate cytochrome *c* reductase (nmol-min^{-1}-mg^{-1} protein)	204 ± 16	49 ± 15[b]	100 ± 12[b,c]
Cytochrome *c* oxidase (ngAO-min^{-1}-mg^{-1} protein)	511 ± 37	221 ± 26[b]	308 ± 21[b,c]
Succinate dehydrogenase (ngAO-min^{-1}-mg^{-1} protein)	94 ± 7	42 ± 5[b]	67 ± 5[b,c]

[a] Enzyme activities were measured as described the subsection on freeze-thawed as described in the section on Materials and Methods. Data are expressed as mean ± SD of three experiments performed on a pool of four animals.
[b] $p < 0.01$ in comparison to control.
[c] $p < 0.05$ in comparison to iron.

of succinate, as well as the lack of the ability to repolarize after the drop due to ADP addition, suggests an irreversible decline in the respiratory chain capability. This view is supported by the finding that the addition of oligomycin, which inhibits both the proton backflow through the ATP synthetase and the ATP synthetase activity (Azzi and Santato, 1970), allows only a complete recovery of $\Delta\Psi$ to the pre-ADP steady state level, but not to that of the control. The low respiratory rate measured in the presence of the uncoupler FCCP, which should bring about the maximum rate of electron flow through the respiratory chain, agrees with the above conclusion.

The reduction in the enzyme activities of all respiratory chain complexes appears to be responsible for the irreversible membrane potential anomalies. In fact, a reduction in the enzyme activity from complex I to complex III (NADH-cytochrome *c* reductase), from complex II to complex III (succinate: cytochrome *c* reductase), of complex IV (cytochrome *c* oxidase), and of complex II (succinate dehydrogenase) has been observed. Many mechanisms may account for the reduction in enzyme activity. Iron-induced peroxidative damage to the polyunsaturated fatty acids of inner membrane phospholipids, especially cardiolipin, may reduce cytochrome *c* oxidase activity (Fry and Green, 1980). The lack of lubrol activation of both cytochrome *c* oxidase and succinate dehydrogenase activity in iron-loaded gerbils, may suggest that the inner membrane organization has been affected. Alternatively, this latter result may indicate that reactive aldehydic products of lipid peroxidation form adducts with proteins, thereby inactivating them. It has been reported that the presence of 4-hydroxy-2-nonenal (Dianzani, 1982), within a micromolar range, strongly impaired mitochondrial oxidative metabolism. In addition, another possibility

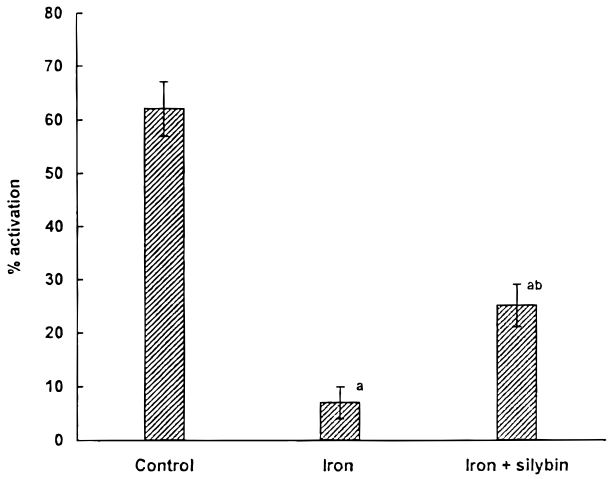

Fig. 2. Extent of lubrol activation of cytochrome *c* oxidase activity in iron-treated gerbils. The percentage changes from the values of enzyme activity measured in the absence of lubrol in the reaction medium are shown. All other conditions as in Table III.

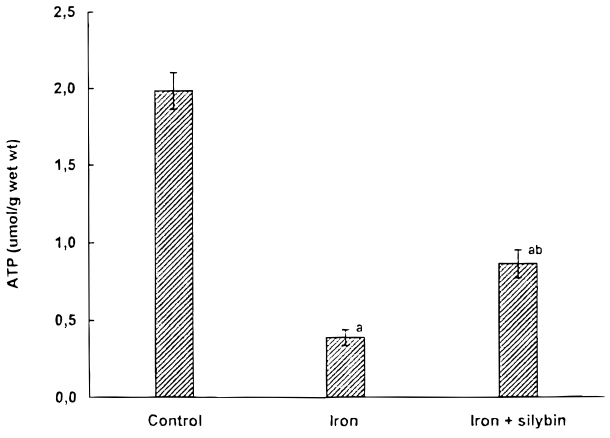

Fig. 3. Adenosine triphosphate level in the hepatic tissue of iron-treated gerbils. Adenosine triphosphate analysis was performed as described in the section on Materials and Methods. Data are expressed as mean ± SD of three experiments performed on a pool of four animals a, $p < 0.01$ in comparison to control; b, $p < 0.05$ in comparison to iron.

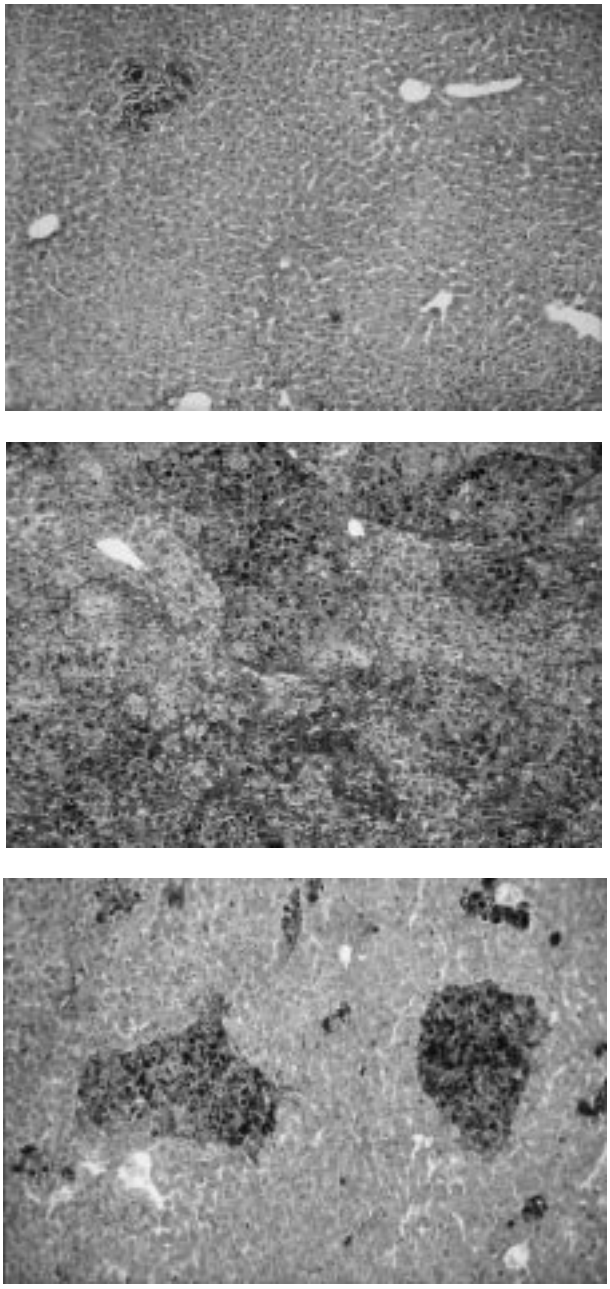

Fig. 4. Collagen accumulation in iron-treated gerbils. Liver sections from control (A), iron-treated (B), and iron-treated silybin-gavaged (C) gerbils were processed for Mallory stain. Iron induced a dramatic accumulation of collagen with typical nodularity appearing after 8 weeks (B). Silybin significantly prevented fibrosis (C). Original magnification: A,B,C = 133×.

is that iron-induced free radicals directly attack the protein components of the enzyme complexes. A reduction in the synthesis of respiratory chain enzymes may be reasonably excluded, at least for cytochrome *c* oxidase and succinate dehydrogenase, as indicated by the lack of lubrol activation.

The depressed mitochondrial oxidative metabolism may reasonably account for the large decrease of 80% in the hepatic ATP concentration observed in iron-treated gerbils. It is worthy to note that in spite of this very low ATP level, no appreciable cell damage is observable as indicated by ALT values. This may suggest that a readjustment in energy metabolism to a lower level has occurred.

Mitochondria represent a primary target of oxidative injury, but at the same time, the main source for reactive oxygen species (ROS)(Chance *et al.*, 1979). It is reasonable to assume that the destabilization of one or more components of the electron transport chain, occurring after the rise in the level of chelatable iron, enhances the potential for autoxidation and increases the production of O^{2-} and other ROS (Dryer *et al.*, 1980), so exacerbating the effects of iron toxicity.

The *in vivo* administration of silybin, a flavonoid with ROS scavenger activity, in combination with dietary iron overload in rats, has been recently reported to fully counteract hepatic mitochondrial oxidative damage and ATP decrease but not to significantly modify the level of total iron (Pietrangelo *et al.*, 1995). These studies further demonstrated that total iron level should not be regarded as the causal factor for oxidant stress. A role for desferrioxamine chelatable iron pool (Thomas *et al.*, 1985; Halliwell and Gutteridge, 1986; Rothman *et al.*, 1992; Ferrali *et al.*, 1990; Kozlov *et al.*, 1992; Breuer *et al.*, 1995) in stimulating lipid peroxidation of hepatic organelles in siderotic rats has been proposed (Britton *et al.*, 1990). The observation that the protective action of silybin against iron-induced oxidative injury strictly depends on its capability to counteract the rise of mitochondrial chelatable iron level, gives direct experimental evidence to the above proposal. These results also give an indication that the well known "antioxidant" activity of silybin, and possibly of other flavonoids, may be due to a "primary" antioxidant effect, that is, chelation of iron, a main catalyst of free-radical reactions in the cell. In this vein, it has been previously shown that α-tocopherol, a secondary antioxidant, is unable to ameliorate hepatic mitochondrial lipid peroxidation in an experimental model of chronic dietary iron overload (Bacon *et al.*, 1989). The partial protective action against oxidant stress exhibited by silybin, may be due to the fact that the hepatic concentration of this antioxidant, resulting from the present treatment, is

able to chelate iron up to a maximum of about 1000 pmol/mg protein.

At variance with other experimental animal models, where hepatic fibrosis has been found after prolonged periods of heavy iron loading (Park *et al.*, 1987; Iancu *et al.*, 1987), in the present gerbil model of iron overload, fibrosis has been demonstrated to occur in 8 weeks, in agreement with previous reports (Carthew *et al.*, 1991; Pietrangelo *et al.*, 1995). The finding that silybin administration significantly ameliorates the severity of fibrosis may suggest that the pathological sequence from chronic iron overload to fibrosis in the liver is via iron-induced lipid peroxidation and cellular organelle damage.

Although more studies are needed to modulate the hepatic silybin concentration *in vivo*, our present findings, in the prospect for the development of an effective control of "free-radical"-associated human diseases, sustain the rationale for the clinical application of silybin during human iron-overload states.

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