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Reduction of Carbon Tetrachloride-Induced Rat Liver Injury by IRFI 042, a Novel Dual Vitamin E-Like Antioxidant

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Carbon tetrachloride (CCl_4)-induced hepatotoxicity is likely the result of a CCl_4-induced free radical production which causes membrane lipid peroxidation and activation of transcription factors regulating both the TNF-α gene and the early-immediate genes involved in tissue regeneration. IRFI 042 is a novel vitamin E-like compound having a masked sulphydryl group in the aliphatic side chain. We studied the effect of IRFI 042 on CCl_4-induced liver injury. Liver damage was induced in male rats by an intraperitoneal injection of CCl_4 (1 ml/kg in vegetal oil). Serum alanine aminotransferase (ALT) activity, liver malondialdehyde (MAL), hydroxyl radical formation (OH·), calculated indirectly by a trapping agent, hepatic reduced glutathione (GSH) concentration, plasma TNF-α, liver histology and hepatic mRNA levels for TNF-α were evaluated 48 h after CCl_4 administration. Hepatic vitamin E (VE) levels were evaluated, in a separate group of animals, 2 h after CCl_4 injection. A control group with vitamin E (100 mg/kg) was also treated in order to evaluate the differences versus the analogue treated groups.

Intraperitoneal injection of carbon tetrachloride produced a marked increase in serum ALT activity (CCl_4= 404.61 ± 10.33 U/L; Controls= 28.54 ± 4.25 U/L), liver MAL (CCl_4= 0.67 ± 0.16 nmol/mg protein; Controls= 0.13 ± 0.06 nmol/mg protein), OH^- levels assayed as 2,3-DHBA (CCl_4= 8.73 ± 1.46 μM; Controls= 0.45 ± 0.15 μM) and 2,5-DHBA (CCl_4= 24.61 ± 3.32 μM; Controls= 2.75 ± 0.93 μM), induced a severe depletion of GSH (CCl_4= 3.26 ± 1.85 μmol/g protein; Controls= 17.82 ± 3.13 μmol/g protein) and a marked decrease in VE levels (CCl_4= 5.67 ± 1.22 nmol/g tissue; Controls= 13.47 ± 3.21 nmol/g tissue), caused liver necrosis, increased plasma TNF-α levels (CCl_4= 57.36 ± 13.24 IU/ml; Controls= 7.26 ± 2.31 IU/ml) and enhanced hepatic mRNA for TNF-α (CCl_4= 19.22 ± 4.38 a.u.; Controls= 0.76 ± 0.36 a.u.).

IRFI 042 (100 mg/kg, 30 min after CCl_4 injection) blunted liver MAL (0.32 ± 0.17 nmol/mg protein), decreased the serum levels of ALT (128.71 ± 13.23 U/L), and restored the hepatic concentrations of VE (9.52 ± 3.21 nmol/g tissue), inhibited OH^- production (2,3-DHBA= 3.54 ± 1.31 μM; 2,5-DHBA= 7.37 ± 2.46 μM), restored the endogenous antioxidant GSH (12.77 ± 3.73 mmol/g protein) and improved histology. Furthermore IRFI 042 treatment suppressed plasma TNF-α concentrations (31.47 ± 18.25 IU/ml) and hepatic TNF-α mRNA levels (11.65 ± 3.21 a.u.). The acute treatment with vitamin E failed to exert any protective effect against CCl_4-induced hepatotoxicity.

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These investigations suggest that IRFI 042 treatment may be of benefit during free radical-mediated liver injury.

Keywords: Liver injury, CCl_4, antioxidants, IRFI 042, lipid peroxidation, free radicals, gene expression, vitamin E, TNF-α

INTRODUCTION

Hepatic failure, caused by several etiologies, produces high morbidity and mortality in the world[1]. In fact, acute alcoholic hepatitis continues to present a substantial mortality rate resistant to therapeutic approaches[2]. Moreover, doses of a large number of chemotherapeutic agents designed to treat cancer or severe infections are limited by hepatic toxicity. These different etiologies of liver injury may be treated with hepatoprotective agents.

Evidence developed over the last several years has suggested that many forms of liver injury may be caused by oxidative stress and subsequent free radical formation[3–6]. It has been shown that free radicals may injure the cell membranes of hepatocytes through lipid peroxidation or by other means[5,7–9]. Free radicals, both *in vitro* and *in vivo* models, have been shown to modify and damage proteins, lipids, carbohydrates and DNA[4–5,7–8]. When the amount of free radicals produced exceed the capacity of the endogenous cellular antioxidant system, significant cellular damage can occur[8–9]. Nevertheless, up till now, a successful therapeutic approach to this pathogenic mechanism in liver disease has not been developed.

Carbon tetrachloride (CCl_4) has been used extensively to induce liver injury in experimental animals. CCl_4 is metabolised by liver microsomal cytochrome P-450 to the trichloromethyl radical ($CCl_3\cdot$), which incorporates O_2 to form the trichloromethylperoxyl radical ($CCl_3O_2\cdot$) which then withdraws allylic hydrogens from polyunsaturated fatty acids (PUFA) to initiate lipid peroxidation[4].

Increasing evidence has indicated that cytokines, particularly tumor necrosis factor α (TNF-α), are important modulators of hepatic injury and repair. In liver injury TNF-α mediates acute phase responses, inflammatory cell infiltration, hyperlipidemia, free oxygen radical generation, fibrogenesis, and cholestasis[10–11]. Elevated TNF-α levels occur in various acute and chronic liver diseases, including viral and alcoholic hepatitis, fulminant liver failure, alcoholic cirrhosis, biliary obstruction, and ischaemia[12–13]. In the last years, elevated TNF-α levels have been observed in experimental animals treated with CCl_4[14,15], nevertheless neither its role in hepatotoxicity nor molecular events responsible for its expression have been well studied.

It has been reported that in rats intoxicated once with CCl_4, hepatic antioxidants decrease with the progression of liver injury[16–18]. It is also known that the hepatic level of vitamin E, a lipid – soluble antioxidant, increases at a progressive stage of liver injury, although the level decreases at an early stage of the injury[19]. In addition, there are several reports showing an increase in the hepatic level of malondialdehyde (MAL) that is a stable intermediate product of lipid peroxidation, which is formed via active oxygen species, with the progression of acute liver injury or at its advanced stage in rats treated with CCl_4[20–23]. The high levels of MAL may be reduced by agents that limit hepatic damage[22,24–27].

The endogenous vitamin E is of great importance in the antioxidant defence system[28]. Vitamin E deficiency, among others, has been shown to exacerbate myocardial injury due to oxidative stress[29]. Acute administration of CCl_4 is associated with an imbalance in the hepatic vitamin E concentrations[18,29–30]. Therefore, the therapeutic use of vitamin E has been considered advantageous over other agents because it is inexpensive, readily used, and essentially non toxic in humans. Nevertheless, the marked lipophilicity of vitamin E has been suggested to explain its slow incorporation into tissues. How-

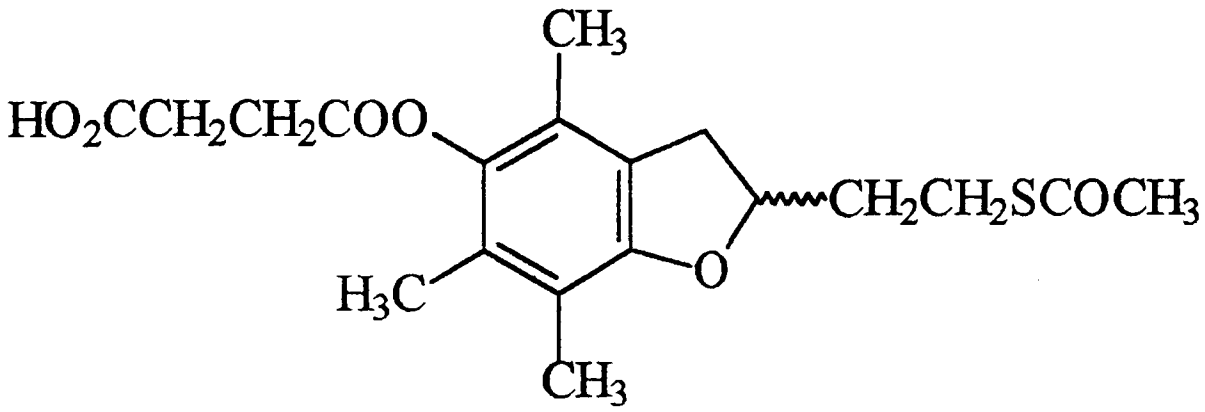


FIGURE 1 Chemical structure of IRFI 042

ever, several experiments carried out with vitamin E supplementation or chronic treatment led to a marked reduction of CCl_4-induced liver injury[25,31,32]. Acute administration of this agent would in fact result in very low levels of vitamin E in the damaged liver[33].

A number of less lipophilic α-tocopherol analogues endowed with radical scavenging activity have been described in the literature. One of them, IRFI 005 has been shown to exert a potent vitamin E-like activity both in chemical and biological systems[34–35]. Its 5-acetoxy prodrug, raxofelast[36–37], is currently being tested in clinical studies aimed to assess its potential for the treatment of vascular diabetic complications.

To our knowledge, no vitamin E-like compounds provided with a sulphydryl moiety have been reported so far. In our mind, the combination in the same molecule of a chain-breaking moiety (characteristic of phenols related to α-tocopherol) with the reducing ability of thiol groups may result in powerful and peculiar biological actions, especially in those oxidative stress-mediated situations (such as the liver injuries) in which a significant depletion of endogenous thiols is observed. IRFI 042, (±)-5-hemisuccinoyl-2-[2-(acetylthio)ethyl]-2,3-dihydro-4,6,7-trimethyl benzofuran (Fig. 1), was designed to this aim; both the 5-OH and the SH groups are acyl-protected and were shown to be easily regenerated in vivo (unpublished data). A previous work shows a protective effect exerted by IRFI 042 on myocardial ischaemia/reperfusion injury[38].

In light of these findings, the aim of the present study was to assess the possible ability of IRFI 042 in limiting the liver injury following CCl_4 administration in rats.

MATERIALS AND METHODS

Animal groups

Experiments were carried out on male Sprague-Dawley rats (230–250 g body weight). Animals were housed at a constant temperature of 22 ± 2°C under a 12 h light-dark cycle with unlimited access to standard laboratory rat chow (0.01 % content of α-tocopherol) and tap water. Rats were subdivided in the following groups: 1)Sham + vehicle; 2)Sham + IRFI 042 (100 mg/kg); 3)CCl_4 + vehicle; 4)CCl_4 + IRFI 042 (25 mg/kg); 5)CCl_4 + IRFI 042 (50 mg/kg); 6)CCl_4 + IRFI 042 (100 mg/kg); CCl_4+ Vitamin E (100 mg/kg).

Induction of acute liver damage

Rats were intraperitoneally injected with CCl_4 (Sigma-Aldrich, Milano, Italy) at a dose of 1.0 ml/kg body weight as a 50% vegetal oil solution and control ones with the same dose of vegetal oil[18]. These animals were maintained on diet and water ad libitum throughout the experiment. Immediately after the treatment, samples of blood were obtained from the main vein of the tail in order to evaluate basal biochemical parameters. They were then sacrificed under ether anaesthesia at 48 h after CCl_4 treatment at which time blood was collected from the inferior vena cava and livers were isolated. A separate group of animals was also sacrificed 2 h after intoxication for hepatic vitamin E determination. The collected blood was separated into serum. The isolated livers were sliced in pieces and washed in ice-cold 0.1 M KCl, blotted on absorbent paper, and then weighed as soon as possible. After weighing, the livers were kept at –80°C until analysis.

Serum ALT evaluation

ALT was measured in samples (0.1ml) of serum obtained 48 h after CCl_4 treatment. The activity was evaluated by using a commercial clinical test kit (ALT/GPT, cod.59–10, Sigma-Aldrich, Milano, Italy).

Malondialdehyde measurement

Determination of the hepatic MAL was carried out in order to estimate the extension of lipid peroxidation in the damaged tissue. Samples of the hepatic tissue obtained 48 h after CCl_4 treatment were frozen at –80°C until the assay. The day of analysis, after thawing, tissue samples were washed in ice-cold 20 mM Tris-HCl, pH 7–4, and blotted on absorbent paper. Each sample was then minced in ice-cold 20 mM Tris-HCl pH 7–4 with butylated hydroxytoluene (BHT) 1 mg/ml and homogenised in a ratio 1:10, w:v by using a teflon pestle. After centrifugation at 3,000 × g for 10 min at 4°C, the clear homogenate supernatant was used for biochemical assay. The assay was carried out by using a colorimetric commercial kit (Lipid peroxidation assay kit, cat.n°437634, Calbiochem-Novabiochem Corporation, USA).

Briefly, 0.65 ml of 10.3 mM N-methyl-2-phenyl-indole in acetonitrile were added to 0.2 ml of homogenate supernatant. After vortexing for 3–4 s and adding 0.15 ml HCl 37%, samples were mixed well and closed with a tight stopper and incubated at 45°C for 60 min. The samples were then cooled on ice and the absorbance was measured spectrophotometrically at 586 nm. A calibration curve of an accurately prepared standard MAL solution (from 10 to 640 nmol/ml) was also run for quantification. The concentration of tissue MAL was expressed as nmol/mg protein.

Measurement of hydroxyl radical (OH·) formation

In order to quantify OH· production during CCl_4-induced liver injury we used the aromatic trap technique[39]. Sodium salicylate serves as a specific trap for hydroxyl radicals because it can react chemically with OH· radicals produced, yielding 2,5-dihydroxybenzoic acid (2,5-DHBA), 2,3-dihydroxybenzoic acid (2,3-DHBA) and catechol as its hydroxylation derivatives in an approximate proportion of 40%, 49% and 11% respectively[40–41]. In the present study we measured both 2,5-DHBA and 2,3-DHBA. To allow the chemical reaction, each group of animals received sodium salicylate (100 mg/kg i.p.)[42] (Janssen Chemical, Beerse, Belgium) one hour before treatment and one hour before the sacrifice. Sodium salicylate by itself did not exert any protective effect in the injured liver. Samples of blood were drawn 5 min and 48 h after CCl_4 administration. The blood was collected in polyethylene tubes with previous addition of 20 µl of heparin solution (16,000 IU). The plasma samples obtained after centrifugation at 3,000 × g for

10 min at 4°C were frozen at –80°C until the assay. To measure 2,5-DHBA and 2,3-DHBA formation, an HPLC method was used[43–44]. Briefly, 250 µl of plasma was treated with 10 µl of 100 µM 2,4-dihydroxybenzoic acid (2,4-DHBA) (Janssen Chemical, Beerse, Belgium), which has been used as internal standard and 10 µl of 40% $HClO_4$. Then the plasma was extracted with 2.5 ml HPLC grade diethylether and mixed on a vortex for 2 min. After centrifugation for 15 min at 15,000 × g at 4°C, the diethylether layer was separated and was then evaporated in a vacuum concentrator system (Heto Lab Equipment, Denmark). The residue obtained was dissolved in 30 µl of 0.1 N HCl and 32.5 µl of mobile phase, and 50 µl of the solution was injected into the HPLC apparatus. The HPLC equipment consisted of a solvent delivery module (Mod. 422 Master, Kontron Instruments, Everett, USA), a programmable wavelength detector (Mod. 165, Beckman Instruments, San Ramon, USA) connected to an automatic integrator (Mod. CR-3A, Shimadzu, Kyoto, Japan). The column used was a Lichrosorb-10-RP_{18}, 10 µ 250x4.6 mm (Labservice Analytica, Milano, Italy), attached to a precolumn (Guard column Water-Millipore, Milford, USA). The mobile phase was 80% 0.03 M citric acid, 0.03 M acetic acid buffer (pH=3.6) and 25% methanol (J.T. Baker, Deventer, Holland) at flow rate of 1.3 ml/min. The detector was set at a wavelength of 315 nm. The concentrations of 2,3-DHBA and 2,5-DHBA were expressed in µM.

Reduced glutathione assessment

Samples of hepatic tissue obtained after 48 h of CCl_4 treatment were frozen at –80°C until the assay. The biochemical analysis was performed by using a spectrophotometric method[45]. Briefly, tissue samples were homogenised in a solution containing 5% trichloroacetic acid and 5 mM ethylenediaminetetracetic acid a 4°C. Then each sample was centrifuged at 15,000 × g for 10 min at 4°C. An aliquot of homogenate supernatant (0.4 ml) was added in a dark polyethylene tube containing 1.6 ml Tris-EDTA buffer 0.4 M pH=8.9. After vortexing, 40 µl of dithiobisnitrobenzoic acid 10 mM in methanol were added. The samples were vortexed again and the absorbance was read after 5 min at 412 nm. The values of unknown samples were drawn from a standard curve plotted by assaying different known concentrations of GSH. The amount of hepatic GSH was expressed as µmol/g protein.

Vitamin E evaluation

Hepatic VE levels were measured in order to determine the oxidative state of the liver following CCl_4 administration. The assay was performed in hepatic tissue obtained 48 h after the CCl_4 treatment. Hepatic specimens were stored at –80°C until the assay. The analysis was carried out by using an HPLC method[46]. Briefly, 0.2 ml of tissue homogenate, contained in polycarbonate tubes lined with tin foil, was treated with 200 µM of tocopherol acetate (25 µg/ml in ethanol), which has been used as an internal standard, and with 400 µl of butanol/ethyl acetate (1:1, v:v). After vortexing for 20 s, 20 mg of sodium sulphate were added and the sample was shaken on vortex mixer for additional 60 s. After centrifugation at 15,000 × g for 5 min at 4°C, the organic layer was recovered and a 50 µl aliquot was injected into the HPLC apparatus. The HPLC equipment consisted of a solvent delivery module (Model 422 Master, Kontron Instruments, Everett, USA), a programmable variable wavelength detector (Spectromonitor 4100, Thermo Separation Products, Florida, USA) connected to an automatic integrator (Model CR-3A, Shimadzu, Kyoto, Japan). The column used was a ultratechsphere C_{18}, 250 × 4.6 mm, 5 µ particle size (HPLC Technology LTD, Macclesfield, Cheshire, UK), attached to a pre-column (Guard column, Water-Millipore, Milford, USA). The mobile phase was methanol/water (97:5, v:v) at a flow rate of 1 ml/min at room temperature. The detector was set at wavelength of 280 nm. VE levels were expressed as nmol/g tissue.

Plasma TNF-α assay

Plasma TNF-α bioactivity was determined by using a highly sensitive method modified from Mosmann[47]. This assay determines TNF α cytotoxicity against WEHI 164 murine fibrosarcoma cells in the presence of actinomycin D and uses MTT (Sigma chemicals, USA) as colorant. Briefly, $5x10^5$ cells were incubated in 96-well microtiter plates at 37°C with 5% CO_2 in 50 µl RPMI 1640 medium (Gibco, Italy) containing 10% calf serum, 1% glutamine and 1% penicillin-streptomycin solution (1:1), actinomycin D (25 µl; 30 mg/ml) was added to each well followed by samples of filtered rat plasma (50 µl) or purified recombinant human TNF-α as standards (R&D Systems, U.K.). The cells were incubated for 24 h at 37°C, and then 25 µl of sterile MTT were added to each well. After incubation for 3 h at 37°C, the reaction was stopped by adding 100 µl lysis buffer and the plates were read at 620 nm by using a spectrophotometric plate reader. One unit of TNF-α activity was defined as the concentration at which 50% of the cells were lysed.

Histology

Histological examination was performed in order to assess the integrity of hepatic cells. The caudal portion of the left lobe from the liver of each rat was removed and fixed by immersion in 10% neutral-buffered formalin. Fixed tissues were embedded in paraffin, cut into 6 mm sections. The sections were stained with hematoxylin-eosin for light microscope examination[48].

Reverse transcriptase-polymerase chain reaction amplification

The chain reaction amplification was performed by using a previous method with some modifications[49]. Briefly, approximately 100 mg of liver tissue were homogenised in 1.0 ml of RNAzol™B (Sigma-Aldrich, Milano, Italy). RNA was solubilized by passing the homogenate few times through the pipette. One-hundred-nanogram samples of mRNA were transcribed into cDNA using 400-U reverse transcriptase (200 U/µl, BRL, U.S.A.) and 0.5 mg oligo (dT) 12–18 primer (BRL, U.S.A.) for 30 min at 37°C. The reaction mixture (50 ml) contained: 25 mM Tris-HCl pH 8.3; 37.5 mM KCl; 10 mM dithiothreitol (DTT); 1.5 mM $MgCl_2$; 10 mM dNTP (Perkin Elmer, U.S.A.). Polymerase chain reaction (PCR) primers for TNF-α, and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) were purchased from Celbio (Italy). G3PDH mRNA transcripts were monitored to determine constitutive expression. An aliquot containing one tenth of the cDNA synthesized from mRNA was amplified in a 50 µl volume with the reagents supplied in the Gene AMP PCR kit (Perkin Elmer, U.S.A.)

Using the OMN-E Hybaid Thermal Cycler (Teddington, U.K.), amplification was initiated by 3 min denaturation at 94°C for one cycle and then followed by three temperature steps for PCR including 3 min of denaturation at 94°C, 100 s of annealing at 55°C and 2 min of polymerization at 72°C. This process was continued for 25, 30 or 35 cycles of amplification depending on the abundance of the message. After the last cycle of amplification, the samples were incubated for 7 min at 72°C. An aliquot of each amplified sample was electrophoresed through 2% agarose (UltraPure, molecular biology grade, Sigma-Aldrich, Italy) and stained with 0.5 mg/ml ethidium bromide in Tris-borate-EDTA buffer.

The resultant gel was illuminated in a darkroom with a fixed camera. The captured image, sent to an image analysis software (Bio-Profil, Celbio, Italy), was subjected to a densitometric analysis and then printed on VPN-120 printer (Celbio, Italty). mRNA levels were expressed as arbitrary units.

TABLE I Changes in serum ALT activities (U/L) in basal conditions and after 18 h from CCl_4 injection

Experimental group	*Basal*	*48 h after CCl_4 injection*
Sham + vehicle (n=7)	30.53±5.62	29.62±5.13
Sham + IRFI 042 (100 mg/kg) (n=7)	29.71±4.67	30.26±4.77
CCl_4 (n=13)	28.54±4.25	404.61±10.33[a]
CCl_4 + IRFI 042 (25 mg/kg) (n=10)	30.51±6.13	357.42±14.65[b]
CCl_4 + IRFI 042 (50 mg/kg) (n=10)	31.44±5.42	267.85±11.42[b]
CCl_4 + IRFI 042 (100 mg/kg) (n=10)	27.63±3.96	128.71±13.23[b]
CCl_4 + Vitamin E (100 mg/kg) (n=10)	28.87±4.21	391.47±17.52

The total number of animals in each group is indicated in parentheses. Values are mean±S.E.M.

a. $p<0.001$ vs. basal.
b. $p<0.001$ vs. CCl4 group.

Drug

IRFI 042 was synthesized according to the reported route[50]. The compound was administered intraperitoneally, 30 min after CCl_4 treatment in dimethylsulphoxide:NaCl 0.9% (1:1, v:v). Vitamin E ((+)-α-tocopherol from vegetable oil) was obtained from Sigma Chemicals (S. Louis, USA), and was administered intraperitoneally, 30 min after CCl_4 treatment, in dimethylsulphoxide: NaCl 0.9% (1:1, v:v). All substances were prepared fresh daily and administered in a volume of 1 ml/kg.

Statistical analysis

Data are expressed as mean ± S.E.M. The difference between the means of two groups was evaluated with an ANOVA and was considered significant when $p<0.05$.

Statement of animal care

The studies reported in this manuscript have been carried out in accordance with the declaration of Helsinki and with the Guide for the Care and Use of Laboratory Animals.

RESULTS

Serum ALT activity

Analysis of serum ALT was carried out in order to evaluate liver damage. Table I reports the changes in ALT activity in serum in basal conditions and 48 h after CCl_4 injection in the considered groups. Low ALT activities were present in each group before CCl_4 administration, and there were no significant differences among any of them. Instead, 48 h after CCl_4 injection, a significant increase of ALT activity was found in the serum of CCl_4-treated rats given vehicle. The administration of IRFI 042 resulted in a dose-dependent blunting of the hepatic ALT depletion. The group given vitamin E did not show any significant decrease in the serum ALT.

Hepatic MAL analysis

Determination of hepatic MAL was performed to estimate free radical damage on biological membranes (Table II). Low levels of MAL were seen in the sham groups 48 h after CCl_4 injection, and these values were considered normal. In contrast, a significant increase in MAL production was found in the liver of CCl_4 treated rats

given with vehicle. The administration of IRFI 042 reduced MAL concentrations by inhibiting lipid peroxidation in hepatic cells with all used doses. No significant effect was seen in the vitamin E treated group.

OH· production

Analysis of 2,3-DHBA and 2,5-DHBA was carried out in order to indirectly assess the detrimental production of OH· radical. Table III reports the levels of 2,3-DHBA and 2,5-DHBA measured in each group under basal conditions and after 48 h from CCl_4 injection. Very low amounts of both acids were detected before CCl_4 treatment in all studied groups (<0.5 mM for 2,3-DHBA and <1.5 for 2,5-DHBA). In contrast, a high amount of both acids was seen 48 h after CCl_4 injection in the vehicle group. The administration of IRFI 042 induced in a dose dependent way a marked reduction in OH· production. No significant effect was observed in the CCl_4 plus Vitamin E group.

TABLE II Hepatic MDA levels (nmol/mg protein) assayed after 48 h from CCl_4 injection

Experimental group	*48 h after CCl_4 injection*
Sham + vehicle (n=7)	0.13 ± 0.06
Sham + IRFI 042 (100 mg/kg) (n=7)	0.17 ± 0.06
CCl_4 (n=13)	0.67 ± 0.16[a]
CCl_4 + IRFI 042 (25 mg/kg) (n=10)	0.46 ± 0.15[b]
CCl_4 + IRFI 042 (50 mg/kg) (n=10)	0.40 ± 0.13[c]
CCl_4 + IRFI 042 (100 mg/kg) (n=10)	0.32 ± 0.17[c]
CCl_4 + Vitamin E (100 mg/kg) (n=10)	0.53 ± 0.14

The total number of animals in each group is indicated in parentheses. Values are mean ± S.E.M.

a. p<0.001 vs. sham + vehicle group.
b. p<0.005.
c. p<0.001 vs. CCl4 group.

TABLE III Plasma amount of 2,3-DHBA and 2,5-DHBA (μM) analysed in basal conditions and after 48 h from CCl_4 injection

Experimental group	2,3-DHBA		2,5-DHBA	
	Basal	48 h	Basal	48 h
Sham + vehicle (n=7)	0.32 ± 0.13	0.38 ± 0.14	1.89 ± 0.65	2.16 ± 0.98
Sham + IRFI 042 (100 mg/kg)(n=7)	0.41 ± 0.18	0.37 ± 0.19	2.24 ± 0.87	2.48 ± 1.12
CCl_4 (n=13)	0.45 ± 0.15	8.73 ± 1.46[a]	2.75 ± 0.93	24.61 ± 3.32[a]
CCl_4 + IRFI 042 (25 mg/kg) (n=10)	0.46 ± 0.11	6.49 ± 1.63[b]	2.87 ± 0.81	17.31 ± 6.65[b]
CCl_4 + IRFI 042 (50 mg/kg) (n=10)	0.37 ± 0.17	5.14 ± 2.13[c]	2.38 ± 0.46	13.41 ± 4.31[c]
CCl_4 + IRFI 042 (100 mg/kg) (n=10)	0.35 ± 0.19	3.54 ± 1.31[c]	2.74 ± 0.93	7.37 ± 2.46[c]
CCl_4 + Vitamin E (100 mg/kg) (n=10)	0.39 ± 0.15	7.29 ± 1.92	2.45 ± 0.73	21.53 ± 3.47

The total number of animals in each group is indicated in parentheses. Values are mean ± S.E.M..

a. p<0.001 vs. basal.
b. p<0.005.
c. p<0.001 vs. CCl4 group.

TABLE IV Hepatic GSH concentrations (μmol/g protein) assayed after 48 h from CCl4 injection

Experimental group	*48 h after CCl_4 injection*
Sham + vehicle (n=7)	17.82 ± 3.13
Sham + IRFI 042 (100 mg/kg) (n=7)	16.94 ± 4.15
CCl_4 (n=13)	3.26 ± 1.85[a]
CCl_4 + IRFI 042 (25 mg/kg) (n=10)	6.13 ± 2.68[b]
CCl_4 + IRFI 042 (50 mg/kg) (n=10)	8.73 ± 3.59[c]
CCl_4 + IRFI 042 (100 mg/kg) (n=10)	12.77 ± 3.73[c]
CCl_4 + Vitamin E (100 mg/kg) (n=10)	4.12 ± 2.17

The total number of animals in each group is indicated in parentheses. Values are mean ± S.E.M..

a. $p<0.001$ vs. sham + vehicle group.
b. $p<0.01$.
c. $p<0.001$ vs. CCl4 group.

Hepatic GSH levels

Table IV reports the changes in GSH content assayed in liver 48 h after CCl_4 injection in the considered groups. In sham rats GSH levels ranged between 16.0 and 18.0 μmol/g protein, and these values were considered normal. In contrast, a marked decrease in GSH concentrations was found in the liver of CCl_4 -treated rats given with vehicle. The treatment with IRFI 042 significantly inhibited the reduction in GSH levels with all used doses. No significant changes occurred in the group given with the vitamin.

Hepatic vitamin E analysis

Table V reports hepatic VE content 2 h after CCl_4 injection in the considered groups. In sham rats, VE amounts ranged between 11.0 and 13.0 nmol/g tissue, and these values were considered normal. Instead, a marked decrease in the vitamin content was observed in the liver of CCl_4-treated rats given with vehicle. The treatment with IRFI 042 significantly limited the loss of VE levels with all used doses. Nevertheless, a non significant increase of the hepatic VE content was found in the vitamin treated group. This increase, however, was not able to afford any protective effect to the damaged liver.

Plasma TNF-α levels

Table VI reports the changes in TNF-α activity assayed in plasma 48 h after CCl_4 injection in the considered groups. In sham rats TNF-α levels ranged between 6.0 and 8.0 IU/ml, and these values were considered normal. In contrast, a marked increase in TNF-α activity was found in the plasma of CCl_4-treated rats given with vehicle. The treatment with IRFI 042 significantly inhibited the increase of TNF-α with all used doses. No significant variations were seen in the group given with vitamin E

TABLE V Hepatic Vitamin E levels (nmol/g tissue) evaluated after 2 h from CCl_4injection

Experimental group	*2 h after CCl_4 injection*
Sham + vehicle (n=7)	13.47 ± 3.21
Sham + IRFI 042 (100 mg/kg) (n=7)	11.95 ± 2.88
CCl_4 (n=13)	5.67 ± 1.22[a]
CCl_4 + IRFI 042 (25 mg/kg) (n=10)	7.63 ± 2.24[b]
CCl_4 + IRFI 042 (50 mg/kg) (n=10)	8.41 ± 2.96[c]
CCl_4 + IRFI 042 (100 mg/kg) (n=10)	9.52 ± 3.21[d]
CCl_4 + Vitamin E (100 mg/kg) (n=10)	7.17 ± 2.78

The total number of animals in each group is indicated in parentheses. Values are mean ± S.E.M.

a. $p<0.01$.
b. $p<0.005$.
c. $p<0.001$ vs. CCl4 group.
d. $p<0.001$ vs. sham + vehicle group.

TABLE VI Plasma TNF-α bioactivity (IU/ml) assayed after 48 h from CCl_4 injection

Experimental group	*48 h after CCl_4 injection*
Sham + vehicle (n=7)	7.26 ± 2.31
Sham + IRFI 042 (100 mg/kg) (n=7)	6.13 ± 2.87
CCl_4 (n=13)	57.36 ± 13.24[d]
CCl_4 + IRFI 042 (25 mg/kg) (n=10)	45.21 ± 14.42[a]
CC_4 + IRFI 042 (50 mg/kg) (n=10)	37.11 ± 17.01[b]

Experimental group	*48 h after CCl_4 injection*
CCl_4 + IRFI 042 (100 mg/kg) (n=10)	31.47 ± 18.25[c]
CCl_4 + Vitamin E (100 mg/kg) (n=10)	47.24 ± 15.19

The total number of animals in each group is indicated in parentheses. Values are mean ± S.E.M.

a. $p<0.05$.
b. $p<0.005$.
c. $p<0.001$ vs CCl4 group.

Hystological analysis

Figure 2 shows the histological changes 48 h after CCl_4 treatment. In panel A is reported the liver section of a sham animal. The cells appear complete, without infiltrations and haemorrhagic signs. In panel B is reported a sections of liver obtained from CCl_4 treated rat given with vehicle. Centrilobular haemorrhagic alterations, hydropic degenerations, fatty change with monocytes, macrophages and Kuppfler cell infiltrations are evident. The necrosis appear massive, involving whole groups of lobules in its entirety. In panel C is reported a section of liver obtained from CCl_4 treated rat given the highest dose of IRFI 042. In this case it is possible to see a marked reduction in hepatocellular lesions and alterations. The hystological report of panel D shows a liver section of a vitamin E treated rat. The presence of haemorrhagic alterations and cell infiltrations, indicated no protective effect exerted by the vitamin E administration.

TNF-α mRNA expression in liver tissue

Table VII reports the amount of hepatic TNF-α mRNA evaluated 48 h after CCl_4 injection. Figure 1 shows the representative amplification of mRNA expression for TNF-α. Increased mRNA levels were found in CCl_4 groups treated with vehicle, the administration of IRFI 042 markedly suppressed hepatic TNF-α expression. A slight suppression of mRNA expression for TNF-α, was observed in the CCl_4 + Vitamin E treated group, nevertheless it was not significant.

TABLE VII Hepatic TNF-α mRNA levels (a.u.) assayed after 48 h from CCl_4 injection

Experimental group	*48 h after CCl_4 injection*
Sham + vehicle (n=7)	0.76 ± 0.36
Sham + IRFI 042 (100 mg/kg) (n=7)	0.83 ± 0.29
CCl_4 (n=13)	19.22 ± 5.38[a]
CCl_4 + IRFI 042 (25 mg/kg) (n=10)	15.31 ± 2.33[b]
CC_4 + IRFI 042 (50 mg/kg) (n=10)	13.21 ± 2.68[c]
CCl_4 + IRFI 042 (100 mg/kg) (n=10)	11.65 ± 3.21[d]
CCl_4 + Vitamin E (100 mg/kg) (n=10)	16.04 ± 2.47

The total number of animals in each group is indicated in parentheses. Values are mean ± S.E.M.

a. $p<0.001$ vs. sham + vehicle group.
b. $p<0.05$.
c. $p<0.005$.
d. $p<0.001$ vs CCl4 group.

DISCUSSION

The present results indicate that a vitamin E analogue compound limits hepatocellular injury in acute CCl_4 intoxication. This effect of the antioxidant IRFI 042 could be attributed both to its ability to limit the propagation of lipid peroxidation and its interaction with superoxide anions and other radicals to produce less toxic species[51–52].

Rapid, extensive lipid peroxidation of the membrane structural lipids has been proposed as the basis of CCl_4 hepatocellular toxicity and the actual activation of CCl_4 has been well documented[4,9,53–54]. In fact, CCl_4 is dehalogenated by cytochrome P-450 to a trichloromethyl free radical which then adds molecular oxygen to yield the trichloromethylperoxyl radical. This reactive compound is capable of removing hydrogen atoms from unsaturated lipids and creating carbon-centered radicals. These lipid radicals add molecular oxygen to produce lipid-peroxyl radicals, which induce the propagation of lipid peroxidation. It is postulated that the membrane damage caused by lipid peroxidation and subsequent toxic lipid peroxidation products, if not halted, results in the death of the

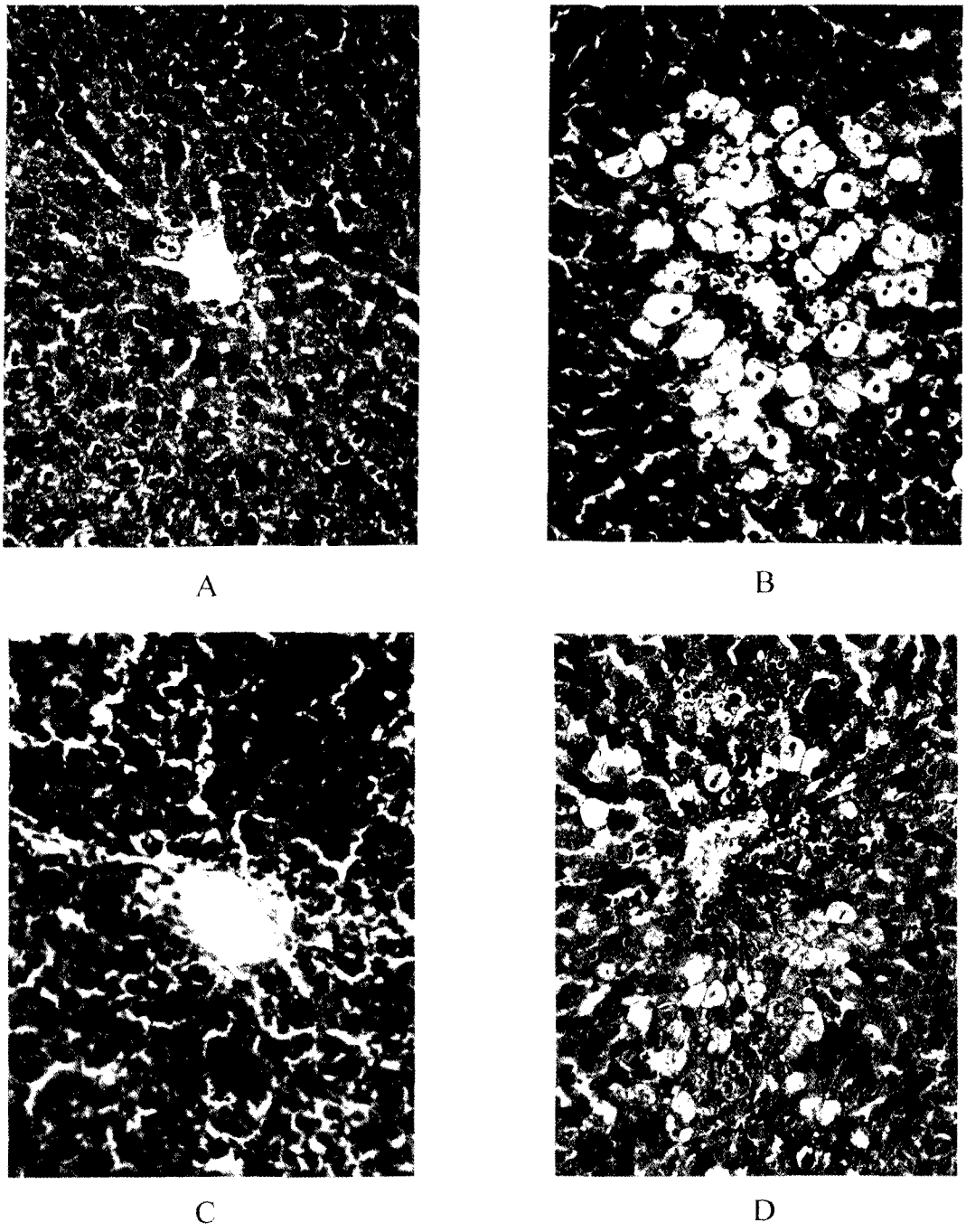
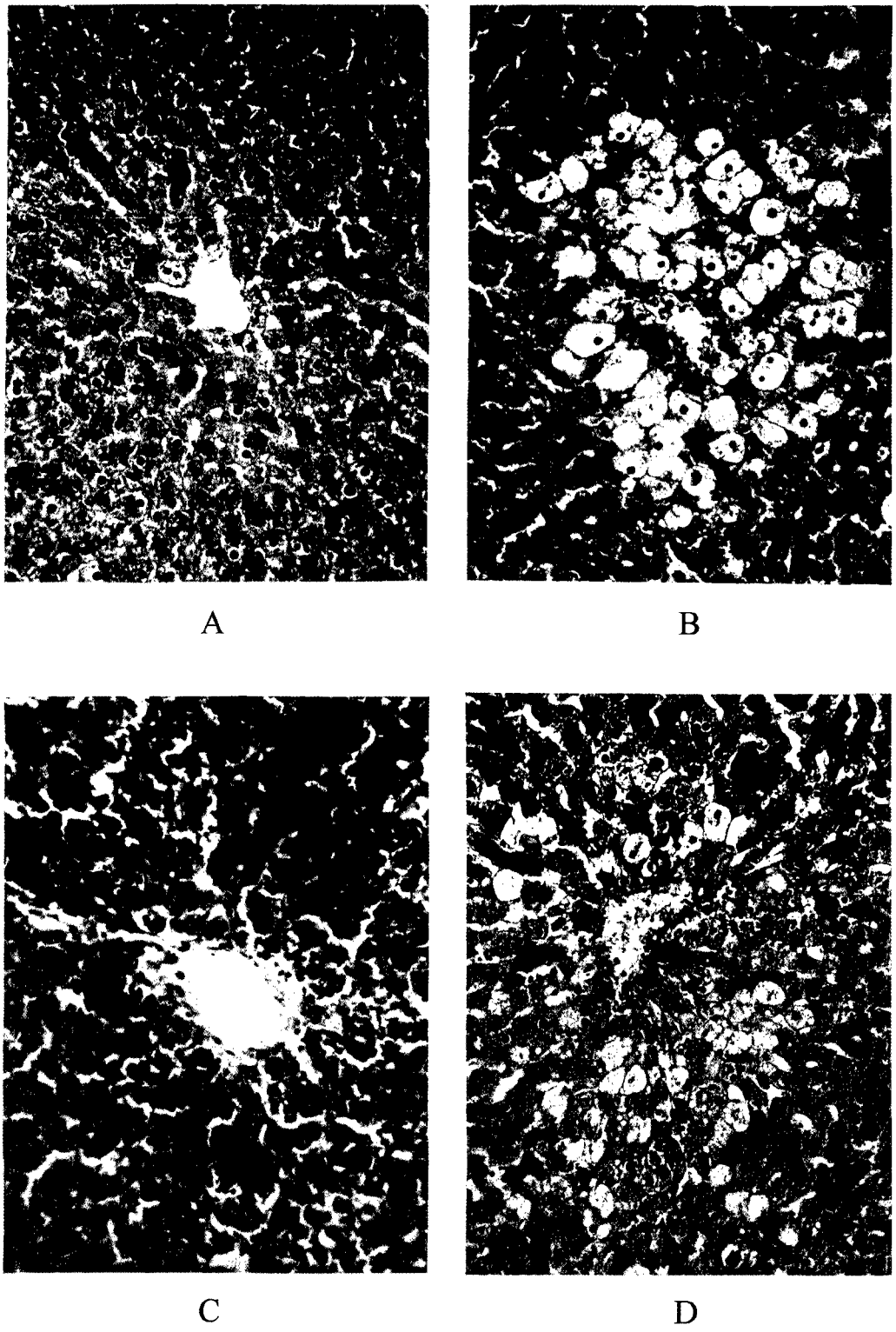


FIGURE 2 Microscopic analysis of hepatic tissue after CCl_4 administration or vehicle, and with IRFI 042 or Vitamin E. A) Sham + vehicle; B) CCl_4 + vehicle; C) CCl_4+ IRFI 042 (100 mg/kg); D) CCl_4 + Vitamin E (100 mg/kg) (see Color Plate I at the back of this issue)



Color Plate I (See page 389, Figure 2) Microscopic analysis of hepatic tissue after CCl_4 administration or vehicle, and with IRFI 042 or Vitamin E. A) Sham + vehicle; B) CCl_4 + vehicle; C) CCl_4 + IRFI 042 (100 mg/kg); D) CCl_4 + Vitamin E (100 mg/kg)

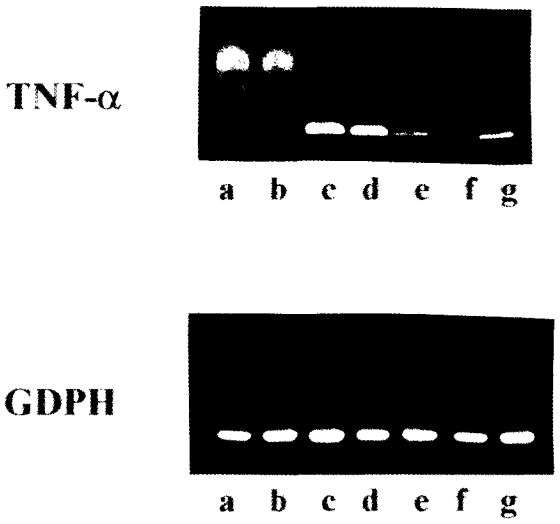


FIGURE 3 Amplification of hepatic mRNA TNF-α transcripts by RT-PCR of rats after CCl_4 administration or vehicle, and with IRFI 042 or Vitamin E. **a**)Sham + vehicle; **b**)Sham + IRFI 042 (100 mg/kg); **c**)CCl_4 + vehicle; **d**)CCl_4 + IRFI 042 (25 mg/kg); **e**) CCl_4 + IRFI 042 (50 mg/kg); **f**)CCl_4 + IRFI 042 (100 mg/kg); **g**)CCl_4+ Vitamin E (100 mg/kg)

cell. α-Tocopherol is considered to be a major inhibitor of oxidative stress in mammalian cells. Its lipophilic C16-phytyl side chain is thought to anchor the molecule to membranes, and the reactive chromanol head group is then oriented at the surface of the membrane[55]. For this reason, it probably inhibits lipid peroxidation by donating a phenolic hydrogen from the chromanol head group to an acyl radical[56,57]. Several authors have shown that prophylactic dietary supplementation with high doses of oral vitamin E can protect animals from the liver disease induced by chronic CCl_4 administration[25,31,58]. The dietary or parenteral approach provides a relatively slow and ineffective means of getting the antioxidant to the target tissue. The amelioration of acute CCl_4 damage has, until these studies, met with limited success. Whereas there have been previous reports of protection from CCl_4 intoxication[5,58], most of the compounds that are cited require high dosage and/or extended pre-treatment to elicit a modest effect. One reason for this lack of effect may be because the CCl_4 model is thought to be primarily a lipid peroxidation injury[4,9]. Vitamin E is the most important naturally occurring chain-breaking antioxidant in blood and erythrocyte membranes[59]. Symptoms of vitamin E deficiency are considered to be the result of free radical-mediated damage[60]. Therefore vitamin E and most of the drugs effective against its damage are lipophilic. As a result, they are not readily available for intravenous use and treatment. Consequently, the main difficulty in the vitamin E treatment is the acute administration, because the high lipophilicity hampers the tissue distribution and therefore cellular bioavailability. In fact, our results did not show any positive effect exerted by the vitamin after a single dose administration.

The α-tocopherol analogue IRFI 042, with less lipophilic character, has been developed with the aim to protect the cells from oxidative injury. The simultaneous occurrence of the (protected) 5-OH phenol group, which is typical of vitamin E-like compound, and the masked sulphydryl in the side chain in position 2, is a distinctive feature of this compound, which may even result in a synergism of action.

In our model, the burst of oxygen-free radicals production that occurs upon the CCl_4 acute treatment in the liver leads to decreased GSH levels as a consequence of its consumption during oxidative stress and cellular lysis[61]. The counteraction of GSH depletion by IRFI 042 is likely to be caused by a combination of two separate mechanisms to be ascribed to the different active moieties of the compound: the free radical scavenging action of the phenolic hydroxyl and the reducing ability of the thiol group, both easily regenerated in vivo. Previous studies reported that hepatic vitamin E content decreased at an early stage of CCl_4-induced acute liver injury in rats and this decreased vitamin E content increased over the original level at a progressed stage of liver injury[16,18,19]. The increased hepatic vitamin E content found at a

progressed stage of CCl_4-induced acute liver injury was maintained during the recovery of the injury. Such a change in hepatic vitamin E content with the progression and recovery of CCl_4-induced acute liver injury in rats seems to be the result of vitamin E supply from adipose and other tissues, as a consequence of antioxidative metabolic adaptation in the rat body. In our study, we measured the VE levels in a early stage of the injury in order to evaluate the restoration of the vitamin as evidence whether the exogenous treatment was effective or not. In fact VE levels were restored by the treatment with the antioxidant compound, and this evidence is the result of a drug-induced preservation of the integrity of cellular membranes in the injured tissue. While the administration of the exogenous VE, at same concentration of the drug, did not restore significantly the endogenous VE levels.

Lipid peroxidation is considered a critical mechanism of the injury occurring after CCl_4-induced acute liver damage[5]. An indicative method of evaluating lipid peroxidation is tissue MAL analysis[62]. The large amount of MAL found in the vehicle group after 48 h from CCl_4 injection, is consistent with the occurrence of a free-radical-mediated damage. The treatment with the antioxidant produced a significant attenuation of membrane injury.

The detection of 2,3-DHBA and 2,5-DHBA is usually considered a bona fide reporter for the flux of hydroxyl radicals which reacted with salicylic acid during oxidative stress[40,63]. However, microsomal fractions from mammals treated with cytochrome P-450 inducers were found to produce 2,5-DHBA, but not 2,3-DHBA from salicylic acid[41]. Then, in this case, since the liver was damaged and cytochrome P-450 is a state of decreased activity, the 2,5-DHBA levels found may come from the reaction between salicylic acid and OH· produced in the liver. The data obtained show that a large amount of OH· radical was produced in rats given CCl_4. The treatment with IRFI 042 reduced both the 2,3-DHBA and 2,5-DHBA formation, perhaps by directly trapping the OH· radical and/or by inhibiting its production. Because salicylic acid makes the stable derivatives by trapping OH·, it may theoretically be considered as a scavenger of OH·. Therefore, we compared the effect of salicylic acid on the considered parameter against control and CCl_4-treated rats given with the drug (data not shown). Salicylic acid failed to exert any beneficial effect on the CCl_4-induced acute liver injury. This suggests that salicylic acid does not have a significant scavenging effect because it can trap only a small portion (11%) of produced OH·[64].

These biochemical parameters were confirmed by the hystological analysis. In fact the treatment with the antioxidant showed a marked reduction in hepatic necrosis and cellular lysis.

Several areas of investigation have indirectly implicated TNF-α as a contributor to the cellular damage in CCl_4-induced liver cell injury. The high levels of this cytokine and the gene expression may be interpreted as a progression of hepatic cell injury[49]. In agreement with these data, our findings indicate both increased levels of TNF-α and a marked TNF-α expression at 48 hrs following the CCl_4injection. The antioxidant activity of the compound may have reduced both plasma TNF-α values and TNF-α mRNA, and consequently hepatic cell damage.

In conclusion, our results obtained by using the novel vitamin E analogue IRFI 042 give strong evidence of its radical scavenging and thiol-maintening activities and suggest that it may have therapeutic potential for hepatoprotective use in acute and chronic hepatitis.

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