MODULATION OF BILE ACIDS INDUCED BY PARAQUAT IN RABBITS

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SUMMARY

Rabbit bile was examined for changes in composition induced by paraquat. Paraquat was administered intraperitoneally and changes in bile components were monitored by high performance liquid chromatography. Alterations in the ratios of total glycine/taurine conjugated bile acids (TGC/TTC), cholic acid/deoxycholic acid (CA/DC), cholic acid/chenodeoxycholic acid (CA/CDC) and cholic acid/cholesterol (CA/CH) were measured as an index of paraquat toxicity. A statistically significant increase in the ratio of TGC/TTC was observed, while CA/DC, CA/CDC and CA/CH showed a decrease. Phospholipids, protein, sugar, bilirubin, β-carotene, vitamin A and vitamin E in the bile and serum of the experimental animals were also monitored. In bile, the levels of cholesterol, phospholipids, protein, sugar, and total bile acids increased while the levels of the antioxidants β-carotene, vitamin A and vitamin E decreased. A decrease in the bilirubin content of the bile was also observed. These modifications may be useful clinically for assessment of paraquat toxicity.

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KEY WORDS

paraquat, bile, bile acids, cholic acid, deoxycholic acid, chenodeoxycholic acid, taurocholic acid, glycocholic acid, total glycocholic acid, total taurocholic acid, total bile acids, lithocholic acid, cholesterol, phospholipids, bilirubin, serum, β-carotene, vitamin A, vitamin E, HPLC, rabbit

INTRODUCTION

Bile acids are synthesized from cholesterol in the liver and secreted into the bile after conjugation with taurine or glycine. Most of these bile acids are absorbed from the gut and returned to the liver via the portal vein. Biliary cholesterol plays an important role in the protection of the intestine from potentially adverse effects of bile acids. 7α-Hydroxylation reactions represent well established metabolic steps in cholesterol disposition and transformation to bile acids. 7α-Hydroxylation of cholesterol is the rate limiting step in the degradation of cholesterol to bile acids. Changes in the profiles of these compounds in the bile and urine may reflect damage at specific intracellular sites. Changes in total bile acids have been indicated as sensitive markers in hepatobiliary diseases /1/.

Abnormal levels of taurine-conjugated bile acids and cholic acid (CA) have been observed in intra- and extra-hepatic cholestasis, whereas glycine-conjugated bile acids and chenodeoxycholic acid (CDC) levels fluctuate in parenchymal liver diseases /2/. An important role in carcinogenesis has also been attributed to bile acids /3/. Secondary bile acids and their metabolic products may promote neoplastic transformation by carcinogens in the colon. Both lithocholic acid (LC) and deoxycholic acid (DC) have been shown to promote formation of colonic adenocarcinomas in conventional and germ free rats /3/.

Paraquat (1,1'-dimethyl-4,4'-bipyridylium dichloride, PQ) has been reported to cause damage to liver, lung and kidney in man /4-6/. PQ toxicity may be mediated by oxygen free radicals produced by cyclic oxidation-reduction reactions /7/. Cellular injury may result from lipid peroxidation through PQ-linked superoxide anions O_2^- /8/. The dose related symptoms of PQ poisoning have been well documented. Large doses of PQ in humans cause death by multi-organ failure, including

the liver /9/. However, the mechanism of PQ induced hepatotoxicity remains controversial. Currently, there are two major hypotheses for hepatocellular injury. One is based on reactive oxygen intermediates formed during PQ metabolism causing cellular death or injury /10,11/, while the other suggests depletion of cellular NADPH and protein SH-groups, thus severely compromising the biosynthetic capabilities of the cell /12,13/. The present investigation was undertaken to study changes in the bile acid profile and other bile components to evaluate the extent of liver damage induced by PQ intoxication. Since bile acids are sensitive clinical markers of hepatobiliary function /14/, changes in the ratio of bile acids in serum may be employed as a marker in PQ toxicity assessment.

MATERIALS AND METHODS

Materials

Paraquat, as Gramoxane solution, was supplied by ICI, U.K. Bile acids, vitamin E as di-α-tocopherol (product No. T-3251), vitamin A as retinol acetate (product No. R-4632) and β-carotene (product No. C-0126) were purchased from Sigma Chemical Co., St. Louis, USA. Precoated silica gel chromatographic plates (Merck, product No. 5553) were used throughout this study.

Animals and Diet

Three month-old male rabbits weighing 1.5-2.9 kg were maintained at 22-24°C with a 12 h light/dark cycle (07:00-19:00 h). A natural diet of green salad and tap water were provided *ad libitum*. The animals were randomly divided into three groups (A-C) of 10-20 animals per group. Control Group A consisted of 20 animals and received daily intraperitoneal (i.p.) injection of normal saline. Group B (10 rabbits) received i.p. injections of 2 mg PQ/kg/day and Group C (10 rabbits) received 4 mg PQ/kg/day for seven days. The animals were deprived of their diet at 10:00 h on the day of sacrifice but were allowed free access to water.

The animals were sacrificed by exsanguination at 14:00 h by sodium pentathol injection (30 mg/kg).

Thirty ml blood was collected in tubes containing 3.8% sodium citrate. Bile was carefully squeezed out from the gallbladder into

preweighed plastic tubes, and the weight of bile was recorded. The bile samples were stored at -40°C.

Determinations

High performance liquid chromotographic analysis of bile acids was carried out using a Waters instrument equipped with a double solvent delivery system, model 510, and a variable wavelength UV detector, model 481, fixed at 200 nm. Chromatography control data station model 840 was used in these investigations. Sample preparation was done according to the method of Swobodnik /15/ with minor modifications. Mobile phase was prepared according to the method of Scacia /16/.

Total bile acid content of the bile was enzymatically assayed using 3α–hydroxysteroid dehydrogenase. A group separation of the bile acids using piperidinohydroxypropyl Sephadex LH-20 (PHP-LH-20) was also carried out as described by Goto *et al.* /17/.

Solubilized and hydrolyzed bile samples were analyzed by thin layer chromatography.

Bile samples were diluted with normal saline keeping wt/vol constant. Total cholesterol was determined from a dried lipid extract (chloroform/methanol 2:1 v/v), and reconstituted with 2-propanol. Enzymatic determination of cholesterol involved the hydrolysis of cholesterol esters by cholesterol esterase, followed by estimation with cholesterol oxidase.

Total phospholipids were extracted by Folch's method /18/. Inorganic phosphate was measured by Bartlett's method /19/, against a standard solution of uridyl monophosphate treated in the same manner. Inorganic phosphorus was converted to phospholipid content by a multiplication factor of 25, based on an average 25 mg phospholipid per milligram of phosphorus /20/. The chromatograms were air dried and then sprayed with Zinzade /21/ ethanol phosphomolybdic acid or Vaskovsky /22/ reagents. A second set of the chromatograms was immersed in 3% cupric acetate in 8% phosphoric acid, blotted carefully and heated at 180°C.

Sugar content of the samples was determined by Anthrone's method /23/. Protein content in the bile was determined by the method of Bardford /24/. Serum and bile β-carotene levels were determined spectrophotometrically by the method described by Yuans /25/.

Vitamin A and E levels in the bile and serum were determined by the fluorometric micro method developed by Hansen /26/ and McLaren /27/ with minor modifications.

Statistical analysis

Student's t-test and paired t-test were used for statistical analysis. Significance was accepted at $p<0.05$.

RESULTS AND DISCUSSION

The intraperitoneal route of PQ administration in preference to the oral route was selected to eliminate the incomplete and variable absorption of PQ from the gastrointestinal tract. Moreover, the oral route would have necessitated studying a larger number of animals because of increased morbidity due to the corrosive effects of PQ on the gastrointestinal mucosa /28/.

Conjugated bile acids (CBA) were resolved by HPLC employing a standard chromatogram of the resolved conjugated bile acids (Figure 1). The major CBA in the control as well as in the PQ-treated animals was identified as glycodeoxycholic acid (GDC). A thin layer chromatographic analysis of the hydrolyzed bile samples showed the presence of cholic acid (CA) and deoxycholic acid (DC) as the major components. These data are in agreement with earlier reports /29,30/. The other CBA found in the gallbladder of the control and PQ-treated rabbits are shown in Table 1.

A significant increase in the total glycoconjugated bile acids (TGC) was observed in Group C, PQ treated with 4 mg/kg/day. This increase in TGC may be responsible for the decreased synthesis of bile acids in the liver. The synthesis of bile salts is regulated through negative feedback inhibition by the bile salts returning to the liver. An increase in glycocholic acid (GC) in the gallbladder has been shown to decrease the hepatic bile salt synthesis pool /31/. Thus an increase in TGC in the PQ-treated animal's gallbladder bile may be responsible for decreased bile salt secretion, resulting in shrunken gallbladder, as observed in the present studies. Hepatocyte structure and function may also be influenced by PQ. Injurious effects of free radicals may lead to cellular damage resulting in conditions including cancer, aging and drug induced toxicity /32,33/. PQ toxicity has been proposed to induce free radicals which in turn may react with molecular O_2 leading to the

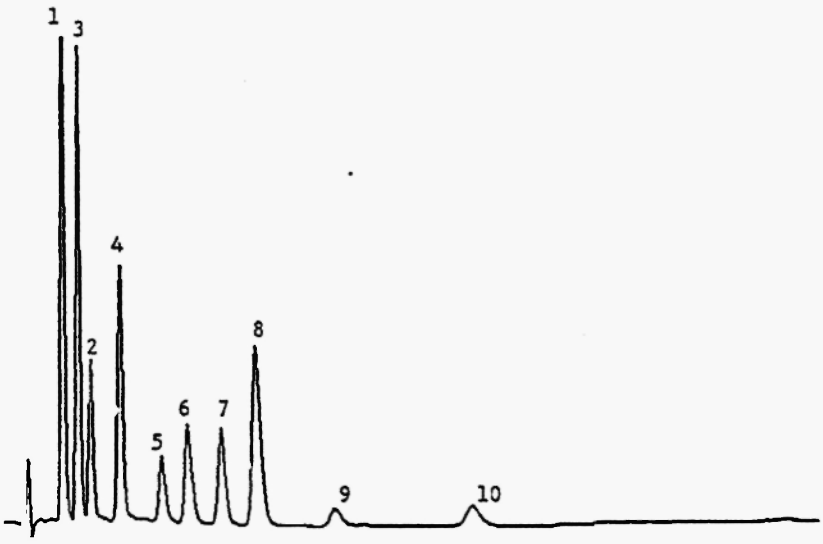

Fig. 1: Separation of a synthetic mixture of conjugated bile acids (*ca.* 0.3-0.6 μg of each component) on two Bondapak C-18 columns, 150x3.9 mm I.D., 5 μm particle size (Waters Associates, Milford, MA, USA). The columns were attached in series.

1. Tauroursodeoxycholic acid	(TUDC)	2. Taurocholic acid	(TC)
3. Glycoursodeoxycholic acid	(GUDC)	4. Glycocholic acid	(GC)
5. Taurochenodeoxycholic acid	(TCDC)	6. Taurodeoxycholic acid	(TDC)
7. Glycochenodeoxycholic acid	(GCDC)	8. Glycodeoxycholic acid	(GDC)
9. Taurolithocholic acid	(TLC)	10. Glycolithocholic acid	(GLC)

Solvent: methanol and 0.2 M sodium acetate buffer pH 4.2 at flow rate 1 ml/min. Detection at 200 nm.

formation of superoxide anion radicals /34/. Superoxide anion radicals or other activated oxygen radical species /35,36/, such as hydroxyl anions and singlet oxygen, may cause peroxidation of polyunsaturated fatty acids /37/ or depletion of cellular reducing equivalents, such as NADPH /38/. A significant decrease in NADPH levels correlated well with intracellular PQ concentration /39/.

Elevated levels of glycine-conjugated bile acids and chenodeoxycholic acid (CDC) have been observed in parenchymal liver diseases

TABLE 1
Conjugated bile acid levels in control and PQ treated rabbits.
% Composition

Treatment	TUDC	GUDC	TC	GDC	TDC	GCDC	TCDC	GLC	GC	TGC	TTC	TGC/TTC
Saline	1.64	0.83	12.69	70.99	6.81	2.34	0.61	0.2.50	6370	83.03	21.75	3.8
	±0.11	±0.11	±018	±1.2	±0.66	±0.15	±0.04	±0.30	±0.29	±0.3	±0 27	±0 20
PQ												
2 mg/kg	1.58	0.81	11.76	70 44	7.10	2.66	0.89	2.77	5.31	81.95	21.33	3.8
	±0 06	±0.11	±0.46	±1.20	±0.56	±0.58	±0.12	±0.19	±0.36	±0.35	±0.32	±0 19
PQ												
4 mg/kg	1.37	0.63	8.80*	71.66	6 21	1.96	0.58	2.55	3.80*	80.6	16.96	4.75
	±0 09	±0.11	±0.33	±1.68	±0 34	±0.26	±0 17	±0.34	±0.29	±0.32	±0.40	±0.32

TUDC = Taurou:sodeo:ycholic acid; GUDC = Gl:coursodeoxycholic acid;
TC = Taurocholic a:id: GDC = Glycodeo:ycholic acid;
TDC = Taurodeoxycholic acid; GCDC = Glycochero leoxycholic a:id;
TCDC = Taurochenodeo:ycholi: acid; GLC = Glyc:lithocholic acid;
GC = G:ychoc:olic a:id; TGC = To:al glycocholate; TTC = Total taurocholate.
Results shown represen: the mean of ten independent experiments (± SEM).
* signifi:ant P < 0.001

/2/. In this study, an increased level of TGC was accompanied by a significant elevation of the TGC/TTC ratio in animals treated with 4 mg PQ/kg/day, as compared to the control and 2 mg/kg/day PQ groups, thus suggesting possible damage to the parenchymal cells of the liver.

It is generally accepted that PQ accumulates in the mucosa and lumen of the small intestine and is excreted from the small intestine /40/. The enterohepatic circulation and the volume of blood flow in the liver may be involved in the distribution kinetics of PQ /41/. Increased mucosal permeability to PQ may be caused by intestinal mucosal damage /42/. Degeneration and necrosis of biliary epithelia in the bile ducts of rats treated with PQ have also been reported /43/. This, in turn, may modify the bile acids/lipids ratio in the gallbladder. An alteration in the permeability of gallbladder mucosa has been suggested as a cause for selective absorption of bile acids relative to cholesterol /44/. The cationic exchange properties of PQ may allow it to bind with some of the tauroconjugated bile acids during their enterohepatic circulation, and may in turn increase the fecal excretion of these bile acids. Clinical significance has been attributed to the modified ratios of taurine and glycine conjugates in the bile. The incidence of gallstones due to the increased proportion of glycine-conjugated bile acids may be controlled by feeding taurine conjugates /45/.

Animals treated with PQ (2 and 4 mg/kg/day) showed a statistically significant decrease in CA levels, whereas DC levels increased only after the 4 mg/kg/day dose (Table 2). A decreased ratio of CA/CDC with a corresponding increase in biliary cholesterol levels in animals treated with 2 mg/kg/day was observed. In contrast, the serum cholesterol level remained unchanged in animals with 2 or 4 mg/kg/day (Table 3) whereas biliary cholesterol increased significantly in the 2 mg/kg/day treated animals (Table 2). The biliary CA/CH ratio decreased significantly with both doses of PQ (2 and 4 mg/kg/day) (Table 2). These modifications may be indicative of Type IV hyperlipoproteinemia induced by PQ, as suggested by Einarsson and Angelin /46,47/.

Passive diffusion has been suggested as the mode of transportation in jejunal uptake of bile acids /30/. It is also known that the preferential substrate for cholic acid synthesis is endogenously synthesized cholesterol /31/. However, the rate of passive uptake is greater for unconjugated than for conjugated bile acids (greater for dihydroxy-

TABLE 2

Total bile acids, their ratios and cholesterol in PQ treated rabbits

Treatment	TBA	CA	DC	CDC	CA/DC	CA/CDC	CII	CA/CII
Saline	10.9	16.96	75.53	2.71	0.23	6.25	2.00	0.24
	±3.0	±0.55	±0.87	±0.16	±0.01	±0.28	±0.12	8.48
PQ 2 mg/kg	19.2	13.93*	78.01	2.55	0.17*	5.46	2.4*	0.21
	±3.2	± 0.46	±1.30	±0.47	±0.01	±0.74	±0.11	5.80*
PQ 4 mg/kg	20.1*	13.58	80.0*	1.97*	0.17*	6.89	2.20	0.18*
	±0.85	±0.70	±1.30	±0.24	±0.01	±0.82	±0.005	6.17

BA = bile acids, CA = cholic acid, DC = deoxycholic acid,
CDC = chenodeoxycholic acid, CH = cholesterol mg/g
Results shown represent the mean of ten independent experiments (± SEM)
* significant $P < 0.001$

TABLE 3
Serum levels of sugar, protein, bilirubin, β-carotene and cholesterol in paraquat treated rabbits

Treatment	Sugar mg/dl	Prot. mg/ml	Bilir. µMol	β-Car. mg/dl	Cholest. mg/dl
Saline	138.25 ±11.30	61.00 ±1.59	4.60 ±0.6	9.800 ±0.94	89.80 ±5.15
PQ 2 mg/kg	156.80 ±6.34	67.80* ±2.45	4.70 ±0.55	11.20 ±0.64	91.80 ±4.84
PQ 4 mg/kg	196.50 ±28.29	68.70* ±1.50	3.10* ±0.44	9.10 ±1.70	101.5 ±4.84

Prot. = protein; Bilir. = bilirubin; β-Car. = β-carotene; Cholest. = cholesterol.
Results shown represent the mean of ten independent experiments (± SEM).
* significant at p = 0.01

TABLE 4
Biliary levels of sugar, protein, phospholipid, bilirubin, vitamin A and E, and β-carotene in PQ treated rabbits

Treatment	Sugar mg/g	Prot. mg/g	P.Lip mg/g	Bilir. µmol	Vit.A µmol	Vit.E mg/Dl	β-car. mg/g.
Saline	0.95 ±.002	6.80 ±0.51	1.01 ±0.13	4.17 ±0.7	2.79 ±0.19	0.67 ±0.01	2.30 ±0.17
PQ 2 mg/kg	1.05 ±0.01	19.10* ±1.70	1.63 ±0.16	2.60 ±0.60	1.60* ±0.35	0.36 ±0.003	0.68 ±0.041
PQ 4 mg/kg	1.15. ±0.1	27.20* ±2.80	1.90 ±0.24	3.20 ±0.80	2.10 ±0.37	0.65 ±0.004	0.61* ±0.03

Prot. = Protein; P.Lip = Phospholipids; Bilir. = Bilirubin; Vit.A = Vitamin A; Vit.E = Vitamin E; β-car. = β-Carotene.
Results shown represent the mean of ten independent experiments (± SEM).
* Significant $P < 0.05$

than for trihydroxy-bile acids) /30/. Thus, a significant decrease in the ratios of CA/DC, CA/CDC and CA/CH (Table 2) is consistent with impaired liver function in PQ treated animals. Abnormal accumulation of specific lipids in either the lumen or wall of the gallbladder has been observed in certain diseases /48/.

Total protein levels in the bile and serum were significantly elevated at both doses of PQ treatment (Tables 3 and 4). Alterations in biliary lipid composition are clinically important, because supersaturation of bile with cholesterol is a prerequisite for gallstone formation. It has been suggested that proteins in the bile may be involved in the solubilization of cholesterol /49/.

There is evidence that the amount of bile acids and cholesterol exert a regulatory influence on the production of phospholipids in the liver /50-52/. In our study, a significant increase in cholesterol was accompanied by a slight increase in total biliary phospholipids (Table 2). The increase in biliary total phospholipids (Table 4) is directly related to an increase in cholesterol level, implying micelle formation. The formation of micelles with relatively fixed proportions of free cholesterol, phospholipids and bile acids, may account for the observed interrelationship between biliary lipids and bile acids. Earlier studies /53/ have suggested that proteins may also be associated in the macromolecular complex formation of biliary phospholipids, bile acids and cholesterol. Hence, an elevated level of protein in bile and serum, as shown by our results (Table 4), is in agreement with previous reports.

Bilirubin has been proposed as a natural antioxidant by Stocker and Ames /54/. Albumin-bound bilirubin efficiently inhibits peroxyl radical-induced oxidation of albumin-bound fatty acids /55/. The serum bile acid concentration is well correlated with serum bilirubin levels, and is a more sensitive index for impaired liver function than bilirubin.

Our investigations indicate that paraquat exposure modifies the biliary composition of lipids and ratios of bile acids, and these may serve as a useful index in the clinical assessment of toxicity from paraquat intoxification.

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