Exp Toxic Pathol 2001; **53**: 199–206
URBAN & FISCHER
http://www.urbanfischer.de/journals/exptoxpath

[1]Dpto Farmacología, Facultad de Farmacia Universidad Complutense de Madrid
[2]Dpto Bioquímica, Facultad de Farmacia Universidad Complutense de Madrid, Spain

Pharmacological modification of endogenous antioxidant enzymes by ursolic acid on tetrachloride-induced liver damage in rats and primary cultures of rat hepatocytes

S. MARTIN-ARAGÓN[1], B. DE LAS HERAS[1], M. I. SANCHEZ-REUS[2], and J. BENEDI[1]

With 5 tables and 2 figures

Received: December 1, 2000; Revised: February 16, 2001; Accepted: March 23, 2001

Address for correspondence: J. BENEDI GONZALEZ, Dpto. Farmacología, Facultad de Farmacia, UCM, 28040 Madrid, Spain; Fax: ++34-91-3941726, e-mail: Jbenedi@eucmax.sim.ucm.es

Key words: Ursolic acid, liver , rats, antioxidant, CCl_4, hepatocytes.

Summary

The purpose of this study was to investigate possible protective effects of ursolic acid against CCl_4-induced alterations of antioxidant defence enzymes *in vivo* as well as its effects against CCl_4-intoxication *in vitro.* Pre-treatment of rats with ursolic acid significantly reduced serum levels of glutamate-oxalate-transaminase and glutamate-pyruvate-transaminase previously increased by administration of CCl_4. Treatment with ursolic acid also significantly reversed the decreased superoxide dismutase, catalase, glutathione reductase, glutathione peroxidase activities and glutathione levels in the liver, as the concentration of reduced glutathione was increased and the content of oxidized glutathione decreased in ursolic acid treated groups. Levels of lipid peroxidation were higher in the CCl_4 group but the increase was also reduced after drug treatment ($p < 0.01$ for 1, 2.5 and 5 mmol/kg). *In vitro* results indicated that addition to the culture medium of ursolic acid ($p < 0.01$ for 500 µM) resulted in a reduction of glutamate-oxalate-transaminase, lactate dehydrogenase activities and in a good survival rate for the CCl_4-intoxicated hepatocytes. Ursolic acid also ameliorated lipid peroxidation in primary cultured rat hepatocytes exposed to CCl_4, as demonstrated by a reduction in malondialdehyde production. Moreover, ursolic acid (50–500 µM) showed radical scavenging properties in terms of hydroxyl formation.

The results obtained suggest that ursolic acid treatment can normalize the disturbed antioxidant status of rats intoxicated with CCl_4 by maintaining the levels of glutathione and by inhibiting the production of malondialdehyde due to its radical scavenging properties.

Introduction

A considerable body of evidences establishes the involvement of uncontrolled oxidative activity as a general mechanism of tissue damage in a variety of pathological conditions such as inflammation, atherosclerosis, carcinogenesis and diseases affecting a great number of organs including the liver [12, 40]. Thus, compounds with ability to reduce lipid peroxidation and radical formation might lead to the control of oxidative damage and tissue protection [23].

Physiological antioxidant defense is offered by preventive enzymes and chain-breaking small molecules, like ascorbic acid, α-tocopherol and glutathione. When more reactive oxygen species are formed than can be counteracted by the defence mechanism of the organism, the therapeutic use of synthetic or natural antioxidants appears to be a rational approach to the management of oxidative-stress-related conditions [18, 14].

Ursolic acid is a pentacyclic triterpenoid obtained from plants. It has long been used in popular medicine and was reported to possess a wide variety of pharmacological effects; as anti-inflammatory [25], anti-arthritic[39], cytostatic and anti-proliferative effects [8, 11], hepatoprotective effects in mice [21], and membrane stabilizing properties[13].

It was previously reported that the reduced viability of hepatocytes [28] and the modifications biochemical parameters in liver and serum after paracetamol and

galactosamine treatments were significantly reversed by ursolic acid [35]. Furthermore, its ability to inhibit peroxidation of microsomal membrane lipids *in vitro* was also described [3, 4,15] and we observed its superoxide radical scavenging activity (unpublished data).

It is well-known that free radical chain reaction leads to lipid peroxidation and finally causes various disorders including liver diseases. In this paper, we wish to report in detail the hepatoprotective effect of ursolic acid on CCl_4-induced hepatotoxicity *in vitro* as well as its effects against CCl_4-intoxication *in vivo*. The hepatoprotective effect of ursolic acid was evaluated *in vivo* in CCl_4-intoxicated rats by measuring serum glutamate oxalate transaminase (sGOT) and serum glutamate pyruvate transaminase (sGPT) activities. In addition, lipid peroxidation levels, liver endogenous antioxidant activities and hepatic total glutathion (GSH) cycle related-enzymes were examined in rats treated with CCl_4, in an effort to define the molecular mechanism involved in the hepatoprotective action. The indices used to evaluate cytotoxicity in hepatocytes were plasma membrane integrity, assessed by GOT and lactate dehydrogenase (LDH) release into the culture medium, and mitochondrial reduction of MTT, as a general measurement of mitochondrial dehydrogenase activity and cell viability. Finally, the *in vitro* radical scavenging activity of this compound was measured in terms of hydroxyl radical formation.

Therefore, in this study we have shown that the lethal injury induced by CCl_4 was effectively inhibited by pretreatment with ursolic acid in rat liver and primary hepatocytes by inhibiting the loss of GSH.

Materials and methods

Chemicals: Ursolic acid was purchased from Extrasynthèse (Genay, France). Williams' medium E. supplements for cell culture and other reagents for the evaluation of enzyme activities were obtained from Sigma (St Louis, MO).

Carbon tetrachloride-induced hepatotoxicity: A rat model of carbon tetrachloride (CCl_4)-induced hepatotoxicity [19], was used for examining *in vivo* antioxidant activities of ursolic acid. Wistar rats, 4-6weeks, 180–230 g were maintained on a 12 hr light/dark cycle at 22 °C and allowed food and water *ad libitum*. In this experiment, animals were randomly assigned into groups of 6 individuals. In the pretreatment groups, animals were treated intragastrically with ursolic acid at a desired daily dose (1, 2.5, 5 mmol/ kg in olive oil) for five days. Control animals received the appropriate vehicle alone (i.e., olive oil, 1 ml/kg). Twenty-four hours after the last dose, an oral dose of CCl_4 (1.0 mmol/kg in olive oil (1% (v/v)) was administered. Twenty-four hours after intoxication, the blood samples were collected and centrifuged at 2000 *g* for 5 min and the serum was separated and kept at 4 °C. sGOT and sGPT activities were measured by UV-Rate method [16] using GOT and GPT test Kits (Wako Pure Chemical Industries Co. Ltd). Results are expressed as Int. units L^{-1}.

Measurement of tissue lipid peroxidation levels

Hepatic tissue samples were homogenized in 154 mM KCl on ice and reflexed for 60 min at 95 °C in the presence of 0,3% (w/v) thiobarbituric acid (TBA) and 7.5% (v/v) acetic acid at pH 3.5. The reaction mixtures were incubated at 37 °C for 10 min and malondialdehyde (MDA) formation, an indirect index of lipid peroxidation was determined following the method of UCHIYAMA and MIHARA [33]. The optical density of the coloured product was read at 532 nm. Tetraethoxypropane was used as the standard curve and the results were expressed as nmol of malondialdehyde per mg of protein. Protein content of liver tissue was determined by the method of LOWRY et al. [22].

Biochemical analyses

• Superoxide dismutase (SOD) activity

The liver was homogenized in 10 mM Tris-HCL buffer (pH 7.4), 0.32 M sucrose and 1 mM EDTA. The homogenate was centrifuged at 15000 *g* for 10 min at 4 °C and the supernatant was collected for the assay. SOD activity was measured as described by SPITZ and OBERTEY [30], a method based on the inhibition of the reduction of nitroblue tetrazolium (NBT) by SOD. MnSOD activity was measured by the addition of 5 mM NaCN into the mixture to inhibit CuZnSOD activity. Cu Zn SOD activity was determined by subtracting MnSOD from total SOD activity. One activity unit is defined as the amount of protein required to give half-maximal inhibition of NBT reduction.

• Glutathione reductase (GR) and glutathione peroxidase (GPx) activities

Each liver was homogenized in 10 mM Tris-HCL buffer (ph 7.4) containing 0.32 M sucrose and 1 mM EDTA using a Teflon-glass Potter homogenizer. The homogenate was centrifuged at 12,000 *g* for 30 min at 4 °C and the supernatant used for GR assay.

Samples for GPx and Catalase were prepared in the same way as for GR activity.

GR activity was measured spectrophotometrically at 340 nm in terms of NADPH oxidation at 30 °C [6]. Briefly, the reaction mixture consisted of 840 µl of 50 mM phosphate buffer (pH 7.6), 10 µl of 1 mM EDTA, 100 µl of 0.15mM NADPH, 20 µl of 1 mM GSSG, 10 µl of 0.1% (w/v) bovine serum albumin (BSA), and 20 µl of the liver supernatant. That activity was expressed as nmol of NADPH oxidized/min/mg protein.

GPx liver activity was assayed by coupling the reduction of *t*-butyl hydroperoxide to the oxidation of NADPH by GR [37]. The assay mixture consisted of 100 µl of 1 M Tris-HCL containing 5 mM EDTA (pH 8.0), 20 µl of 0.1 M GSH, 100 µl GR (10 U/ml), 100 µl of 2 mM NADPH, 650 µl distilled water, 10 µl of 7 mM *t*-butyl hydroperoxide and 10 µl of the sample solution. Oxidation of NADPH at 37 °C was determined spectrophotometrically at 340 nm. One unit of activity was defined as the amount of GPx required to oxidize 1 mM of NADPH per min.

• Catalase activity (CAT)

CAT activity was determined at 37 °C by following the decomposition of H_2O_2 at 240 nm. [1]. The assay mixture consisted of 50 µl of 1 M Tris-HCL containing 5 mM EDTA (pH 8.0), 900 µl of H_2O_2, 30 µl distilled water, and 10 µl of liver supernatant. The activity was calculated

using a molar extinction coefficient of 7.1×10^{-3} M^{-1} cm^{-1}. In all assays, each reaction mixture was incubated at 37 °C for 5 min. The reaction was started by adding the supernatant prepared as described before.

• Glutathione determination

Total glutathione (GSH) was determined by the method described by AKERBOOM and SIES [2] based on the spectrophotometric evaluation of the reduction rate of 5,5`-dithiobis-2-nitrobenzoic acid into 5-thio-2-nitrobenzoate. Values were determined by comparing the reduction rate against a standard curve of glutathione. Oxidized glutathione (GSSG) was determined under the same conditions after adjusting pH with ethanolamine and trapping GSH with 2-vinyl-pyridine added to the sample. Glutathione levels were expressed as µmoles/ mg protein.

Isolation of hepatocytes: Rat liver parenchymal cells were isolated with a collagenase perfusion method following the procedure of SEGLEN [29]. In brief, the liver was pre-perfused *in situ* with Ca^{2+}-free HBSS (Hank`s balanced salt solution) containing 0.5% BSA and 0.5 mM EGTA, then recirculated with collagenase solution composed of Ca^{2+}-free HBSS, 0.075% collagenase, 4 mM $CaCl_2$, and 0.005% trypsin inhibitor.

Cell viability was determined by incubating the cell suspension (100 µl) with 0,4% trypan blue (25 µL) and phosphate buffered saline (PBS, 375 µL) and then counting the number of cells excluding the dye and the number of cells stained. Then, cells were suspended in William´s E medium supplemented with 10% calf serum, 50 µg/ml gentamycin, 1 µM dexamethasone, and 10 nM insulin. Cells seeded in 24-well plastic plates at a density of 1×10^6 cell/well were used for the CCl_4-induced hepatocytes lipid peroxidation assay, while 2×10^5 cells/well were taken for the hepatocyte necrosis assay. Cells were incubated in a 95% air, 5% CO_2 atmosphere at 37 ºC for 2 h and then medium was changed. After preculture, the cells were exposed to fresh medium (1 ml) containing CCl_4 (10 mM) in ethanol (0.01 ml) with or without test samples dissolved in DMSO (0.01 ml). Ursolic acid was added to hepatocyte cultures at the concentrations of 50, 100, 200 and 500 µM. Control cultures contained the same amount of vehicle.

Lipid peroxidation levels, GOT and LDH activities in the medium and MTT reaction were measured 60 min after intoxication.

In vitro assessment of hepatotoxic activity: GOT and LDH activities in the medium were measured 60 min after CCl_4 challenge, as indicators of hepatocytes necrosis and plasma membrane integrity. After incubation, the media which containing detached cells were collected and centrifuged at 50 *g* at 4 °C for 2 min. The supernatant was used to assay GOT and LDH activities by an autoanalyzer COBAS MIRA (Roche) using commercial Kits based on the GOT and LDH assay methods. Data are expressed in Int. units L^{-1}. Generally, experiments are valid only if the background activity of control cells is ≤ 25% of total available intracellular activity. So, for control cells, the total intracellular enzyme content was determined after treatment of cells with 0.05% Triton X-100 detergent to induce 100% lysis [32].

MTT reduction assay: Mitochondrial respiration, an indicator of cell viability, was assessed by the mitochondrial-dependent reduction of MTT (3 (4,5-dimethylthiazol-2 yl)-2,5-diphenyltetrazolium bromide) to formazan [24].One day after the isolated rat hepatocytes were plated, the cultured cells were exposed to the above-prepared medium containing CCl_4 for 1 h to induce cytotoxicity. The test compound was dissolved in DMSO and added to the hepatocytes simultaneously. One hour after the CCl_4 challenge, MTT assay was performed. The extent of reduction of MTT to formazan within cells was quantitated by measurement of OD540 with a microplate reader. Viability was set as 100% in untreated cells. The cell survival rate was expressed as percentage formazan production by treated samples compared with control samples.

Hepatocytes lipid peroxidation: The level of malondialdehyde was measured by a modification of the method described by Buege and Aust[7]. Briefly, 0.8 ml of culture supernatant was added to 80 µl 50% TCA, vortexed and centrifuged at 2500 *g* for 5 min. 0.6 ml of supernatant was added to 75 µl of 90 mM butylated hydroxytoluene (BHT), 0.6 ml of 0.2 M phosphoric acid and 75 µl of 0.11 M thiobarbituric acid. The reaction was stopped by placing the tubes on ice-cold water and 0.15 ml of saturated NaCl and 1.5 ml of 1-butanol were added. The mixture was then shaken and centrifuged at 2500 *g* for 10 min. The amount of MDA formed in each sample was assessed by measuring the optical density of the supernatant at 535 nm. Results were expressed as mmol MDA/10^6 cells using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}.

Hydroxyl radical formation: Formaldehyde formed during DMSO oxidation by the ascorbic acid/Fe^{3+} system was used to detect hydroxyl radicals [27]. Reaction mixture contained EDTA (0.1mM), Fe^{3+} (as a 1:2 mixture with EDTA) (167 mM), H_2O_2 (1 mM), 2,8 deoxyribose and phosphate buffer (20 mM, pH 7.4). Different concentrations of ursolic acid were added (50, 100, 250, 500 µM) and the reaction was started by addition of ascorbic acid (2 mM). Hydroxyl radicals were generated by incubating the mixtures at 37 °C for 60 min. [33]. Mannitol (5 mM) was used as reference substance. The extent of deoxyribose degradation by the formed hydroxyl radicals was directly measured in the aqueous phase by the thiobarbituric acid test [26].

Statistical analysis: Data values are given as arithmetic mean ± standard deviation (s.d.). Student's *t*-test was used to compare unpaired means of two data sets. *P* values < 0.05 were considered statistically significant.

Results

In vivo

The results obtained in the evaluation of the hepatoprotective activity of ursolic acid are shown in figure 1. CCl_4 treatment (1.0 mmol/kg *p.o.)* caused hepatocellular damage in rats, as shown by a drastic increase in plasma sGOT and sGPT activities. In contrast, a significant decrease in these enzymes levels was observed in all the

Table 1. Effects of drug administration on the specific activities of enzymes and levels of thiobarbituric acid reactive substances in the liver.

Treatment	Enzyme activity (units /mg liver protein^{-1})				TBA (nmol MDA mg liver protein^{-1})
	SOD	Gpx	GR	CAT	
Control (non-CCl_4)	64.45 ± 8.35	0.862 ± 0.023	1.041 ± 0.041	156.71 ± 7.12	0.94 ± 0.34
CCl_4-olive oil	44.13 ± 4.50†	0.646 ± 0.021†	0.946 ± 0.032†	83.21 ± 9.04†	2.56 ± 0.03†
Ursolic acid (1 mmol/kg)	48.45 ± 4.11	0.724 ± 0.012*	0.881 ± 0.010	96.11 ± 4.61	1.57 ± 0.10*
Ursolic acid (2.5 mmol/kg)	50.78 ± 1.32	0.802 ± 0.021*	0.872 ± 0.012	126.52 ± 5.12*	1.42 ± 0.13*
Ursolic acid (5 mmol/kg)	63.54 ± 0.91*	0.824 ± 0.031*	1.021 ± 0.032*	149.61 ± 5.30*	1.40 ± 0.12*

Changes in enzymes activities in the liver are expressed as the means ± s.d. n = 6; † $p < 0.01$ significantly different from control, *$p < 0.01$, significantly different from CCl_4-treated rats.

Table 2. Changes in the total glutathione content in CCl_4- induced on liver injury after pretreatment with ursolic acid.

Treatment	GSH (nmol/mg protein)	GSSG (nmol/mg protein)	GSH/GSSG ratio
Control (non-CCl_4)	22.30 ± 4.13	0.31 ± 0.02	71.11 ± 0.41
CCl_4-olive oil	12.51 ± 1.24†	1.12 ± 0.04†	11.12 ± 0.32†
Ursolic acid (1 mmol/kg)	14.32 ± 6.11	0.96 ± 0.61	14.80 ± 1.54
Ursolic acid (2.5 mmol/kg)	16.61 ± 2.45	0.61 ± 0.21	14.92 ± 0.23
Ursolic acid (5 mmol/kg)	29.82 ± 1.10*	0.49 ± 0.24*	60.80 ± 1.01*

The results are the mean ± s.d. for 4–6 rats. * $p < 0.01$, significantly different from the value of CCl_4-treated rats; † $p < 0.01$, significantly different from the control group.

Table 3. Effect of ursolic acid on CCl_4-induced liver injury in vitro and the survival rate of hepatocytes.

Treatment	GOT (units L^{-1})	LDH (units L^{-1})	Cell survival (% of cells surviving)
Control (non-CCl_4)	21.12 ± 4.30	82.48 ± 11.61	100.00 ± 0.00
CCl_4	92.22 ± 14.51†	267.21 ± 3.21†	74.42 ± 1.87†
Ursolic acid (50 µM)	75.71 ± 2.63*	241.14 ± 5.43*	78.70 ± 1.41
Ursolic acid (100 µM)	54.36 ± 4.67*	154.35 ± 2.12*	82.41 ± 1.87
Ursolic acid (200 µM)	61.42 ± 1.24*	178.58 ± 1.87*	81.01 ± 2.12
Ursolic acid (500 µM)	51.75 ± 1.13**	156.49 ± 3.67**	89.66 ± 3.87*

Each value is mean ± s.d. of results from two experiments in triplicate. † $P < 0.01$ with respect to control ; * $P < 0.05$, ** $P < 0.01$ with respect to CCl_4-group.

groups treated with ursolic acid compared with untreated controls exposed to CCl_4 alone. This effect was more marked at the concentration of 5 mmol/kg of ursolic acid.

Activities of GR, SOD, CAT and GPx were decreased significantly in CCl_4 -intoxicated rats compared with the control group (table 1). Meanwhile, pre-treatment of ursolic acid (1 and 2.5 mmol/kg) did not cause a significant decrease in the activity of glutathione reductase and superoxide dismutase. Only the pre-treatment with the highest dose of ursolic acid (5 mmol/kg) resulted in a significant partial recovery of the activity of these enzymes.

Pre-treatment with 1, 2.5 and 5 mmol/kg of ursolic acid reverted the decreased GPx activity, when compared with the CCl_4 intoxicated group, showing protection percentages of 36.3%, 73.6% and 82.8%, respectively. At the highest dose, the level of glutathione peroxidase activity almost reached control levels. Administration of ursolic acid to rats also caused a significant dose dependent recovery of CAT activity.

A significant increase of malondialdehyde levels in liver homogenates was observed after CCl_4 administration, indicating the occurrence of lipid peroxidation. Pre-treatment with ursolic acid dose-dependently inhibited CCl_4-mediated lipid peroxidation of microsomal

Table 4. Effects of pretreatment with ursolic acid on CCl_4-induced lipid peroxidation in primary cultured rat hepatocytes.

Treatment	MDA (nmol/10^6 cells)	Inhibition (%)
Control (non-CCl_4)	0.56 ± 0.13	–
CCl_4	3.12 ± 0.09†	–
Ursolic acid (50 µM)	3.09 ± 0.11	1.1
Ursolic acid (100 µM)	2.87 ± 0.03*	9.7
Ursolic acid (200 µM)	2.69 ± 0.11*	16.7
Ursolic acid (500 µM)	2.30 ± 0.09*	32.1

MDA values were expressed as mean ± s.d. † $P < 0.01$ with respect to control group. * $p < 0.01$, significantly different from the value of CCl_4-group.

membranes when compared with the CCl_4-intoxicated group showing a 71.1% protection at 5 mmol/kg of ursolic acid (table 1).

Following CCl_4 intoxication, hepatic mitochondrial glutathione redox status was greatly impaired, as indicated by a drastic decrease in mitochondrial GSH levels and a large increase in the GSSG levels. Treatment with ursolic acid (5 mmol/kg) caused a significant increase in the mitochondrial GSH level and a reciprocal decrease in the GSSG level; with a resultant elevation in the mitochondrial GSH/GSSG ratio, used as index of glutathione redox status (table 2).

In vitro

The extent of liver cell injury, expressed in terms of GOT and LDH levels released into the medium is shown in table 3. GOT and LDH release in CCl_4-treated group was significantly increased ($P < 0.01$ compared with control group) after CCl_4 exposure for 1h. Ursolic acid had a significant hepatoprotective activity as demonstrated by the reduction of GOT levels in cells treated with this compound ($P < 0.05$ with 50–200 µM and $P < 0.01$ with 500 µM). The results obtained indicate that ursolic acid also caused a significant reduction on LDH leakage ($P < 0.01$ is achieved only at the highest dose).

The survival rate of hepatocytes, expressed as a percentage of MTT reduction relative to control, was measured 60 min after CCl_4 exposure in cells pretreated with ursolic acid and in untreated cells (100% viability). As shown in Table III cells survival rate, was reduced to 74% in the CCl_4-treated cells, remaining over 78% in all the groups treated with ursolic acid. The effect of ursolic acid on lipid peroxidation was quantified by measuring the production of malondialdehyde. At the concentrations of 100–500 µM ursolic acid significantly prevented CCl_4-induced hepatocytes lipid peroxidation (table 4).

Finally, ursolic acid was tested for its ability to scavenge hydroxyl radicals. The effect of removing the hydroxyl radical exerted by this compound was small and detectable starting from the concentration of 50 µM in the reaction mixture (fig. 2).

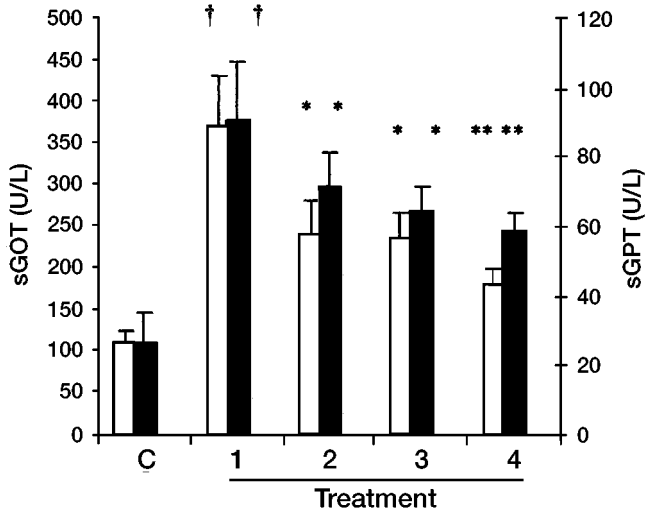

Fig. 1. Effect of ursolic acid on serum GOT and GPT levels in rats intoxicated by carbon tetrachloride. Result are expressed as mean ± s.d. (n = 6). C: control group, 1: group treated with carbon tetrachloride (1 mmol/kg), 2: group pre-treated with ursolic acid (1 mmol/kg, for 5 days) and treated with CCl_4 (1 mmol/kg, the last day), 3: group pre-treated with ursolic acid (2.5 mmol/kg, for 5 days) and treated with CCl_4 (1 mmol/kg, the last day), 4 group pre-treated with ursolic acid (5mmol/kg, for 5 days) and treated with CCl_4 (1 mmol/kg, the last day). † $p < 0.01$ compared with control, * $p < 0.05$, ** $p < 0.01$ compared with CCl_4-treated. □ sGOT; ■ sGPT

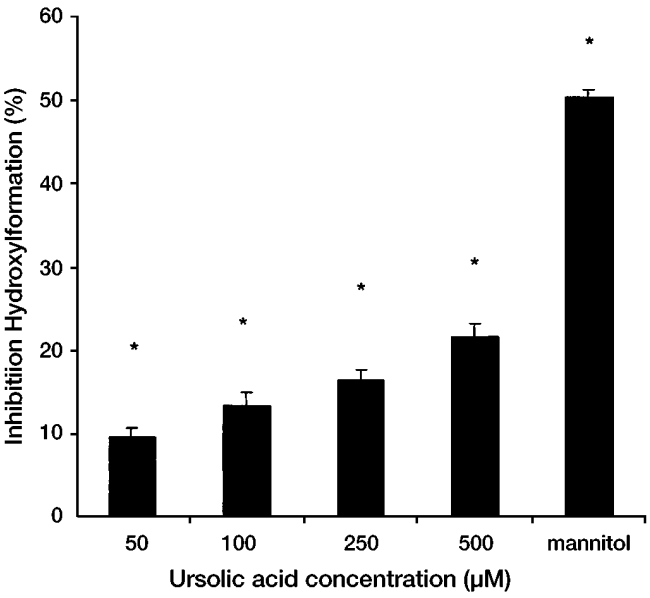

Fig. 2. Effect of ursolic acid on hydroxyl radical formation. Percent inhibition is based on absorbance values of samples with ursolic acid or reference compound (mannitol 5 mM) against controls containing an equal volume of the solvent. Each column represents the mean value ± s.d. (n = 6), * $p < 0.01$.

Discussion

It is well-recognised that free radicals are critically involved in various pathological conditions such as inflammation, liver diseases, etc [10]. Chemicals such as CCl_4-catabolized radicals induce lipid peroxidation, damage the membranes of liver cells and organelles, cause the swelling and necrosis of hepatocytes and result in the release of cytosolic enzymes such as GOT, GPT and LDH into the blood circulation. Therefore, CCl_4-induced liver injury has been employed as a convenient model for investigating radical-induced damage and its prevention in animals [17].

Some compounds, natural or synthetic, with antioxidant properties, would contribute towards the partial or total alleviation of this damage. In a previous paper [3], it has been shown that ursolic acid decreases microsomal lipid peroxidation *in vitro* by using two different standard systems: ascorbic acid, and ADP/Iron to induce lipid peroxidation in isolated rat liver microsomes *in vitro*. We found that ursolic acid had a strong inhibitory activity against superoxide formation and also showed superoxide scavenging activity and because of these activities, it was expected that ursolic acid exerted hepatoprotective activity against CCl_4-intoxication.

On the basis of these results, the present study was designed to determine if ursolic acid can affect the antioxidant enzymes activities and levels of thiobarbituric acid reactive substances in rat liver homogenates, since to the best of our knowledge the literature does not provide any information on this subject. Pre-treatment of rats with different doses of ursolic acid caused a significant reduction in hepatic injury, as a result of its ability to stimulate or to protect hepatic drug-metabolizing enzymes. In CCl_4-induced acute liver damage, elevated levels of GOT and GPT in serum are indicative of cellular leakage and loss of functional integrity of cell membranes in the liver [9]. The reduction in the levels of sGOT and sGPT after administration of ursolic acid could indicate stabilization of the plasma membrane and the repair of hepatic tissue damage caused by CCl_4. This is in agreement with the commonly accepted view that serum levels of transaminases return to normal with healing of hepatic parenchyma and the regeneration of the hepatocytes [31]. The hepatoprotective activity of ursolic acid against CCl_4-intoxication was also confirmed in experiments performed *in vitro* on isolated rat hepatocytes. Ursolic acid significantly reduced GOT and LDH release into the medium. The results obtained on the MTT assay confirm the hepatoprotective activity of this compound.

Glutathione is present at a large concentration in the liver and plays a major role in the elimination of a large number of nucleophilic exogenous toxicants. Therefore, it is involved in the detoxification of many chemical agents. Glutathione metabolism is also important in quenching the reactive intermediates and radical species generated during oxidative toxicity.

Enzymes such as GPx, CAT and SOD also play important roles in protecting against free radical damage and are considered the primary antioxidant enzymes since they are involved in direct elimination of active oxygen species. Glutathione reductase participates in maintaining adequate levels of GSH by reducing GSSG to GSH [12]. A toxicant itself can be a nucleophile or converted to a nucleophile by microsomal enzymes. After the administration of toxicants such as CCl_4, glutathione levels are drastically decreased and, very often, the enzymes responsible for protecting against nucleophilic damage are also affected. Pretreatment with ursolic acid (5 mmol/kg) significantly preserved levels of total glutathione and preventing decreased by CCl_4 intoxication, showing an increase in the GSH/GSSG ratio, an index of glutathione redox status.

Moreover, in the present study, we have shown that pre-treatment of rats with ursolic acid (5 mmol/kg) significantly preserved the activities of the antioxidative enzymes SOD, GR, GPx, and CAT. In rats treated with ursolic acid (5 mmol/kg), GR activity was increased compared with CCl_4 group, and this effect could be considered as a supercompensation in glutathione redox status which protects against the increased production of hydroperoxides during the hepatotoxical process.

The results obtained suggest that the maintenance of reduced glutathione by ursolic acid was mainly due to inactivation of reactive oxygen species via retention of antioxidative enzymes such as CAT and SOD [5]. The ability of ursolic acid to remove the hydroxyl radical was small and detectable at concentrations higher than 50 µM in the reaction mixture.

Therefore, it was presumed that the effects of ursolic acid might be related to a normalization mechanism by maintaining adequate levels of GSH for detoxification of xenobiotics.

Depletion of glutathione by CCl_4 ultimately resulted in lipid peroxidation, which is believed to be responsible in part for subsequent hepatocellular damage [38]. Ursolic acid significantly ameliorated lipid peroxidation *in vivo* and *in vitro*, as demonstrated by the reduction in MDA production, which suggest that ursolic acid may prevent the initiation and propagation of the lipid peroxidation process by scavenging free radical via conjugation with glutathione. This tissue protection was more evident with the highest dose of ursolic acid. The beneficial effect, therefore, was associated with a significant enhancement of hepatic glutathione status, as indicated by the substancial increase in tissue GSH levels and the GSH/GSSG ratio, the corresponding decreases in GSSG levels and the levels of TBA. We found an inverse correlation between the content of GSH and the levels of MDA, which agrees with results obtained by other authors [40, 36]. Thus, the hepatoprotection afforded by ursolic acid pre-treatment may be attributed to an enhancement in the functioning of the hepatic GSH antioxidant system, possibly by stimulating the activities of the related enzymes of GSH.

Other antioxidant compounds have also shown hepatoprotective activity. Thus, it has been reported that oleanolic acid [20, 21, 34], a triterpenoid compound with a structure closely related to ursolic acid exerts hepatoprotective effects on CCl_4-treated rats.

According to our data and those previously reported in the literature, the antioxidant properties together with the hepatoprotective activity both *in vivo* and *in vitro* in terms of inhibition of lipid peroxidation could explain the beneficial action of ursolic acid against pathological alterations caused by the presence of free radicals which occur in certain hepatic disorders.

Acknowledgments: This work was supported by project PR 486/97-7488, from University Complutense Madrid, Spain.

References

1. AEBY H: Catalase in vitro. Methods Enzymol 1984; **105**: 121–126.
2. AKERBOOM TP, SIES H: Transport of glutathione, glutathione disulfide, and glutathione conjugates across the hepatocyte plasma membrane. Methods Enzymol 1989; **173**: 523–534.
3. BALANERHU S, NAGARAJAN B: Protective effect of oleanolic acid and ursolic acid against lipid peroxidation. Biochem Int 1991; **24**: 981–990.
4. BALANERHU S, NAGARAJAN B: Intervention of adriamycin induced free radical damage. Biochem Int 1992; **28**: 735–744.
5. BARJA DQ, GIL P, LOPEZ-TORRES M: Physiological significance of catalase and glutathione peroxidases, and in vivo peroxidation, in selected tissues of the toad Discoglossus pictus (Amphibia) during acclimation to normobaric hyperoxia. J Comp Physiol [B] 1988; **158**: 583–590.
6. BEUTLER E: Effect of flavin compounds on glutathione reductase activity: in vivo and in vitro studies. J Clin Invest 1969; **48**: 1957–1966.
7. BUEGE JA, AUST SD: Microsomal lipid peroxidation Methods Enzymol 1978; **52**: 302–310.
8. CHA HJ, PARK MT, CHUNG HY, et al.: Ursolic acid-induced down-regulation of MMP-9 gene is mediated through the nuclear translocation of glucocorticoid receptor in HT1080 human fibrosarcoma cells. Oncogene 1998; **16**: 771–778.
9. DROTMAN RB, LAWHORN GT: Serum enzymes as indicators of chemically induced liver damage. Drug Chem Toxicol 1978; **1**: 163–171.
10. ELSTNER EF: Oxygen radicals—biochemical basis for their efficacy. Klin Wochenschr 1991; **69**: 949–956.
11. ES-SAADY D, SIMON A, JAYAT-VIGNOLES C, et al.: MCF-7 cell cycle arrested at G1 through ursolic acid, and increased reduction of tetrazolium salts. Anticancer Res 1996; **16**: 481–486.
12. HALLIWELL B, GUTTERIDGE JM.: The antioxidants of human extracellular fluids. Arch Biochem Biophys 1990; **280**: 1–8.
13. HAN SK, KO YI, PARK SJ, et al.: Oleanolic and ursolic acid stabilize liposomal membranes. Lipids 1997; **32**: 769–773.
14. HOULT JR, PAYA M: Pharmacological and biochemical actions of simple coumarins: natural products with therapeutic potential. Gen Pharmacol 1996; **27:** 713–722.
15. JUNG HA, PARK JC, CHUNG HY, et al.: Antioxidant flavonoids and chlorogenic acid from the leaves of Eriobotrya japonica. Arch Pharm Res 1999; **22**-2: 213–218.
16. KARMEN A, WROBLEWSKI F, LA DUE JS: Transaminase activity in human blood. J Clin Invest 1955; **34**: 126–131.
17. KISO Y, TOHKIN M, HIKINO H, et al.: Mechanism of antihepatotoxic activity of glycyrrhizin. I: Effect on free radical generation and lipid peroxidation. Planta Med 1984; **50**: 298–302.
18. KITANI K, KANAI S, IVY GO, CARRILLO MC: Pharmacological modifications of endogenous antioxidant enzymes with special reference to the effects of deprenyl: a possible antioxidant strategy. Mech Ageing Dev 1999; **111**: 211–221.
19. KO KM, IP SP, POON MK, et al.: Effect of a lignan-enriched fructus schisandrae extract on hepatic glutathione status in rats: protection against carbon tetrachloride toxicity. Planta Med 1995; **61**: 134–137.
20. LIU J: Pharmacology of oleanolic acid and ursolic acid. J Ethnopharmacol 1995; **49**: 57–68.
21. LIU J, LIU Y, MAO Q, KLAASSEN CD: The effects of 10 triterpenoid compounds on experimental liver injury in mice. Fundam. Appl Toxicol 1994; **22**: 34–40.
22. LOWRY OH, ROSEBROUGH NJ, FARR AL, RANDALL RJ: Protein measurement with the Folin phenol reagent. J Biol Chem 1951; **193**: 265–275.
23. MARTIN-ARAGON S, BENEDI JM, VILLAR AM: Effects of fraxetin on glutathione redox status. Z Naturforsch *[C]* 1997; **52**: 55–59.
24. MOSMANN T: Rapid colorimetric assay for cellular growth and survival. Application to proliferation and cytotoxicity assays. J Inmunol Method 1983; **65**: 55–63s.
25. NOGUERA B, CAMACHO M, MATA R, et al.: Constituents of Kholeria deppeana. Fitoterapia 1994; **65** 2: 182–5.
26. PAYA M, HALLIWELL B, HOULT JR: Interactions of a series of coumarins with reactive oxygen species. Scavenging of superoxide, hypochlorous acid and hydroxyl radicals. Biochem Pharmacol 1992; **44**: 205–214.
27. REKKA A, KOUROUNAKIS L, KOUROUNAKIS P: Antioxidant activity of and interleukin production affected by honey bee venom. Arzneimittelforschung 1996*;* **40** 8: 912–913.
28. SARASWAT B, VISEN PK, AGARWAL DP: Ursolic acid isolated from Eucaliptus tereticornis protects against ethanol toxicity in isolated rat hepatocytes. Phytother Res 2000; **14**: 163–166.
29. SEGLEN PO: Preparation of isolated rat liver cells. Methods Cell Biol 1976; **13**: 29–83.
30. SPITZ DR, OBERLEY LW: An assay for superoxide dismutase activity in mammalian tissue homogenates. Anal Biochem 1989; **179**: 8–18.
31. THABREW MI, JOICE PD, RAJATISSA W: A comparative study of the efficacy of Pavetta indica and Osbeckia octandra in the treatment of liver dysfunction. Planta Med 1987; **53**: 239–241.

32. TYSON CA, HAWK-PRATHER K, STORY DL, GOULD DH: Correlation of in vitro and in vivo hepatotoxicity for five haloalkanes. Toxicol Appl Pharmacol 1998; **70**: 289–302.
33. UCHIYAMA M, MIHARA M: Determination of malondialdehyde precursor in tissues by thiobarbituric acid test. Anal Biochem 1977; **86**: 271–278.
34. VISEN PK, SARASWAT B, DHARWAN BN: Curative effect of pricoliv on primary cultured rat hepatocytes against different hepatotoxins: an in vitro study. J Pharmacol Toxicol Methods 1998; **40** 3: 302–310
35. VISEN PK, SHUKLA B, PATNAIK GK, DHAWAN BN: Prevention of galactosamine-induced hepatic damage by picroliv: study on bile flow and isolated hepatocytes (ex vivo). Planta Med 1993; **59**: 37–41.
36. WESTERVELD GJ, SCHEEREN RA, DEKKER I, et al.: Antioxidant actions of oxymethazoline and xylomethazoline. Eur J Pharmacol 1995; **291**: 27–31.
37. YAMAMOTO Y, TAKAHASHI K: Glutathione peroxidase isolated from plasma reduces phospholipid hydroperoxides. Arch Biochem.Biophys 1993; **305**: 541–545.
38. YASUDA H, IZUMI N, SHIMADA, et al.: The protective effect of tinoridine against carbon tetrachloride hepatotoxicity. Toxicol Appl Pharmacol 1980; **52**: 407–413.
39. YESILADA Z, SIZIK E: Screening of some turkish medicinal plants used in the treatment of rheumatoid arthritis and inflammatory diseases for their anti-inflammatory activities. Planta med 1990; **56**: 65–69
40. YU BP: Cellular defences against damage from reactive oxygen species. Physiol Rev 1994; **74**: 139–162.