Cyclosporine A toxicity and effect of the S-adenosylmethionine

Toxicidad de la ciclosporina A y efecto de la S-adenodilmetionina

GALÁN, A.; FERNÁNDEZ, E.; FELIPE, A.; MORÓN, D.; MUÑOZ, M. E. AND JIMÉNEZ, R.

Department of Phisiology and Pharmacology, Universidad de Salamanca
37007 – Salamanca, Spain

ABSTRACT

We studied the simultaneous changes undergone by the main indicators of hepatotoxicity, nephrotoxicity and diabetogenicity in rats treated with cyclosporine A (CyA) for one, two, three and four weeks, using the dose of 10 and 20 mg/kg/day. The effects of the drug on the biliary excretion of several biliary compounds as well as on the bile flow fractions - dependent and independent- of the biliary secretion of bile acids and glutathione were also studied. A further aim of this research was to evaluate the hepatoprotective effect of S-adenosylmethionine (SAMe) against the action of CyA. Our results show that CyA treatment alters hepatic, renal and pancreatic functions rapidly and simultaneously; the changes were slightly more intense with the higher dose but they did not become more pronounced when the treatment was prolonged for 4 weeks. The cholestatic effect of the drug is a multifactorial phenomena and develops accompanied by simultaneous decreases in the biliary secretion of bile acids, lipid, glutathione and proteins. SAMe plus CyA cotreatment antagonizes the main hepatotoxic effects of CyA in this species. This hepatoprotective effect of SAMe could be related to its regulatory function as regards membrane lipid composition and fluidity and to its key role in promoting the hepatic synthesis of thiol compounds.
KEY WORDS: Bile acid, bile flow, cholestasis, cyclosporine A, glutathione, lipids, SAMe

RESUMEN

Hemos estudiado los cambios simultáneos experimentados por los principales indicadores de hepatotoxicidad, nefrotoxicidad y diabetogenicidad en ratas tratadas con ciclosporina A (CyA) durante una, dos, tres y cuatro semanas, utilizando dósis de 10 y 20 mg/kg/día. También fueron estudiados los efectos de la droga sobre la excreción biliar de varios compuestos biliares, así como sobre las fracciones de flujo biliar –dependiente e independiente- de la secreción biliar de ácidos biliares y glutation. Otro objetivo de este estudio fue la evaluación del efecto hepatoprotector de la S-Adenosilmetionina (SAMe) frente a la acción de la CyA. Nuestros resultados mostraron que el tratamiento con CyA alteran las funciones hepática, renal y pancreática rápida y simultáneamente; los cambios cambios fueron ligeramente más intensos con una dosis más alta pero no se volvieron más pronunciados cuando el tratamiento se prolongó hasta las cuatro semanas. El efecto colestático de la droga es un fenómeno multifactorial y se desarrolla junto a una disminución de la secreción biliar de ácidos biliares, lípidos, glutation y proteinas. El tratamiento conjunto de SAMe y CyA antagoniza los principales efectos hepatotóxicos del CyA en estas especies. Este efecto hepatoprotector del SAMe podría estar relacionado con su función reguladora al preservar la composición y fluidez de la membrana lipídica y con su papel clave en la promoción de la síntesis de componentes tiol.
PALABRAS CLAVE: Ácido biliar, flujo biliar, colestasis, ciclosporina A, glutation,, lípidos, SAMe

INTRODUCTION

Cyclosporine A (CyA), a powerful immunosuppressor that improves survival in solid organ transplantation, is known to induce side effects in animals and humans; among them re-

nal, hepatic and pancreatic dysfunctions, gastrointestinal and neurological complications, bone effects, etc. have been reported (1-3). Data relating to the chronological evolution of these dysfunctions, especially CyA-induced changes in several plasma markers of nephrotoxicity, hepatotoxicity and diabetogenicity in the rat are discrepant and contradictory (4-6).

The hepatotoxic effects of the drug elicit alterations in bile formation, the capacity of the liver to excrete organic anions and xenobiotics and changes in the hepatic content of glutathione (GSH) (5, 7-11). Regarding bile formation, it has been reported that CyA treatment induces a cholestatic syndrome with increased plasma levels of bilirubin and bile acids (BA). We have recently reported that acute and long-term CyA administration reduces the formation of what is operationally defined as the bile acid-dependent bile flow (BADF) and bile acid-independent bile flow (BAIF) (10, 12). The hepatocellular mechanisms responsible for decreases in BADF-associated cholestasis are still unclear, but some evidence suggests that decreases in the sinusoidal and canalicular transport of BA, secondary both to inhibition of membrane BA carriers (13-15) and to disturbances in hepatocyte plasma membrane composition and fluidity (4, 16, 17) may be involved. Inhibition of the biliary secretion of BA could also interfere in biliary lipid (BLP = cholesterol and phospholipid) secretion, since it is well known that BLP secretion is related to and dependent upon BA secretion (18, 19). Thus, the vectorial transport of BA from blood to bile is a critical process not only for BADF formation but also for the removal of BLP from the canalicular membrane and their later secretion into and solubilization in the bile canaliculus (19, 20). However, the effects of CyA on BLP secretion are debatable since decreases (10, 21, 22) or no changes (5) in their secretion have been observed in the rat and the mechanisms of this effect of the drug have not been clarified either.

The causes of CyA-induced inhibition of the BAIF and its contribution to cholestasis are unknown. Although many reports concerning the mechanisms involved in BAIF formation have been made, such mechanisms remain to be fully elucidated. However, different studies have suggested biliary GSH as one of the solutes responsible for the BAIF (23-25). The transport of GSH into bile is mediated, as in the case of BA, by specific carriers embedded in the canalicular membrane of hepatocytes (25, 26) their activity depending, among other factors, on membrane composition and fluidity (26-28). Studies addressing the effect of CyA on rat liver GSH contents have afforded conflicting results since no changes (29, 30) or reductions (9, 11) have been reported. Moreover, the effects of the drug on the biliary secretion of GSH are unknown, as is the possible relationship between such effects and the decreases observed in the BAIF after CyA treatment.

S-adenosyl-L-methionine (SAMe) is a physiological compound that plays a key role in liver transmethylation and transsulphuration reactions. Through SAMe-dependent methylation of membrane phospholipid, the compound modulates and/or restores liver plasma membrane fluidity and reverses the cholestatic effects of several hepatotoxins (31-34), including the CyA-induced cholestasis in the rat (35, 36). It has also been described that, through SAMe-dependent transsulphuration reactions, SAMe antagonizes the GSH depletion caused by different agents in several experimental models, thus improving the detoxifying capacity of hepatocytes (31, 34, 37-39). However, it remains to be seen whether SAMe antagonizes the hepatic depletion of GSH caused by CyA, as well as the possible alterations induced by the immunosuppressor on the biliary secretion of GSH, and hence, on BAIF.

On the basis of these considerations we assessed the simultaneous changes undergone by the main indicators of nephrotoxicity, hepatotoxicity and diabetogenicity. We also studied the effects of the drug on bile flow and BA, BLP, protein and GSH secretion as well as on the bile flow fractions -dependent and independent- of the biliary secretion of BA and GSH. Finally, a further aim of our investigations was to evaluate the ability of SAMe to prevent or antagonize the adverse effects of the CyA on the different parameters evaluated in our study.

MATERIALS AND METHODS

Adult male Wistar rats were randomly divided into eleven groups (6 to 8 rats in each group) and treated as follows: One group received physiological saline over four weeks and was used as Controls; another group (VE group) was given the CyA-vehicle (olive oil) for four weeks; eight groups were treated with CyA, at doses of 10 mg/kg per day (the C groups) or 20 mg/kg per day (the CC groups), for one (C1 and CC1), two (C2 and CC2), three (C3 and CC3) and four (C4 and CC4) weeks. Finally, the S1C1 rat group was cotreated with SAMe and CyA over one week, at doses of 10 mg/kg/12 hours and 10 mg/kg/day, respectively. CyA, olive oil and saline were administered i.p., and SAMe s.c. Twelve hours after the treatments had ended, rats were anaesthetized with sodium pentobarbital (50 mg/kg b.wt, i.p.). After tracheotomy and laparotomy the animals were fitted with a cannula in the common bile duct and a catheter in the left carotid artery for collecting bile and blood samples, respectively. Thirty minutes after the end of the surgical procedure four bile samples were collected at 15 min intervals. Biliary GSH glutathione was determined before and after hepatic gamma glutamyl transferase (ã-GT) activity was inhibited. Irreversible inhibition of this enzyme was achieved by means of a retrograde intrabiliary infusion of acivicin following the method reported by Sztefko

et al. (40). At the end of the assays a blood sample was taken; after this, the rats were killed by exsanguination, the livers were quickly removed and weighed and small pieces were harvested for biochemical determinations and histological examination using light and electron microscopy.

Bile flow was determined gravimetrically. Total BA concentrations in plasma and bile (12) and cholesterol and phospholipid concentrations in bile (22) were determined enzymatically. Acid phosphatase (ACP) and ã-GT activities in bile were evaluated by the method reported previously (22). Total GSH in bile and liver was evaluated by the enzymatic recycling procedure using glutathione reductase and 5,5«-dithio-bis (2-nitrobenzoic acid) (41, 42). Plasma activities, at 30¡C, of ACP, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase and lactate dehydrogenase, the plasma concentrations of bilirubin, glucose, creatinine, urea and total protein and the hepatic protein contents were measured by routinely used optimized methods on an automated analyzer (Hitachi, mod. 717).

Data were analyzed statistically by the Kruskal-Wallis test and the Wilcoxon test or the Mann-Whitney U test for paired and unpaired values. P values of 0.05 or less were considered statistically significants. Results are expressed as means ± Standard Error of Means (S.E.M.).

RESULTS

Effects of treatment with CyA on hepatic, pancreatic and kidney function indicators and on hepatic morphology. All animals appeared to be healthy and none died during the treatments although those receiving CyA gained weight at a rate significantly lower than the controls. Despite this, the mean values of the hepatosomatic ratio (liver weight as % of body weight) at the end of the treatments were similar in all rat groups (data not shown).

Treatment with the CyA-vehicle did not result in any significant changes in any of the parameters evaluated in plasma, liver and bile. This is consistent with the findings of previous studies in which olive oil treatment did not lead to observable effects in the parameters evaluated in our study (4, 10, 36). By contrast, CyA treatment

did elicit significant changes in the most sensitive biochemical indicators of hepatic, pancreatic and kidney function in all experimental groups. In this sense, it increased BA, bilirubin, glucose, urea and creatinine plasma levels but reduced total proteins in plasma and liver (Table 1, fig. 1). The rises and falls were evident with both doses, the changes being slightly more intense with the higher dose. However, they did not become more pronounced as treatment progressed. Chromatography studies revealed that hyperbilirubinemia was due to increases in the concentrations of both bilirubin metabolites: unconjugated and conjugated bilirubin. Transaminase activities and those of the other hepatic-function indicator enzymes evaluated were unmodified (data not shown).

Table 1. Effects of long-term CyA treatment and CyA plus SAMe cotreatment on plasma concentration of bile acids, bilirubin, glucose, urea, creatinine and proteins (mg/dl) and the hepatic content of total proteins (mg/g liver). Rats were treated for four weeks with physiological saline (Control) or the CyA-vehicle (VE). The C_1, C_2, C_3 and C_4 rats groups were treated with 10 mg of CyA/kg/day for one, two, three or four weeks, respectively. The S_1C_1 rats group was cotreated with CyA (10 mg/kg/day) plus SAMe (10 mg/kg/12 hours) for one week. Values are given as means ± SEM for 6-8 rats in each group; $p < 0.05$, significantly different from the Control group (*) or C_1 group (#).

|	Control	VE	C_1	C_2	C_3	C_4	S_1C_1
Bile acids	0.59 ± 0.11	0.70 ± 0.11	8.01 ± 1.18*	9.03 ± 1.02*	10.1 ± 1.4*	12.0 ± 3.1*	3.06 ± 0.32* #
Bilirubin	0.08 ± 0.01	0.09 ± 0.01	0.21 ± 0.03*	0.20 ± 0.02*	0.20 ± 0.01*	0.19 ± 0.01*	0.11 ± 0.01 #
Glucose	149 ± 5	176 ± 10	266 ± 29 *	273 ± 40 *	273 ± 45 *	282 ± 18 *	194 ± 13
Urea	35.0 ± 2.6	41.7 ± 2.2	77.3 ± 2.6*	71.6 ± 6.5*	81.6 ± 3.9*	73.8 ± 2.9*	91.4 ± 5.9*
Creatinine	0.41 ± 0.02	0.42 ± 0.02	0.59 ± 0.03*	0.61 ± 0.06*	0.68 ± 0.06*	0.67 ± 0.05*	0.63 ± 0.01*
Plasma proteins	5390 ± 200	5060 ± 70	4520 ± 140 *	4380 ± 180 *	4510 ± 60 *	4500 ± 100 *	5150 ± 190 #
Hepatic proteins	90.4 ± 6.2	91.0 ± 4.9	75.9 ± 3.2*	73.1 ± 4.6*	74.8 ± 2.3*	82.8 ± 5.1	93.3 ± 6.6 #
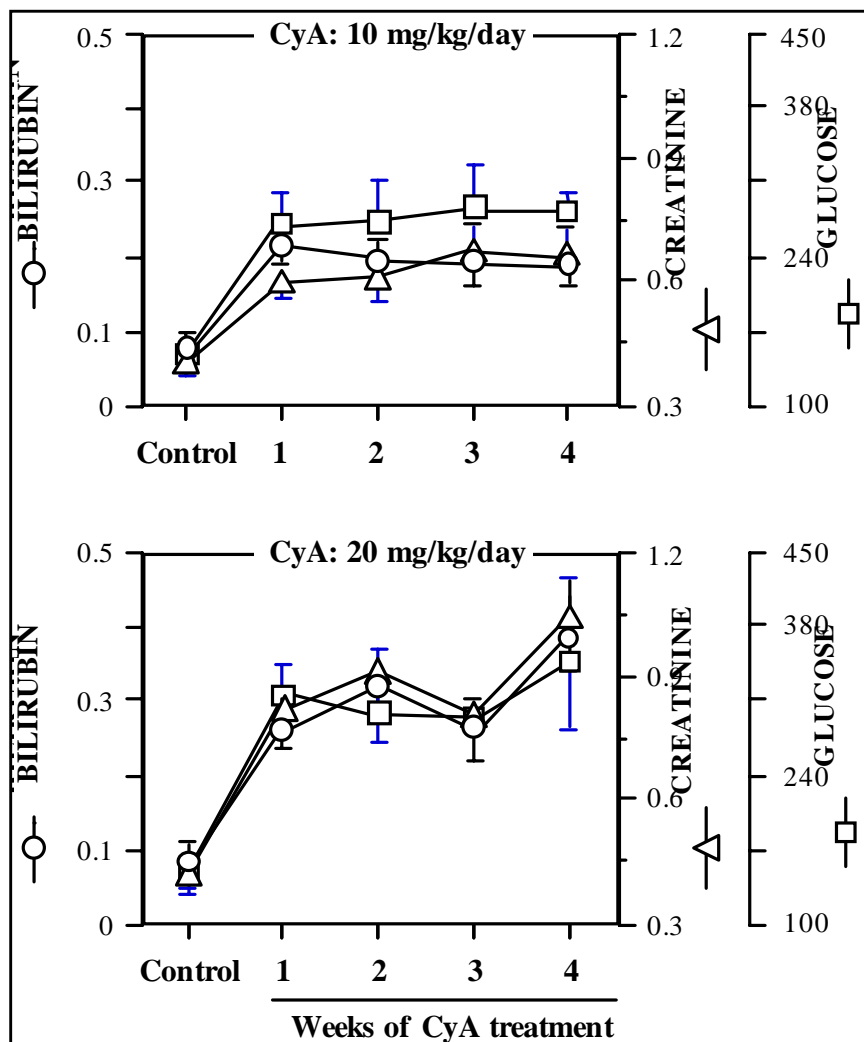
Figure 1: Effects of long-term CyA treatment on one plasma indicator of hepatotoxicity (bilirubin) and its relationship over time with those observed in the plasma indicators of renal (creatinine) and pancreatic (glucose) toxicity. Rats were treated for four weeks with physiological saline (Control) or CyA for one, two, three or four weeks. CyA was given at doses of 10 or 20 mg/kg/day. Plasma concentrations of the three indicators of toxicity are expressed in mg/dl and are given as means ± SEM for 6-8 rats in each group.

Regarding changes in hepatic morphology, Stone et al. (5) have not observed architectural alterations in rats treated for 4 weeks; however, we observed that CyA treatment, among other effects, induced a dilation of the endoplasmic reticulum, an increase in the number of autophagic vacuoles, a slight degree of mitochondrial degeneration, lysosomal labilization, the presence of fat droplets in the cytosol and focal fat degeneration of perivascular hepatocytes. In some animals, inflammatory infiltration and necrosis of individual hepatocyte were observed. All these findings were generally more prominent in the livers of rats treated for 4 weeks, in accordance with previous reports (43).

Effects of the treatment with CyA on biliary secretion. The mean values of bile flow and of the biliary secretion rates of BA, cholesterol, phospholipids as well as those of the biliary activity and secretion rates of two marker proteins ($\tilde{a}$-GT -a canalicular membrane marker enzyme- and ACP -a lysosomal marker-) in the Control, CyA-vehicle- and CyA treated rat groups are shown in table 2 and figure 2. The mean values of these parameters in the CyA-vehicle-treated group were similar to those obtained in the controls and lay within the physiological ranges for this species. By contrast, CyA treatment induced significant reductions in bile flow and in the biliary secretion rates of BA, BLP and proteins (Table 2, fig. 2).

Table 2. Effects of long-term CyA treatment and CyA plus SAMe cotreatment on bile flow and the biliary activity and secretion rates of the marker enzymes γ-GT and ACP. Rats were treated for four weeks with physiological saline (Control) or the CyA-vehicle (VE). The C_1, C_4, CC_1 and CC_4 rats groups were treated with CyA for one (C_1 and CC_1) or four (C_4 and CC_4) weeks, using the dose of 10 mg/kg/day (C_1 and C_4 groups) or 20 mg/kg/day (CC_1 and CC_4 groups). The SC_1 rats group was cotreated with CyA (10 mg/kg/day) plus SAMe (10 mg/kg/12 hours) for one week. Values are given as means $\pm$ SEM for 6-8 rats in each group; $p < 0.05$, significantly different from the Control group (*) or C_1 group (#).

|	BILE FLOW	γ-GT		ACP	
	(μl/min/g liver)	(IU/l)	(μIU/min/g liver)	(IU/l)	(μIU/min/g liver)
Control	1.64 ± 0.08	149 ± 27	373 ± 64	19.1 ± 6.8	39.0 ± 15.1
VE	1.61 ± 0.04	156 ± 18	339 ± 48	21.1 ± 5.3	40.2 ± 9.3
C_1	1.27 ± 0.06*	17.6 ± 4.2*	22.1 ± 5.1*	0.23 ± 0.14*	0.34 ± 0.20*
C_4	1.32 ± 0.04*	14.4 ± 5.2*	18.1 ± 6.0*	1.34 ± 0.78*	1.69 ± 0.98*
CC_1	1.43 ± 0.06*	25.8 ± 6.0*	38.6 ± 9.9*	3.04 ± 1.15*	4.72 ± 1.85*
CC_4	1.29 ± 0.04*	19.8 ± 4.6*	25.0 ± 6.8*	1.60 ± 1.00*	2.06 ± 1.34*
S_1C_1	1.45 ± 0.05* #	43.0 ± 14.7* #	68.0 ± 17.1* #	15.3 ± 6.5 #	20.6 ± 8.6 #
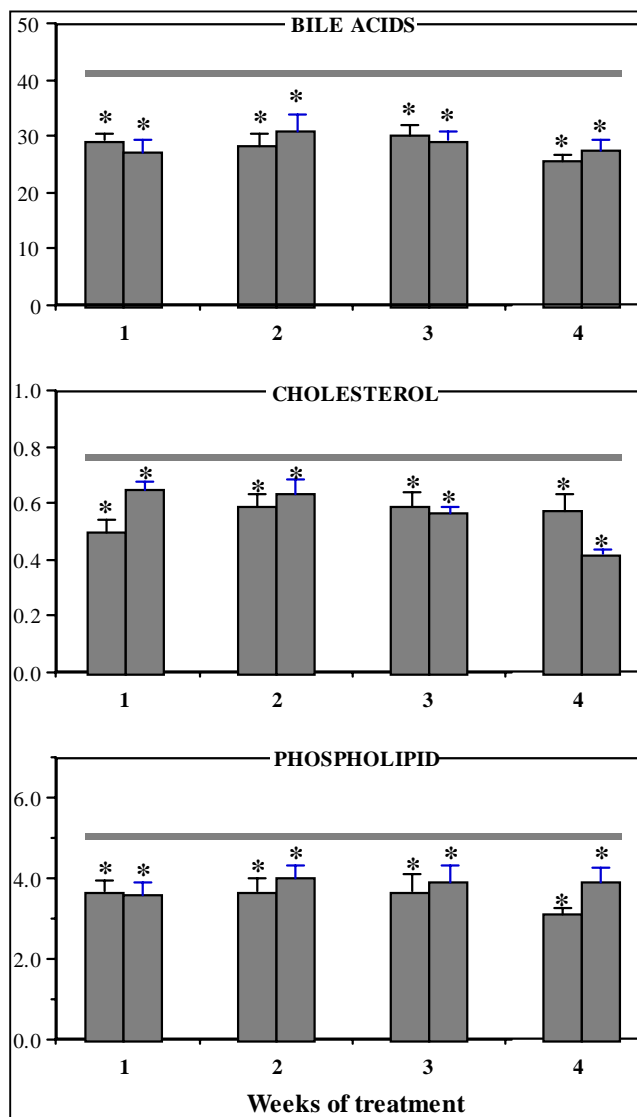
Figure 2: Effects of long-term CyA treatment on biliary secretion rates (nmol/min/g liver) of bile acids, cholesterol and phospholipid. Rats were treated with physiological saline or with CyA, at the doses of 10 mg/kg/day () or 20 mg/kg/day (), for one, two, three or four weeks. Values are given as means $\pm$ SEM for 6-8 rats in each group. Lines over the columns represent the means values in the controls. * $p < 0.05$, significantly different from the controls.

The decreases observed in the biliary parameters were significant for both doses as from the first week of treatment. When CyA treatment was prolonged for 2, 3 or 4 weeks the cholestasis and the inhibitory effect of the drug on the biliary secretion rate of BA, BLP and proteins were similar to those observed during the first week, regardless of the dose used and the duration of the treatment (Fig. 2).

Biliary and hepatic GSH was only evaluated in the Control, VE and C1 groups. We observed that acivicin administration induced significant increases in the biliary concentrations and secretion

rates of GSH in all these rats, although the mean values in the C1 group were significantly lower than in the controls and CyA-vehicle-treated rats both before (data not shown) and after acivicin administration (Fig. 3). Finally, our study revealed that CyA treatment significantly reduced the hepatic pool of GSH (Fig. 3).

In an attempt to analyze the origin and dynamics of the cholestatic phenomenon and to estimate the relative contribution of BA and GSH to bile flow, we evaluated the relationships between bile flow and BA secretion and between bile flow and GSH secretion, following the method reported
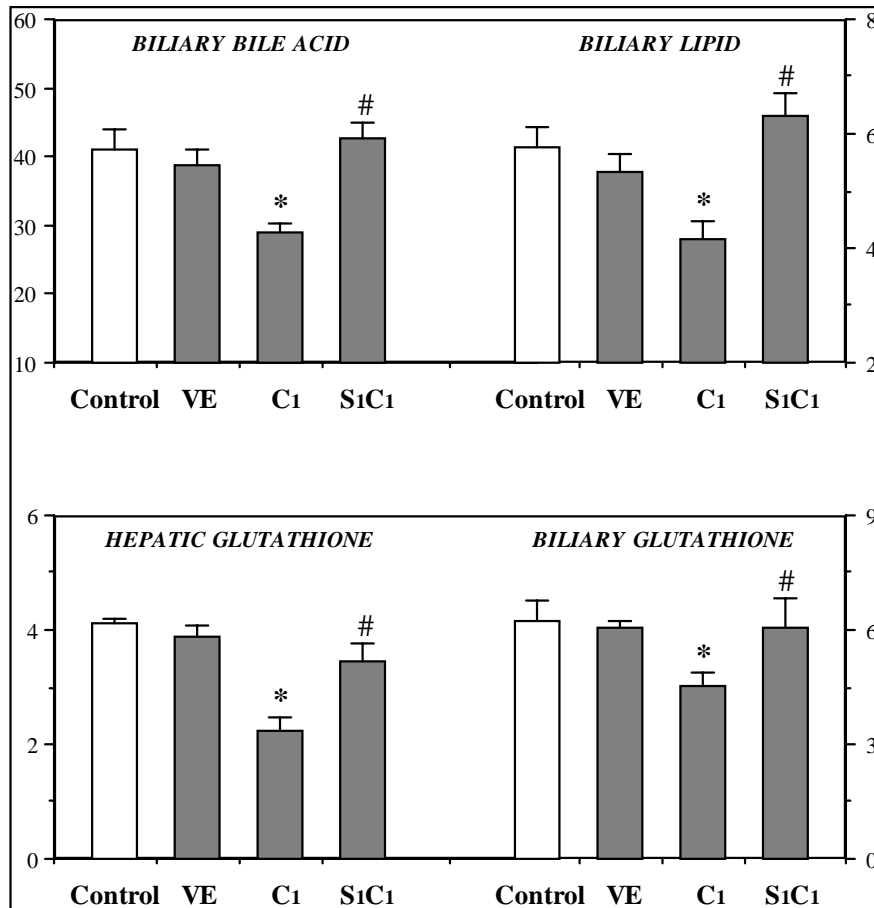
Figure 3: Effects of CyA plus SAMe cotreatment on biliary secretion rates of bile acids, lipid (cholesterol plus phospholipid) and glutathione (nmol/min/g liver) and on hepatic content of glutathione (µmol/g liver). Rats were treated with physiological saline (Control), the CyA-vehicle (VE), CyA (C1) or CyA+SAMe (S1C1). CyA and SAMe were coadministered for one week using doses of 10 mg/kg/day and 10 mg/kg/12 hr, respectively. Values are given as means ± SEM for 6-8 rats in each group; $p < 0.05$, significantly different from the Control (*) and CyA-treated rats (#).

previously (10). This mathematical approach revealed that the apparent choleretic activities of BA and GSH (slopes of the regression lines) in controls and CyA-treated rats were similar. Nonetheless, CyA treatment decreased the BADF (37%) and GSDF (36%), supporting the view that cholestasis would be attributable to both the inhibition of the biliary output of BA and to the fall in the biliary secretion of GSH. The BAIF and GSIF (determined by the y-intercept) were also reduced (18% and 21%, respectively) whereas the contribution to total bile flow of the bile flow fraction generated independently of BA and GSH (the non-BA non-GSH dependent bile flow) was not significantly modified after CyA treatment.

Effects of short-term SAMe plus CyA cotreatment on the CyA-induced alterations. When

rats were simultaneously treated with CyA plus SAMe (S1C1 group), the main alterations observed in the biochemical indicators of CyA-induced nephrotoxicity and diabetogenicity were not abolished. By contrast, the CyA-induced increases in plasma bilirubin and BA were totally or partially eliminated and the adverse effects of the drug on total protein concentrations in plasma and liver were totally suppressed (Table 1); additionally, the cholestasis was significantly attenuated (Table 2) and the CyA-induced falls in the biliary secretion of BA and lipids were totally antagonized (Fig. 3). Finally, the biliary outputs of ã-GT and ACP were also improved (Table 2).

Regarding GSH, we observed that the hepatic depletion of this compound caused by CyA treatment was blocked when the animals were cotreated with SAMe and CyA; additionally, as

in the case of the biliary secretion of BA and BLP, the inhibitory effect of the immunosuppressor on the biliary secretion of GSH was totally antagonized (Fig. 3).

Finally, the four bile flow fractions evaluated in the study, BADF, BAIF, GSDF and GSIF were significantly higher in the rats treated concurrently with SAMe plus CyA than in the rats treated with CyA alone. Although the values of these four bile flow fractions were slightly lower than in the controls, they did differ significantly from those of CyA-treated rats (data not shown).

DISCUSSION

Our study confirms that CyA treatment does not produces severe abnormalities in the general physiology of the rat and neither does it lead to systemic toxicity. However, the results also demonstrate that this drug rapidly, simultaneously and significantly alters hepatic, renal and pancreatic function in this species, inducing a moderate degree of toxicity that, in general, depends on dose but not on the duration of treatment. Our observations also demonstrate that subchronic treatment in the rat induces the well known cholestatic effect, but also reduces the biliary secretion of BA, cholesterol, phospholipids, GSH and proteins, as well as the four conventional bile flow fractions, the BADF, BAIF, GSDF and GSIF. Finally, it is interesting that SAMe and CyA cotreatment partially suppresses or totally antagonizes the adverse effect of CyA on the different biliary and hepatic parameters evaluated in our study, and significantly improves the plasma parameters indicators of cholestasis.

Previous studies by our team (10, 12) and other authors (5, 44) have demonstrated that the impairment of bile secretion in CyA-treated rats is mainly associated with reductions in the BADF and BAIF. It is widely documented that BADF-related cholestasis is mainly due to an inhibition of the hepatobiliary transport of BA, which would explain not only the decreases observed in their biliary secretion rates but also the increases in their plasma levels after treatment, as well as the reductions observed by us in BLP secretion. Inhibition of the basolateral and canalicular plasma membrane BA transporters (13-15), secondary to alterations in hepatocyte membrane composition and fluidity (4, 16, 17), have been postulated as the possible causative cellular mechanisms for BADF-related cholestasis and inhibition of the hepatobiliary transport of BA.

Regarding the BLP, it is well known that their secretion into bile is primarily determined by quantitative changes in the biliary secretion rates of BA because when bile acids are secreted into bile, apart from generating an osmotic water flow toward the canalicular lumen, they also promote the removal of cholesterol and phospholipid from the canalicular membrane and the secretion of these into bile. Accordingly, a possible explanation for the CyA-induced inhibition of BLP secretion is that it would be, at least partly, secondary to the inhibition of the hepatobiliary transport of BA caused by the drug since the decreases in the biliary secretion of BA observed by us coincided with what has been reported previously in the sense that acute or prolonged CyA administration impairs the vectorial transport of BA through the enterohepatic cycle -ileal absorption (45), hepatocyte uptake (5, 12, 15, 27) and canalicular secretion (13, 14, 27)-.

Despite this, CyA induces a series of modifications that suggest that the drug might also inhibit BLP secretion directly. In this context, we have previously observed (22) that after a single i.v. administration of CyA a) the biliary secretion of cholesterol and phospholipid is uncoupled from the biliary secretion of BA and, b) the inhibitory effect of the drug on phospholipid secretion is higher than its effect on cholesterol secretion. Additionally, the lithogenic index of bile in CyA treated rats is increased. All these findings favour the hypothesis that CyA would decrease biliary cholesterol and phospholipid secretion directly, and not only secondarily to the fall in BA secretion, and also suggest that other mechanisms, involved in the transcellular and/or canalicular transport processes of BLP, could be altered by the drug. Rom‡n et al. (12) and Gal‡n et al. (22) have demonstrated that CyA blocks the transcytosis processes in the rat, and it is well known that intracellular precursors of BLP are transported by vesicle-mediated process to the canalicular membrane to which they would fuse until bile acids secreted into the canaliculus cause the removal and release of membrane

components (19). Accordingly, either by altering transcytosis and/or the fusion of lipid vesicles with the canalicular membrane, CyA might reduce membrane recycling/repair and the removal of BLP from it.

In addition, it is likely that CyA might also act at canalicular membrane level since 1) its molecule is so lipophilic that it is rapidly incorporated into the membrane lipid bilayer (46), and 2) Whitington et al. (17) and our team (unpublished data) have demonstrated that CyA alters hepatocyte plasma membrane lipid composition and reduces its fluidity; so, it is possible that CyA and/or its metabolites, which are eliminated through the biliary route in high percentages (3, 43), could become incorporated into BA micelles or lipid vesicles inside the canalicular lumen (47, 48). Thus, competition between lipid and CyA for association in mixed micelles/vesicles could prevent the formation of mixed micelles/vesicles and would reduce the membrane-solubilizing capacity of BA and hence BLP secretion.

Regarding biliary GSH, it is known that transport of this compound across the canalicular membrane of hepatocytes plays an important role in several processes and also contributes to canalicular bile formation. In this sense, recent studies carried out on several experimental models (23, 26, 49-51) have provided direct evidence to suggest that GSH would be the primary osmotic driving force in BAIF formation. Accordingly, CyA-induced alterations in the hepatic contents and canalicular transport of GSH could potentially compromise not only hepatic GSH homeostasis but also bile formation. We have previously reported that, administered as a single i.v. dose, CyA induces an abrupt and simultaneous fall in biliary GSH and bile flow, suggesting a possible cause-effect relationship between the inhibition in GSH secretion and cholestasis. In this study, when the rats were treated with CyA for 7 days, a similar decrease in bile flow and GSH secretion was observed. When the changes in bile flow and GSH secretion rates were related under control conditions and in the CyA-treated rats, a positive correlation was obtained, suggesting that a certain portion of total bile flow, the GSDF, would indeed be proportional to the biliary secretion of GSH. Our results also clearly indicate that CyA significantly reduces (36%) the absolute contribution of the GSDF to total bile flow,

demonstrating that cholestasis may be mediated, at least partially, by the inhibition of GSH biliary secretion. Additionally, CyA-treatment caused a decline not only in the GSDF and BADF, as indicated above, but also in the GSIF and BAIF. Similar decreases in the BAIF after CyA treatment have been previously observed in rats by different authors (5, 10, 12). By contrast, the slopes of the regression lines -that is, the apparent choleretic activity of GSH in the controls and CyA-treated rats- were not statistically different. This absence of changes in the apparent choleretic activity of GSH and in that of BA suggests that the drug does not affect the paracellular permeability pathway, in agreement with previous report (52, 53). All these results, together with the absence of changes in the contribution to total bile flow of the bile flow fraction generated independently of BA and GSH -the non-BA non-GSH dependent bile flow-, clearly indicate that CyA specifically reduces the formation of bile dependent on GSH secretion and suggest that although secondary alterations may lead to reductions in the four bile flow fractions, inhibition of the canalicular secretion of GSH could also be involved in CyA-induced cholestasis in this species. The mechanisms responsible for this inhibition, and hence for the reduction in the GSDF, are not readily apparent from this study, although they could theoretically be attributed to different alterations. Firstly, a shift or reflux of GSH from the bile canaliculus to the blood secondary to increases in junctional permeability can be ruled out since plasma GSH concentrations were not increased either after acute CyA administration or in the subchronically treated rats. Also, the apparent choleretic activity of both GSH and BA after CyA treatment were similar to those observed in controls.

Another mechanism that could be invoked to explain our observations on GSH secretion would be related to the hepatic depletion of GSH in the rats treated for 7 days. It has been widely documented that when the hepatic content of GSH decreases the biliary concentrations and secretion rates of the tripeptide also decrease (25, 54, 55). However, previous studies carried out by our team have demonstrate that a single i.v. dose of CyA rapidly and markedly reduces the biliary secretion of GSH whereas the hepatic contents of GSH remain unaffected (unpublished data). Accordingly, at least under these

conditions, the lower GSH efflux into bile seems to occur unaccompanied by changes in liver GSH contents. A third potential cause of decreases in biliary GSH would be changes in ã-GT activity since increases in this activity could intensify the intrabiliary hydrolysis of GSH, thus reducing the biliary contents of GSH (49, 54, 55). Nonetheless, the immediacy with which the fall in biliary GSH occurs in the acute assays and, especially, the fact that the biliary secretion rate of GSH in the rats treated for 7-days was lower than in the controls, both before and after ã-GT inhibition by acivicin administration, argue against such a hypothesis.

Finally, the most probable cause of the falls in biliary GSH would be a direct inhibition of the transport of GSH into bile, probably secondary to the well known CyA-induced interferences in the structure/fluidity/function of the hepatocyte membrane. It is known that GSH is transported into bile through two different carrier-mediated mechanisms: a low affinity electrogenic carrier for reduced GSH and an ATP-driven multispecific organic anion transporter, the cMOAT, for oxidized GSH (25, 26, 28, 54) and it has been observed that CyA markedly depresses at least the activity of the cMOAT in vesicles obtained from canalicular membranes (13, 14, 27). Therefore, the CyA-induced inhibition in GSH canalicular carriers could account for the reduction observed by us in the biliary secretion of GSH, and hence in the GSDF.

Our results on SAMe plus CyA-treated rats suggest that the beneficial effect of SAMe on BA and BLP output as well as on bile flow and BADF might be due to an increased bile acid-dependent lipid and water secretion secondary to the normalization of the hepatobiliary transport of bile acids found in SAMe-cotreated rats. Normalization of the plasma levels and biliary secretion of bile acids clearly suggests that SAMe would antagonize the CyA-induced alterations in the sinusoidal and canalicular transport systems of BA. Currently, it is unknown whether CyA alters the hepatic SAMe synthesis or its utilization, but on the basis of its key role in transmethylation reactions in the liver, we believe that the beneficial effects of exogenous SAMe administration on the

hepatobiliary transport of BA, and hence on the biliary secretion of lipids, bile flow and BADF, might be related to the improvement in hepatocyte plasma membrane recycling/repair and fluidity through methylation of membrane phospholipids. In this sense, it has been reported that SAMe antagonizes/prevents alterations in liver membrane fluidity and normalizes the activity of different membrane transporters, thus normalizing bile formation (31-34).

Regarding the beneficial effect of SAMe on biliary and hepatic GSH, we observed that CyA administration reduces the biliary secretion of GSH and depletes its hepatic pool whereas SAMe plus CyA cotreatment antagonizes both adverse effects. In the light of the effects of the CyA on the liver plasma membrane and in view of our own results on the hepatic and biliary secretion of GSH in CyA plus SAMe- cotreated rats, we believe that these effects of SAMe might be related to an improvement in the canalicular transport of GSH as a consequence of a normalization of hepatocyte plasma membrane fluidity. Furthermore, our results on the hepatic contents of GSH clearly suggest that the beneficial effect of SAMe might also involve transsulphuration reactions and hepatic synthesis of GSH; the hepatic pool of GSH was depleted by CyA treatment but became totally normalized when the rats were cotreated with SAMe. In this regard, it has been reported that, as a precursor for GSH biosynthesis SAMe prevents, restores or antagonizes the depletion of the hepatic pool of GSH caused by agents such as phalloidin, ethynylestradiol, ethanol, paracetamol, carbon tetrachloride, á-naphthylisothiocyanate and other hepatotoxins (31, 34, 37-39). In summary, although further studies should be performed to clearly identify the mechanisms by which exogenous SAMe administration antagonizes the hepatotoxic effects of CyA in the rat, our results suggest that the beneficial effect could be related, at least partially, to the known ability of SAMe to modulate liver membrane lipid composition, fluidity and membrane carriers activity, and also to its key role in the hepatic synthesis of GSH since both the canalicular transport and hepatic contents of the tripeptide were rendered totally normalized when the rats were cotreated with both drugs.

ACKNOWLEDGMENTS

REFERENCES

CADRA NEL, J.F., BABANY, G., OPOLON, P., ERLINGER, S.: PharmacocinZtique et effets hZpatiques de la ciclosporine A. Gastroenterol. *Clin. Biol.* (1992), **16**: 314-321.

FAULDS, D., GOA, K.L., BENFIELD, P.: Cyclosporin. A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic use in immunoregulatory disorders. *Drugs* (1993), **45**: 953-1040.

KAHAN, B.D.: Cyclosporine. *N. Engl. J. Med.* (1989), **321**: 1725-1738.

FARTHING, J.G., CLARK, M.L.: Nature of the toxicity of cyclosporin A in the rat. *Biochem. Pharmacol.* (1981), **30**: 3311-3316.

STONE, B.G., UDANI, M., SANGHYI, A., WARTY, V., PLOCKI, K., BEDETTI, C.D., VAN THIEL, D.H.: Cyclosporin A-induced cholestasis. The mechanism in a rat model. *Gastroenterology* (1987), **93**: 344-351.

WHITING, P.H., SIMPSON, J.G., DAVIDSON, R.J.L., THOMPSON, A.W. Pathological changes in rats receiving cyclosporine A at immunotherapeutic dosages for seven weeks. *Br. J. Exp. Pathol.* (1983), **64**: 437-444.

CADRANEL, J.F., DUMONT, M., MESA, V., DEGOTT, C., TAILLANDIER, J., ERLINGER, S.: Effet de l'administration chronique de ciclosporine A sur la cholZr_se chez le rat. *Gastroenterol. Clin. Biol.* (1989), **13**: 779-782.

CADRANEL, J.F., DUMONT, M., MESA, V.A., DEGOTT, C., TOUCHARD, D., ERLINGER, S.: Effect of chronic administration of cyclosporin A on hepatic uptake and biliary secretion of bromosulfophthalein in rat. *Dig. Dis. Sci.* (1991), **36**: 221-224.

DURUIBE, V.A., OKONMAH, A., BLYDEN, G.T.: Effect of ciclosporin on rat liver and kidney glutathione content. *Pharmacology* (1989), **39**: 205-212.

GALÁN, A.I., FERNÁNDEZ, E., MORÓN, D., MU„OZ, M.E., JIMƒNEZ, R.: Cyclosporine A hepatotoxicity: effect of prolonged treatment with cyclosporine on biliary lipid secretion in the rat. *Clin. Exp. Pharmacol. Physiol.* (1995), **22**: 260-265.

MORÓN, D., GALçN, A.I., FERNÁNDEZ, E., MORENO, C., JIMƒNEZ, R., MU„OZ, M.E.: Changes in glutathione hepatic content and ã-glutamyl-cycle enzymes during CyA long term treatment in the rat. *J. Hepatol.* (1995), **23** (Suppl. 1): 160.

ROMÁN, I.D., MONTE, M.J., GONZÁLEZ-BUITRAGO, J.M., ESTELLER, A., JIMƒNEZ, R.: Inhibition of hepatocytary vesicular transport by cyclosporin A in the rat: relationship with cholestasis and hyperbilirrubinemia. *Hepatology* (1990), **12**: 83-91.

B…HME, M., M†LLER, M., LEIER, I., JEDLITSCHKY, G., KEPPLER, D.: Cholestasis caused by inhibition of the adenosine triphosphate-dependent bile salt transport in rat liver. *Gastroenterology* (1994), **107**: 255-265.

KADMON, M., KL†NEMAN, C., B…HME, M., ISHIKAWA, T., GORGAS, K., OTTO, G., HERFART, C., KEPPLER, D.: Inhibition by cyclosporine A of adenosine triphosphate dependent transport from the hepatocyte into bile. *Gastroenterology* (1993), **104**: 1507-1514.

MOSELEY, R.H., JOHNSON, T.R., MORRISSETTE, J.M.: Inhibition of bile acid transport by cyclosporine A in rat liver plasma membrane vesicles. *J. Pharmacol. Exp. Ther.* (1990), **253**: 974-980.

ROSSARO, L., HO, C., VAN THIEL, D.H.: 19F nuclear magnetic resonance studies of cyclosporine and model unilamellar vesicles: where does the drug sit with the membrane? *Transplant. Proc.* (1988), **20**: 41-45.

WHITINGTON, P.F., DUDEJA, P., HECHT, J.R., WHITINGTON, S.H., BRASITUS, T.A.: Lipid alterations in the hepatocyte basolateral sinusoidal membrane (BLM) domain may explain cyclosporine (CsA) induced cholestasis in the rat. *Hepatology* (1988), **8**: 1363.

HOFMANN, A.F.: Bile acid secretion, bile flow and biliary lipid secretion in humans. *Hepatology* (1990), **12**: 175-255.

COLEMAN R., RAHMAN, K.: Lipid flow in bile formation. *Biochim. Biophys. Acta* (1992), **1125**: 113-133.

LOWE, P.J., BARNWELL, S.G., COLEMAN, R.: Rapid kinetic analysis of the bile salt-dependent secretion of phospholipid, cholesterol and a plasma membrane enzyme into bile. *Biochem. J.* (1984), **222**: 631-637.

CHANUSSOT, F., BOTTA-FRIDLUND, D., DE LA PORTE, P.L., SBARRA, V., PORTUGAL, H., PAULI, A.M., HAUTON, J., GAUTHIER, A., LAFONT, H.: Effects of cyclosporine and corticosteroids on bile secretion in the rat. *Transplantation* (1992), **54**: 226-231.

GALÁN, A.I., ROMÁN, I.D., MU„OZ, M.E., CAVA, F., GONZçLEZ-BUITRAGO, J.M., ESTELLER, A., JIMƒNEZ, R.: Inhibition of biliary lipid and protein secretion by ciclosporine in the rat. *Biochem. Pharmacol.* (1992), **44**: 1105-1113.

BALLATORI, N., TRUONG, A.T.: Relation between biliary glutathione excretion and bile acid-independent bile flow. *Am. J. Physiol.* (1989), **256**: G22-G30.

BALLATORI, N., TRUONG, A.T.: Glutathione as a primary osmotic driving force in hepatic bile formation. *Am. J. Physiol.* (1992), **263**: G617-G624.

FERNÁNDEZ-CHECA, J.C., LU, S., OOKHTENS, M., DELEVE, L., RUNNEGAR, M., YOSHIDA, H., SAIKI, H., KANNAN, R., MADDATU, T., GARCIA-RUIZ, C., KUHLENKAMP, J., KAPLOWITZ, N.: Regulation on hepatic glutathione. In: *Hepatic Transport and Bile Secretion. Physiology and Pathophysiology* (1993). Pp: 363-395, eds. N. Tavoloni and P.D. Berk, Raven Press, Nueva York.

FERNÁNDEZ-CHECA, J.C., TAKIKAWA, H., HORIE, T., OOKHTENS, M., KAPLOWITZ, N.: Canalicular transport of reduced glutathione in normal and mutant Eisai hyperbilirubinemic rats. *J. Biol. Chem.* (1992), **267**: 1667-1673.

ARIAS, I.M.: Cyclosporin, the biology of the bile canaliculus, and cholestasis. *Gastroenterology* (1993), **104**: 1558-1560.

BALLATORI, N., DUTCZAK, W.J.: Identification and characterization of high and low affinity transport systems for reduced glutathione in liver cell canalicular membranes. *J. Biol. Chem.* (1994), **269**: 19731-19737.

EDKINS, R.D., RYBAK, L.P., HOFFMAN, D.W.: Comparison of cyclosporine and FK506 effects on glutathione levels in rat cochlea, brain, liver and kidney. *Biochem. Pharmacol.* (1992), **43**: 911-913.

MAYER, R.D., COCKETT, A.T.K.: Cyclosporin-mediated increase in kidney glutathione and effects on ã-glutamyl-cycle enzymes. *J. Biochem. Toxicol.* (1988), **3**: 213-221.

ALMASIO, P., BORTOLINI, M., PAGLIARO, L., COLTORTI, M.: Role of S-adenosyl-L-methionine in the treatment of intrahepatic cholestasis. *Drugs* (1990), **40** (Suppl. 3): 111-123.

BOELSTERLI, U.A., RAKHIT, G., BALAZS, T. Modulation of S-adenosyl-L-methionine of hepatic Na+,K+-ATPase, membrane fluidity, and bile flow in rats with ethinyl estradiol-induced cholestasis. *Hepatology* (1983), **3**: 12-17.

FRICKER, G., LANDMANN, L., MEIER, P.J. Ethynil-estradiol (EE) induced structural and functional alterations of rat liver plasma membranes and their reversal by S-adenosyl-L-methionine (SAMe) in vitro. *Hepatology* (1988), **8**: 1224.

FRIEDEL, H.A., GOA, K.L., BENFIELD, P.: S-adenosyl-L-methionine. A review of its pharmacological properties and therapeutic potential in liver dysfunction and affective disorders in relation to its physiological role in cell metabolism. *Drugs* (1989), **38**: 389-416.

FERNçNDEZ, E., MU„OZ, M.E., ROMçN, I.D., GALçN, A.I., GONZçLEZ-BUITRAGO, J.M., JIMƒNEZ, R.: Cyclosporin A-induced cholestasis in the rat. Beneficial effects of S-adenosyl-L-methionine. *Drug Invest.* (1992), **4** (Suppl. 4): 54-63.

FERNçNDEZ, E., GALçN, A.I., MORçN, D., GONZçLEZ-BUITRAGO, J.M., MU„OZ, M.E., JIMƒNEZ, R.: Reversal of cyclosporine A-induced alterations in biliary secretion by S-adenosyl-L-methionine in rats. *J. Pharmacol. Exp. Ther.* (1995), **275**: 442-449.

CORRALES, F., GIMƒNEZ, A., çLVAREZ, L., CABALLERêA, J., PAJARES, M.A., ANDREU, A., MATO, J.M., RODƒS, J.: S-adenosylmethionine treatment prevents carbon tetrachloride-induced S-adenosylmethionine synthetase inactivation and atenuates liver injury. *Hepatology* (1992), **16**: 1022-1027.

GARCêA-RUIZ, C., MORALES, A., COLELL, A., BALLESTA, A., RODƒS, J., KAPLOWITZ, N., FERNçNDEZ-CHECA, J.C.: Feeding S-adenosyl-l-methionine attenuates both ethanol-induced depletion of mitochondrial glutathione and mitochondrial dysfunction in periportal and perivenous rat hepatocytes. *Hepatology* (1995), **21**: 207-214.

JOVER, R., PONSODA, X., FABRA, R., TRULLENQUE, R., GîMEZ-LECHîN, M.J., CASTELL, J.V.: S-adenosyl-L-methionine prevents intracellular glutathione depletion by GSH-depleting drugs in rat and human hepatocytes. *Drug Invest.* (1992), **4** (Suppl. 4): 46-53.

SZTEFKO, K., LI, P., BALLATORI, N., CHEY, W.Y.: CCK-receptor antagonists proglumide and loxiglumide stimulate bile flow and biliary glutathione excretion. *Dig. Dis. Sci.* (1994), **39**: 1974-1980.

GRIFFITH, O.W.: Determination of glutathione and glutathione disulfide using gutathione reductase and 2-vinylpyridine. *Anal. Biochem.* (1980), **106**: 207-212.

TIETZE, F.: Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. *Anal. Biochem.* (1969), **27**: 502-522.

WHITING, P.H., THOMPSON, A.W., SIMPSON, J.G. Cyclosporine: toxicity, metabolism and drug interactions-implications from animal studies. *Transplant. Proc.* (1985), **17**: 134-144.

EL-NAGGERE, M., BOUCHARD, G., TUCHWEBER, B., YOUSEF, I.M.: Partial protection by taurocholic and ursodeoxycholic acids of cyclosporin A-induced cholestasis in isolated perfused rat liver. *Gastroenterology* (1995), **108**: A1062.

SAUER, P., KL…TERS-PLACHKY, P., GUTZLER, F., KOMMERELL, B., STIEHL, A.: Cyclosporin A inhibits the active absorption of bile acids in the ileum of the rat. *XII International Bile Acid Meeting,* Basel (1992): 26.

ZIEGLER, K., POLZIN, G., FRIMMER, M. Hepatocellular uptake of cyclosporin A by simple diffusion. *Biochim. Biophys. Acta* (1988), **938**: 44-50.

LE GRUE, S.J., FRIEDMAN, A.W., KAHAN, B.D.: Binding of cyclosporine by human lymphocytes and phospholipid vesicles. *J. Immunol.* (1983), **131**: 712-718.

MEHTA, M.U., VENKATARAMANAN, R., BURCKART, G.J., PTACHCINSKI, R.J., DELAMOS, B., STACHAK, S., VAN THIEL, D.H., IWATSUKI, S., STARZL, T.E.: Effect of bile on cyclosporin absorption in liver transplant patients. *Br. J. Clin. Pharmacol.* (1988), **25**: 579-584.

BALLATORI, N., JACOB, R., BARRETT, C., BOYER, J.L. Biliary catabolism of glutathione and differential reabsorption of its amino acid constituents. *Am. J. Physiol.* (1988), **254**: G1-G7.

BOUCHARD, G., YOUSEF, I.M., TUCHWEBER, B.: Decreased biliary glutathione content is responsible for the decline in bile salt-independent flow induced by ethinyl estradiol in rats. *Toxicol. Lett* (1994), **74**: 221-233.

MOHAN, P., LING, S.C., WATKINS, J.B.: Ontogeny of hepatobiliary secretion: role of glutathione. *Hepatology* (1994), **19**: 1504-1512.

ERLINGER, S.: Bile flow. In: *The Liver: Biology and Pathobiology* (1994). Pp: 769-786, eds. I.M. Arias, J.L. Boyer, N. Fausto, W.B. Jacoby, D. Schachter and D.A. Shafritz, Raven Press, Nueva York.

HARDISON, W.G.M.: Hepatocellular tight junctions. Role of canalicular permeability in hepatobiliary transport. In: *Hepatic Transport and Bile Secretion: Physiology and Pathophysiology* (1993). Pp. 571-587, eds. N. Tavoloni and P.D. Berk, Raven Press, New York.

DELEVE, L.D., KAPLOWITZ, N.: Importance and regulation of hepatic glutathione. *Semin. Liver Dis.* (1990), **10**: 251-266.

LAUTERBURG, B.H.: Biliary excretion of glutathione and glutathione adducts. *Progr. Pharmacol. Clin. Pharmacol.* (1991), **8**: 201-213.