JPET Fast Forward. Published on September 27, 2004 as DOI: 10.1124/jpet.104.075051
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Long-term effect of treating pregnant rats with ursodeoxycholic acid on the congenital impairment of bile secretion induced in the pups by maternal cholestasis

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Short Title: Impaired bile secretion in the offspring of cholestatic rats

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Abbreviations:
Basolateral liver plasma membrane, bLPM; Bile acid, BA; Canalicular liver plasma membrane, cLPM; Intrahepatic cholestasis of pregnancy, ICP; Obstructive cholestasis during pregnancy, OCP; Ursodeoxycholic acid, UDCA; Taurocholic acid, TCA.

Financial Support:

This study was supported in part by the Junta de Castilla y León (Grants SA017/03 and SA013/04); Fondo de Investigaciones Sanitarias, Ministerio de Sanidad y Consumo, Spain, co-funded by the FEDER-FSE Program of the E.U. (Grant 01/1043); Ministerio de Ciencia y Tecnología, Plan Nacional de Investigación Científica, Desarrollo e Innovación Tecnológica (Grant BFI2003-03208), Spain. The group is member of the Spanish Network for Cooperative Research on Hepatitis, Instituto de Salud Carlos III, FIS (Grant G03/015).

Text pages: 23
Tables: 2
Figures: 5
References: 29
Words in the Abstract: 250
Words in the Introduction: 495 (<750)
Words in the Discussion: 1323 (<1500)

Recommended Section: **Gastrointestinal, Hepatic, Pulmonary, and Renal**

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ABSTRACT

Transient latent cholestasis in young rats born from mothers with obstructive cholestasis during pregnancy (OCP) has been reported. The cause of this congenital impairment and the long-term effect on the pups of treating their mothers with ursodeoxycholic acid (UDCA) during pregnancy were investigated. Complete biliary obstruction was imposed on day 14 of pregnancy and UDCA treatment begun on day 15. Serum bile acids (BAs) concentrations were elevated in 4-wks-old pups born from OCP, but not OCP+UDCA, mothers. However, GC/MS analysis of BA species in basal bile indicated the presence of significant differences among all experimental groups (Control, OCP and OCP+UDCA). Canalicular plasma membrane fluidity was reduced in OCP, but not in OCP+UDCA, pups. Screening by real-time quantitative RT-PCR of the steady-state levels of mRNA of genes related to hepatobiliary function revealed changes (up-regulation of *Cyp7a1* and *Mrp1* and down-regulation of *Abcg5* and *Abcg8*) in OCP group, which were prevented by UDCA treatment. Electron microscopy examination showed multilamellar bodies occupying part of the canalicular lumen in OCP pups. Their number and size were reduced in animals born from OCP+UDCA mothers. In OCP, but not OCP+UDCA, the stimulation of bile flow and BA output induced by taurocholate administration were reduced and cholesterol/BA output ratio was increased, whereas phospholipid/BA output ratio was enhanced in both groups (OCP>OCP+UDCA). In conclusion, UDCA treatment of rats with cholestasis during pregnancy has long-term beneficial effects on their offspring by preventing in part the congenital impairment in hepatobiliary function of the pups that affects their biliary lipid secretion.

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INTRODUCTION

Intrahepatic cholestasis of pregnancy (ICP) is a reversible form of cholestasis that may develop during the second or third trimester of pregnancy and usually resolves soon after delivery. Although it is regarded as a benign condition for the mothers, it is associated with premature delivery and increased risk of fetal mortality during late pregnancy (for a review, see Lammert et al., 2003).

Ursodeoxycholic acid (UDCA), a hydrophilic bile acid (BA) widely used in the treatment of several cholestatic liver diseases, has beneficial effects on patients with ICP by improving pruritus, hypercholanemia and other biochemical parameters, and preventing prematurity in these pregnancies (Palma et al., 1992; 1997). Moreover, it has been reported that UDCA restores the ICP-induced impaired transport capabilities of the placenta (Serrano et al., 1998). Previous studies have suggested that treatment of pregnant women with UDCA is not harmful for the fetus (Mazzella et al., 2001). However, there are no studies at mid-term on children born from pregnant women with ICP, with or without UDCA treatment.

Elevated serum BA concentrations have been detected both *in utero* (Macias et al., 2000) and at birth (Monte et al., 1996) in offspring born from pregnant rats with obstructive cholestasis during the last third of pregnancy and the lactation period (OCP). Congenital alterations in the hepatobiliary function of these animals have been detected during juvenile life (4-wk); these were characterized by a partial impairment in the ability of the liver to secrete BAs and bromosulfophthalein whereas the BA-induced biliary secretion of phospholipids, but not that of cholesterol, was markedly enhanced (Monte et al., 1996; El-Mir et al., 1997).

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These alterations, which were transient and no longer detectable at 8-wk of age (Monte et al., 1996; El-Mir et al., 1997), have been associated in part with delayed maturation of the mechanisms involved in hepatocyte transcytosis (Monte et al., 2003), as well as with the presence of multilamellar bodies in the bile canaliculi that might act as plugs hindering bile flow (El-Mir et al., 1997). By contrast, no alterations in the expression of the basolateral transporters Oatp1 -now named Oatp1a1- and Ntcp (Arrese et al., 1998) and in the efficiency of ATP-dependent BA transport across the canalicular membrane (Serrano et al., 1997) were found in these animals.

Recently, we have found that OCP causes a moderate accumulation of BAs in the fetal compartment that is sufficient to induce marked oxidative damage and apoptosis in the fetal liver through stimulation of the mitochondrial pathway. Treatment of pregnant rats with UDCA has beneficial effects by lowering the exposure of the fetus to toxic BAs, restoring the levels of glutathione in the fetal liver, preventing lipid peroxidation and protein carbonylation, and correcting pro-apoptotic alterations in the Bax-α/Bcl-2 ratio (Perez et al., 2004).

The aim of the present study was to gain insight on the mechanisms responsible for the latent cholestasis of pups born from cholestatic mothers and to elucidate the long-term repercussions on the pups of treating their mothers with UDCA during pregnancy.

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MATERIALS AND METHODS

Materials

Taurocholic acid (TCA) sodium salt, ursodeoxycholic acid (UDCA), 5β-cholestane, protease inhibitor cocktail, 3α-hydroxysteroid dehydrogenase, diaphorase and resazurin were obtained from Sigma-Aldrich (Madrid, Spain). All other reagents were from Merck (Barcelona, Spain).

Animals

Pregnant Wistar rats and their offsprings received standard humane care as outlined in the "Guide for the Care and Use of Laboratory Animals" (National Institute of Health Publication No. 80-23, revised 1985). Experimental protocols were approved by the ethical committee of the University of Salamanca for the use of laboratory animals. At birth, the litter size was cut down to eight. The pups were assigned to the following experimental groups according to whether their mothers had undergone: i) sham operation (Control); ii) complete obstructive cholestasis during the last week of pregnancy (OCP), which was performed surgically on day 14 of pregnancy as described elsewhere (Monte et al., 1996) and maintained until weaning, i.e., on day 21 after birth, or; iii) in addition to OCP, they received UDCA (60 μg/100 g b.w./day, i.g.) from day 15 of gestation until weaning (OCP+UDCA). This apparently low dose of UDCA was selected based on previous studies using this experimental model, in which maternal serum BA concentrations were 16-fold higher in OCP than in Controls and, after receiving approximately 3 μmol UDCA over the last week of pregnancy, maternal serum BA concentrations were further increased by approximately 20% in the OCP+UDCA group (Serrano et al., 2003).

"In vivo" studies

Experiments on bile secretion in anaesthetized pups were performed on day 28 after birth after a 24 h fasting period as previously described (Marin et al., 1988). After an equilibration period (10

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min), the bile flowing through a catheter implanted in the common bile duct was collected in pre-weighed vials. A basal bile sample was collected for 60 min, and then during 2 h of step-wise increase in the dose (0.3, 0.6, 0.9, 1.2, 1.5, 1.8, 2.1, 2.4, 2.7, 3.0, 3.3 and 3.6 μmol/min; 10 min each) of TCA infusion via a catheter implanted into the left jugular vein. At the end of the experimental period the livers were removed and weighed.

Canalicular plasma membrane fluidity

In a different set of 28 day-old overnight-fasted rats their livers were removed after being perfused through the portal vein with ice-cold saline. The organs were immediately frozen in liquid nitrogen and stored at -80ºC until they were used to prepare enriched canalicular liver plasma membrane fractions. Approximately 10-20 g from 6-8 livers were pooled together, homogenized and subjected to centrifugation in a discontinuous sucrose density gradient by a previously reported (Serrano et al., 1997) modified combination of the methods described by others (Touster et al., 1970; Meier et al., 1984). Alkaline phosphatase activity (Bretaudiere and Spilmann, 1984) used as a marker of canalicular membrane, was corrected by protein concentrations as determined by a modification of the method of Lowry et al. (Markwell et al., 1978) using bovine serum albumin as standard. This marker was enriched 110-fold in the canalicular liver plasma membrane fraction. The fluidity of these membranes was estimated by the 1,6-diphenyl-1,3,5-hexatriene (DPH) fluorescence depolarization method (Van Blitterswijk et al., 1981). The values of steady-state fluorescence anisotropy were used to calculate the membrane-order parameter P-value, which is inversely proportional to plasma membrane fluidity (Van Blitterswijk et al., 1981). Fluorescence anisotropy was measured using a Hitachi F4010 device equiped with spectrofluorometer polarizers (Hitachi, Tokio, Japan).

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Real-time quantitative RT-PCR

Total RNA was isolated from livers using RNeasy spin columns from Qiagen (Izasa, Barcelona, Spain), treated with DNAse I (Roche, Barcelona, Spain), and measured with the RiboGreen RNA-Quantitation kit (Molecular Probes, Leiden, The Netherlands). RT was carried out using random nonamers and the Enhanced Avian RT-PCR kit (Sigma-Genosys, Cambridge, UK). Real-time quantitative PCR (ABIPrism-5700 Sequence Detection System, Perkin-Elmer Applied Biosystems) was performed using the primers listed in Table 1 for the following proteins: Ntcp, sodium-taurocholate cotransporting polypeptide or Slc10a1; Cyp8b1, sterol 12α-hydroxylase; Cyp27, sterol 27-hydroxylase; Cyp7a1, cholesterol 7α-hydroxylase; Bsep, bile salt export pump or Abcb11; Mdr2, multidrug resistance protein-2 or Abcb4; cholesterol half-transporters Abcg5 and Abcg8, FXR, farnesoid X receptor and SHP, small heterodimer primer. Or primers previously reported (Serrano et al., 2003) for: multidrug resistance associated proteins Mrp1 (Abcc1), Mrp2 (Abcc2) and Mrp3 (Abcc3) and the organic anions transporting polypeptides Oatp1 (Oatp1a1), Oatp2 (Oatp1a4) and Oatp4 (Oatp1b2). Since amplification products were detected using SYBR Green I, it was ascertained previously that no non-specific products were formed during PCR in any case (data not shown). Total liver RNA from a healthy male adult rat was used in all determinations as an external calibrator. 18S rRNA was measured using the TaqMan® Ribosomal RNA Control Reagents kit (Perkin-Elmer Applied Biosystems) to correct the differences of RNA extraction in each sample.

Analytical, histological and statistical methods

Bile flow was determined gravimetrically. Enzymatic methods based on 3α-hydroxysteroid dehydrogenase were used to measure total BA concentrations in bile (Talalay, 1960) and in serum after liquid-solid extraction (Mashige et al., 1976) as described previously (Monte et al., 1996). Gas chromatography-mass spectrometry (GC/MS) was used for the determination of individual BAs in basal bile as previously described (El-Mir et al., 1997). Biliary cholesterol and

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diacylphosphatidylcholines (lecithins) concentrations were determined enzymaticaly using commercial kits (Biomérieux, Marcy l'Etoile, France). Three samples collected under stimulation of bile secretion by taurocholate administration in each experimental group were analyzed by HPLC as previously reported (Villanueva et al., 1990) to confirm that lecithins accounted for most of the phospholipids secreted in bile in all cases (data not shown). Other parameters were measured in serum by routine automated methods (Hitachi 747, Roche, Barcelona, Spain).

Examination of livers using transmission electron microscopy (Zeiss EM 900) was carried out as previously reported (Monte et al., 1996).

Results are expressed as means±SEM. To calculate the statistical significance of differences among groups, the Bonferroni method of multiple-range testing was used.

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RESULTS

Morphological, biochemical, and histological changes

The morphological and biochemical characteristics of OCP rats have been described elsewhere (Serrano et al., 2003). In the offspring of these animals, body weight was significantly lower than in normal pups of the same age (Table 2). Liver weight was also slightly, but not significantly, lower in OCP. The OCP-induced changes observed in the biochemical parameters of the offspring included elevated serum levels of BAs and alkaline phosphatase. UDCA treatment of the mothers prevented these alterations in the pups.

Electron microscopy examination confirmed previous observations (El-Mir et al., 1997) indicating the presence of multilamellar bodies in the hepatocytes of 4-wk-old animals born from OCP mothers that were absent in the Control group. These structures were located both intracellularly and within the bile canaliculi. In the present study we have found that their abundance and size were reduced by treatment of OCP mothers with UDCA. Thus, in OCP they were seen in almost all hepatocyes and intracanalicular plugs occupied most of the canalicular lumen, whereas in the OCP+UDCA group they were found in approximately 1 out of 3 hepatocytes and, when they were found within the canaliculus, less than half of canalicular lumen was occupied by them (Figure 1).

BA profile and expression of enzymes involved in BA synthesis

Analysis by GC-MS of bile samples collected for the 60-min non-stimulated period (Figure 2) revealed that the major change in the BA profile in basal bile from OCP offspring was a decrease in the proportion of cholic acid (CA). Treatment with UDCA did not restore CA bile output, but did decrease that of deoxycholic acid and other minor BAs, whereas that of α-muricholic acid was enhanced. In general, the proportion of α- and β-muricholic acids in bile was enhanced in

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OCP pups and further so in the OCP+UDCA group. The levels of mRNA for the major BA sensor, farnesoid nuclear receptor (FXR) and those for small heterodimer partner (SHP), were similarly increased in OCP and OCP+UDCA groups (Figure 3). Those of cholesterol-7α-hydroxylase (*Cyp7a1*), the limiting step of the neutral pathway for BA synthesis, were elevated in OCP and partly restored to Control values in OCP+UDCA. The expression of other key enzymes for 12α-hydroxylation (*Cyp8b1*) and BA synthesis through the acidic pathway (*Cyp27*) were not significantly altered (Figure 3).

Expression of sinusoidal and canalicular transporters

Regarding changes in the expression levels of sinusoidal BA carriers in offspring livers, in agreement with previous studies by others (Arrese at al., 1998), OCP did not affect those of Ntcp (*Slc10a1*) and Oatp1 or Oatp1a1 (*Slco1a1*) (Figure 3). *Oatp2* or Oatp1a4 (*Slco1a4*) and Oatp4 or Oatp1b2 (*Slco1b2*) steady-state mRNA levels were not affected by OCP either. Moreover, treatment of the mothers with UDCA had no effect on the expression of any of these genes (Figure 3).

Steady-state mRNA levels of the bile salt export pump (*Bsep*) and phospholipid flipase (*Mdr2*) were not significantly affected by OCP or OCP+UDCA. A tendency to be increased in OCP, but normalized by UDCA, was observed for members *Mrp2* and *Mrp3* of the multidrug resistance-associated protein family. This became more marked and statistically significant only for *Mrp1*. The abundance of mRNA for the cholesterol transporters Abcg5 and Abcg8 was significantly decreased by OCP. This down-regulation was completely (*Abcg5*) or partly (*Abcg8*) prevented by treating pregnant OCP rats with UDCA.

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Bile flow, biliary lipids output and plasma membrane fluidity

No significant differences were found among the three experimental groups as regards basal bile flow and bile output of BAs, cholesterol and phospholipids (Figure 4).

However, in agreement with previous studies (El-Mir et al., 1997), a latent impairment became evident under TCA stimulation of bile secretion. Thus, TCA-induced bile flow and BA output were lower in OCP, while cholesterol output was not significantly reduced. By contrast, phospholipid output was significantly enhanced. Treatment of OCP mothers with UDCA partly restored the biliary response of the pups to TCA administration regarding bile flow and BA output. In contrast, in the OCP+UDCA animals cholesterol output was slightly reduced, whereas over-stimulated phospholipid output was not prevented. Accordingly, the bile output ratios for phospholipid/BA, cholesterol/BA, and phospholipid/cholesterol were higher in OCP animals than in the Controls (Figure 5). These differences were partly or completely prevented by treatment with UDCA, except for the phospholipid/cholesterol output ratio, which was further increased in OCP+UDCA.

OCP also induced a significant change in the fluidity of canalicular plasma membrane. Thus, the P-values of DPH anisotropy at 37ºC were increased in OCP (Figure 5D), i.e., canalicular plasma membrane fluidity was lower in OCP than in Control pups. This change was completely prevented by UDCA treatment of OCP mothers.

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DISCUSSION

OCP causes oxidative stress and apoptosis in both the rat placenta and fetal liver (Perez et al., 2004), which impairs their normal development and reduce the number of viable fetuses per pregnancy. Treatment with UDCA improves pregnancy outcome, in part due to an improvement in placental function (Serrano et al., 2003). Similar effect of UDCA treatment has also been observed in placentas from patients with ICP (Serrano et al., 1998). The present study revealed that the beneficial effect of maternal treatment during pregnancy is extended to the offspring at long-term after birth by partly preventing the congenital impairment of bile secretion that is more evident in response to the stimulation of bile formation by a BA load. A similar situation can also be expected to occur during postprandrial periods. In fact, likely due to the partial impairment in bile secretion present in these animals, serum BA concentrations were elevated in pups born from mothers with OCP. However, since infant rats were investigated shortly after weaning, the possibility that BA supply with maternal milk could contribute to hypercholanemia of these pups cannot be ruled out.

Several possible mechanisms that could be involved in the latent cholestasis of the offspring as well as in the response to treating their mothers with UDCA have been explored in the present study and the results are discussed below.

In adult rats fed with an excess of BAs, changes in the expression of the sinusoidal carriers involved in BA uptake have been reported (Rost et al., 2003). However, this did not occur in OCP pups, and hence impaired liver uptake probably did not account for the reduction in TCA-stimulated bile secretion. Nevertheless, regarding the control of the expression of both carriers and enzymes involved in BA transport and biosynthesis, respectively, some alterations were detected. Thus, the nuclear receptor FXR, which is the major sensor involved in up- or down-

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regulation of several of these proteins, was up-regulated and the levels of its ligands enhanced, however no change (e.g., Ntcp, Bsep and Cyp8b1) or change in the opposite direction to that expected (e.g., Cyp7a1) in the abundance of mRNA for genes controlled by FXR was found. Most of the effects of FXR are mediated by controlling the expression of SHP. As expected, when BA levels and FXR expression were enhanced, SHP was up-regulated. These findings imply that under the experimental circumstances of the present study the difference with the mechanism present in the healthy adult rat liver was probably due to elements responsible for the sensitivity to SHP.

The expression of Bsep, the major mechanism involved in active BA transport into bile (for review see Kullak-Ublick et al., 2004), was not significantly affected by OCP either. Although changes in plasma membrane fluidity may affect the function of canalicular transporters (Sinicrope et al., 1992), the functional data obtained with isolated plasma membrane vesicles in previous studies (Serrano et al., 1997) did not support the existence of OCP-induced impairment in ATP-dependent BA transport across the canalicular membrane.

The abundance of mRNA for other transporters involved in either the canalicular secretion of dianionic BAs, such as Mrp2 (Kullak-Ublick et al., 2004) or the efflux of BAs across the basolateral membrane of the hepatocyte, such as Mrp1 and Mrp3, was not decreased. By contrast, their expression was moderately (Mrp2 and Mrp3) or markedly (Mrp1) enhanced. This situation is consistent with a typical response to the accumulation of BAs and/or the release of certain cytokines (Kullak-Ublick et al., 2004) rather than to be the cause of impaired bile secretion. Moreover, whether compensatory proliferation after birth in response to enhanced apoptosis that has been detected in fetal liver at term in rats with OCP (Perez et al., 2004) is involved in up-regulation of Mrp1 is not known.

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The presence of multilamellar aggregates in the canaliculi of adult rats with a massive secretion of biliary cholesterol had previously been reported by other authors (Rigotti et al., 1993), who suggested that these structures might be formed in a process resembling the budding of viruses from the cell surface. Lamellar particles could remain as proteoliposomes within the canaliculus and would mix with lipids reaching the bile via other hepatocellular mechanisms. This is in part consistent with our results, which revealed the coexistence of multilamellar bodies in bile together with a normal basal secretion of biliary lipids. Moreover, complete normalization of serum levels of BA and alkaline phosphatase in OCP+UDCA group, even though smaller multilamellar aggregates were still present in some canaliculi suggests that these structures did not cause a significant obstruction to bile flow in this group, even under stimulation of bile formation by TCA administration.

Increased proportions of more hydrophilic BAs, such as muricholic acids, with a lower ability to emulsify lipids, had been previously suggested to play a role in the formation and/or delay of removal from the canaliculi of multilamellar bodies (El-Mir et al., 1997). The present study indicates that there were indeed changes in the synthesis of BA and its regulation in OCP pups. However, impaired BA metabolism was probably the consequence rather than the cause of reduced bile secretion. Accordingly, the proportions of α- and β-muricholates in bile were further increased in OCP+UDCA offspring, even though bile secretion was partly restored. Moreover, changes in the biliary profile of BAs cannot explain the reduction of multilamellar bodies in OCP+UDCA offspring in terms of the different detergent capabilities of the BA pool.

The observation that multilamellar bodies are also present within the cells suggests that intracellular mechanisms, such as those responsible for the transcytosis of canalicular membrane constituents, must be also involved. The transcytotic machinery in the offspring of OCP mothers is indeed altered (Monte et al., 2003). Thus, the transfer of biliary lipids from intracellular

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compartments toward the canalicular membrane is probably also affected. It has been reported that the rates of liposomal fusion to membranes are decreased in cholestatic rats and that a redistribution of phospholipid species within canalicular membranes is associated with decreased transcytotic vesicle fusion (Hyogo et al., 1999). This suggests the existence of alterations in the physical-chemical properties of transcellular/canalicular membranes, which may be involved in both the formation of multilamellar bodies and their intracellular location.

In fact, increased cholesterol contents in the canalicular membrane of the cholestatic rats have been reported and the hypothesis of the existence of decreased membrane fluidity has been entertained (Hyogo et al., 1999). This was later confirmed in acute cholestasis induced by selective ligation of some bile duct branches (Kanno et al., 2003). In opposite situations, such as in rats receiving diosgenin, canalicular membrane fluidity is enhanced and this is accompanied by enhanced cholesterol output and bile flow (Yamaguchi et al., 2003). In OCP offspring, the fluidity of the canalicular membrane decreased and the expression of cholesterol transporters Abcg5/Abcg8 was down-regulated. Moreover, TCA-stimulated cholesterol output remained unchanged or was slightly decreased when phospholipid output was markedly enhanced, which led to a higher phospholipid/cholesterol ratio in the bile. Although there is probably a relationship between these changes and the formation of multilamellar bodies as well as with the reduction of them in OCP+UDCA pups, it is not possible at present to identify among these findings the causes and the consequences.

Since the expression of Mdr2 seemed to be unaltered, the question arises as to whether changes in i) cholesterol dynamics at the canalicular pole, ii) canalicular membrane fluidity or iii) an enhanced release from multilamellar bodies might be related to over-stimulation of phospholipid output. Our results suggest a negative answer to the first two possibilities but do not rule out the third one. Thus, UDCA treatment completely restored Abcg5/Abcg8 expression levels, as well as

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canalicular membrane fluidity, but only partly prevented the formation of multilamellar bodies and over-stimulation of phospholipid output by BAs.

In conclusion these results indicate that UDCA treatment of pregnant rats with OCP does not aggravate but instead has a beneficial effect on the transient congenital impairment of hepatobiliary function of their offspring.

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Acknowledgments

The authors thank L. Muñoz, J.F. Martin, J. Villoria, N. Gonzalez and E. Vallejo for care of the animals. Secretarial help by M.I. Hernandez, technical help by E. Flores, assistance in ETM by M.T. Sanchez Montero, in plasma membrane fluidity measurement by M. Llanillo, and in the English revision of the manuscript by N. Skinner are also gratefully acknowledged.

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REFERENCES

Arrese M, Trauner M, Ananthanarayanan M, Boyer JL, Suchy FJ (1998) Maternal cholestasis does not affect the ontogenic pattern of expression of the Na^+/taurocholate cotransporting polypeptide (Ntcp) in the fetal and neonatal rat liver. *Hepatology* **28**:789-795.

Bretaudiere JP, Spilmann T (1984) *Alkaline phosphatase. In: Methods of Enzymatic Analysis* Bergmeyer HU, Bergmeyer J, Grabl M, eds.. Weinheim: Verlag Chemie, 75-82.

El-Mir MY, Monte MJ, Morales AI, Arevalo M, Serrano MA, Marin JJG (1997) Effect of maternal cholestasis on biliary lipid and bile acid secretion in the infant rat. *Hepatology* **26**:527-536.

Hyogo H, Tazuma S, Kajiyama G (1999) Transcytotic vesicle fusion is reduced in cholestatic rats: redistribution of phospholipids in the canalicular membrane. *Dig Dis Sci* **44**:1662-1668.

Kanno K, Tazuma S, Niida S, Chayama K (2003) Unique reciprocal changes of hepatocellular membrane transporter expression and fluidity in rats with selective biliary obstruction. *Hepatol Res* **26**:157-163.

Kullak-Ublick GA, Stieger B, Meier PJ (2004) Enterohepatic bile salt transporters in normal physiology and liver disease. *Gastroenterology* **126**:322-342.

Lammert F, Marschall HU, Matern S (2003) Intrahepatic cholestasis of pregnancy. *Curr Treat Options Gastroenterol* **6**:123-132.

Macias RIR, Pascual MJ, Bravo A, Alcalde MP, Larena MG, St-Pierre MV, Serrano MA, Marin JJG (2000) Effect of maternal cholestasis on bile acid transfer across the placenta-maternal liver tandem. *Hepatology* **31**:975-983.

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Marin JJG, Villanueva GR, Esteller A (1988) Diabetes-induced cholestasis in the rat. Possible role of hyperglucemia and hypoinsulinemia. *Hepatology* **8**:332-340.

Markwel MAK, Haas SM, Bieber LL, Tolbert ME (1978) A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal Biochem* **87**:206-210.

Mashige U, Imai K, Osuga T (1976) Simple and sensitive assay of serum total bile acids. *Clin Chim Acta* **70**:79-86.

Mazzella G, Rizzo N, Azzaroli F, Simoni P, Bovicelli L, Miracolo A, Simonazzi G, Colecchia A, Nigro G, Mwangemi C, Festi D, Roda E (2001) Ursodeoxycholic acid administration in patients with cholestasis of pregnancy: effects on primary bile acids in babies and mothers. *Hepatology* **33**:504-508.

Meier PJ, Sztul ES, Reuben A, Boyer JL (1984) Structural and functional polarity of canalicular and basolateral plasma membrane vesicles isolated in high yield from rat liver. *J Cell Biol* **98**:991-1000.

Monte MJ, Morales AI, Arevalo M, Alvaro I, Macias RIR, Marin JJG (1996) Reversible impairment of neonatal hepatobiliary function by maternal cholestasis. *Hepatology* **23**:1208-1217.

Monte MJ, Villanueva GR, Macias RIR, Vazquez DJ, Toledo M, Dominguez M, Marin JJG (2003) Effect of maternal obstructive cholestasis during pregnancy on the biliary transport of horseradish peroxidase in the rat offspring. *Clin Sci* **105**:347-353.

Palma J, Reyes H, Ribalta J, Iglesias J, Gonzalez MC, Hernandez I, Alvarez C, Molina C, Danitz AM (1992) Effects of ursodeoxycholic acid in patients with intrahepatic cholestasis of pregnancy. *Hepatology* **15**:1043-1047.

Palma J, Reyes H, Ribalta J, Hernandez I, Sandoval L, Almuna R, Liepins J, Lira F, Sedano M, Silva O, Toha D, Silva JJ (1997) Ursodeoxycholic acid in the treatment of cholestasis

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of pregnancy: a randomized, double-blind study controlled with placebo. *J Hepatol* **27**:1022-1028.

Perez MJ, Serrano MA, Duran C, Macias RIR, Briz O, Marin JJG (2004) Maternal cholestasis induces oxidative stress and cell death in rat placenta and foetal liver. Protective effect of ursodeoxycholic acid. *J Hepatol* **40**:26.

Rigotti A, Nunez L, Amigo L, Puglielli L, Garrido J, Santos M, Gonzalez S, Mingrone G, Greco A, Nervi F (1993) Biliary lipid secretion: immunolocalization and identification of a protein associated with lamellar cholesterol carriers in supersaturated rat and human bile. *J Lipid Res* **34**:1883-1894.

Rost D, Herrmann T, Sauer P, Schmidts HL, Stieger B, Meier PJ, Stremmel W, Stiehl A (2003) Regulation of rat organic anion transporters in bile salt-induced cholestatic hepatitis: effect of ursodeoxycholate. *Hepatology* **38**:187-195.

Serrano MA, Monte MJ, Martinez-Diez MC, Marin JJG (1997) Effect of maternal cholestasis on the kinetics of bile acid transport across the canalicular membrane of infant rat livers. *Int J Exp Pathol* **78**:383-390.

Serrano MA, Brites D, Larena MG, Monte MJ, Bravo MP, Oliveira N, Marin JJG (1998) Beneficial effect of ursodeoxycholic acid on alterations induced by cholestasis of pregnancy in bile acid transport across the human placenta. *J Hepatol* **28**:829-839.

Serrano MA, Macias RIR, Vallejo M, Briz O, Bravo A, Pascual MJ, St-Pierre MV, Stieger B, Meier PJ, Marin JJG (2003) Effect of ursodeoxycholic acid on the impairment induced by maternal cholestasis in the rat placenta-maternal liver tandem excretory pathway. *J Pharmacol Exp Ther* **305**:515-524.

Sinicrope FA, Dudeja PK, Bissonnette BM, Safa AR, Brasitus TA (1992) Modulation of P-glycoprotein-mediated drug transport by alterations in lipid fluidity of rat liver canalicular membrane vesicles. *J Biol Chem* **267**:24995-25002.

Talalay P (1960) Enzymatic analysis of steroid hormones. *Meth Biochem Anal* **8**:119-143.

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JPET Fast Forward. Published on September 27, 2004 as DOI: 10.1124/jpet.104.075051
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Touster O, Aronson N, Dulaney JT, Hendrickson H (1970) Isolation of rat liver plasma membranes. *J Cell Biol* **47**:604-618.

Van Blitterswijk WJ, van Hoeven RP, van der Meer BW (1981) Lipid structural order parameters (reciprocal of fluidity) in biomembranes derived from steady-state fluorescence polarization measurements. *Biochim Biophys Acta* **644**:323-332.

Villanueva GR, Herreros M, Perez-Barriocanal F, Bolanos JP, Bravo P, Marin JJG (1990) Enhancement of bile acid-induced biliary lipid secretion by streptozotocin in rats. Role of insulin-deficiency. *J Lab Clin Med* **115**:441-448.

Yamaguchi A, Tazuma S, Ochi H, Chayama K (2003) Choleretic action of diosgenin is based upon the increases in canalicular membrane fluidity and transporter activity mediating bile acid independent bile flow. *Hepatol Res* **25**:287-295.

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FIGURE LEGENDS

Figure 1. Representative transmission electron microscopy images of liver sections obtained from rats at 4 wk of age belonging to the experimental groups: Control (A, sham-operated mothers), OCP (B, mothers with obstructive cholestasis), and OCP+UDCA (C and D, mothers with obstructive cholestasis treated orally with 60 μg UDCA/100 g b.wt./day). Arrowheads show multilamellar bodies within hepatocytes (C) or bile canaliculi (B and D).

Figure 2. Bile output of individual bile acid (BA) species as measured by GC-MS in 60-min basal samples collected from 4-wk-old rats belonging to the experimental groups: Control (n=8), OCP (n=10), and OCP+UDCA (n=8). Values are means±SEM. *, $p<0.05$ by comparing with OCP using the Bonferroni method of multiple range testing. CA: cholic acid, CDCA: chenodeoxycholic acid, DCA: deoxycholic acid, UDCA: ursodeoxycholic acid, MCA: muricholic acid.

Figure 3. Steady-state levels of mRNA as determined by real-time quantitative RT-PCR in the liver of 4-wk-old rats belonging to the experimental groups: Control, OCP, and OCP+UDCA, for plasma membrane transporters: Ntcp, Oatp1 or Oatp1a1, Oatp2 or Oatp1a4, Oatp4 or Oatp1b2, Mrp1, Mrp2, Mrp3, Mdr2, Bsep, Abcg5 and Abcg8, key enzymes involved in bile acid biosynthesis: Cyp8b1, Cyp27 and Cyp7a1, and major nuclear receptors controlling the expression of several of these genes in response to bile acid levels: FXR and SHP. Inter-reaction variability was corrected using total RNA from an adult rat liver as calibrator, and the levels of 18S rRNA in each sample were used to normalize the results. Values are means±SEM of eight samples analyzed in triplicate for each experimental group.

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*, p<0.05 by comparing with OCP using the Bonferroni method of multiple range testing.

Figure 4. Bile flow (A), bile acid output (B), phospholipid output (C) and cholesterol output (D) in rats of 4 wk of age belonging to the experimental groups: Control (n=8), OCP (n=10), and OCP+UDCA (n=8). Taurocholic acid (TCA) was i.v. infused (step-wise from 0.3 to 3.6 μmol/min) from min 60 to 180. Basal, cumulative values from min 0 to min 60; +TCA, cumulative values determined over the following 2 h of TCA infusion. Values are means±SEM. *, p<0.05 by comparing with OCP using the Bonferroni method of multiple range testing.

Figure 5. Ratios of taurocholic acid (TCA)-stimulated bile output of cholesterol/bile acids (A), phospholipids/bile acids (B), and phospholipids/cholesterol (C) in rats of 4 wk of age belonging to the experimental groups: Control (n=8), OCP (n=10), and OCP+UDCA (n=8). Stimulated values were calculated by subtracting those measured under TCA administration and those determined in the basal period. (D) Canalicular plasma membrane fluidity was calculated as the inverse of DHP anisotropy values at 37°C measured in 4 preparations from each group. Values are means±SEM. *, p<0.05 by comparing with OCP using the Bonferroni method of multiple range testing.

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Table 1. Oligonucleotide sequences of primers used in real-time quantitative RT-PCR.

Target Sequence	Primer Sequence (5'-3')		Amplicon (bp)		GenBank
	Forward	Reverse	Position	Length	Accesion No.
Slc10a1	CGTTGCCGGAATGTTTGTCT	TGCCCTTCTGTCTCAGTTCATG	1253-1327	75	NM_017047
Cyp8b1	GTACACATGGACCCCGACATC	GGGTGCCATCAGGGTTGAG	1195-1270	76	AB009686
Cyp27	CCTTTGGGACTCGCACCA	GCCCTCCTGTCTCATCACTTG	748-818	71	M73231
Cyp7a1	GCTTTACAGAGTGCTGGCCAA	CTGTCTAGTACCGGCAGGTCATT	987-1078	92	NM_012942
Bsep	GCCATTGTGCGAGATCCTAAA	TGCAGGTCCGACCCTCTCT	3956-4073	118	NM_031760
Mdr2	ACTGTCCGGAATGCAGATGTC	TCTTTATCAGCTCACTGTGGCTT	1800-1881	82	NM_012690
Abcg5	GGCTCGGCACAGCTTAGG	CTGGCATGATTTGATGTTCCA	216-300	85	NM_053754
Abcg8	CCCTGATCCGTCGTCCAGATT	AGAGACATCAGGCAGGCTTCT	1309-1393	85	AF351785
FXR	GTGACAAAGAAGCCGCGAAT	GCAGGTGAGCGCGTTGTAAT	481-594	114	U18374
SHP	CTCGGTTTGCATACAGTGTTTGAC	GCATATTGGCCTGGAGGTTTT	920-994	75 bp	D86580

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Table 2. Effect of obstructive cholestasis during pregnancy (OCP) and treatment of pregnant rats with ursodeoxycholic acid (OCP+UDCA) on the morphological and biochemical parameters of their offspring at 4 wk of age.

	Control (n=44)	**OCP** (n=22)	**OCP+UDCA** (n=40)
Morphological Parameters			
Body Weight (g)	71±2[a]	59±2	69±2[a]
Liver Weight (g)	2.8±0.2	2.3±0.2	2.6±0.2
Liver Weight/Body Weight Ratio (%)	4.0±0.3	3.9±0.2	4.1±0.3
Serum Biochemical Parameters			
Total Bile Acids (μmol/L)	49±5[a]	71±9	41±5[a]
Total Bilirubin (mg/dL)	0.07±0.01	0.08±0.01	0.09±0.01
GOT (UI/L)	145±6	128±7	144±8
GPT (UI/L)	33±2	39±2	36±1
Alkaline Phosphatase (UI/L)	319±8[a]	419±12	343±9[a]
Cholesterol (mg/dL)	80±2	77±2	84±2
HDLc (mg/dL)	48±2	42±2	46±2
Triglycerides (mg/dL)	100±8	95±8	98±11

OCP was induced surgically in pregnant rats on day 14 of pregnancy. UDCA treatment (60 μg/100 g b.wt./day, i.g.) was administered from day 15 of pregnancy until weaning (21 days after birth). Values are mean±SEM. [a], $p<0.05$, as compared to OCP by the Bonferroni method of multiple range testing.

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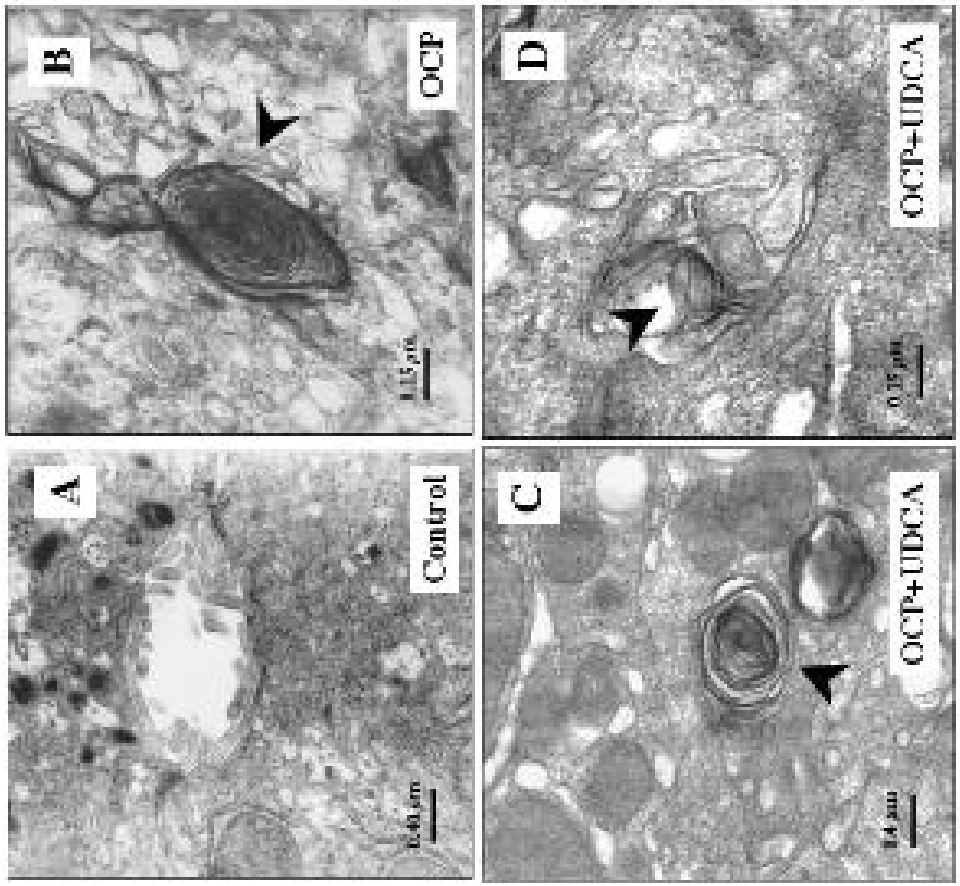

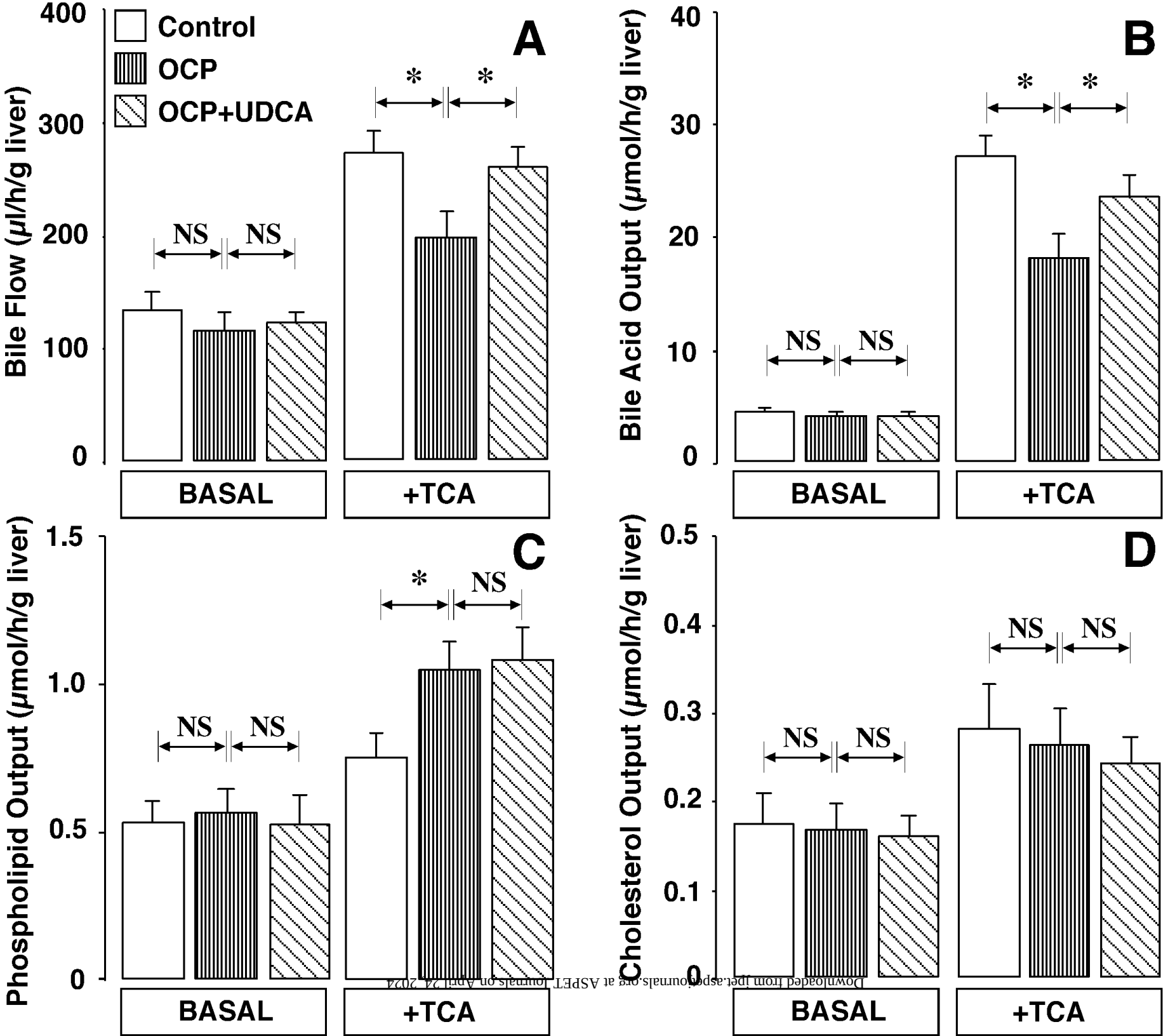

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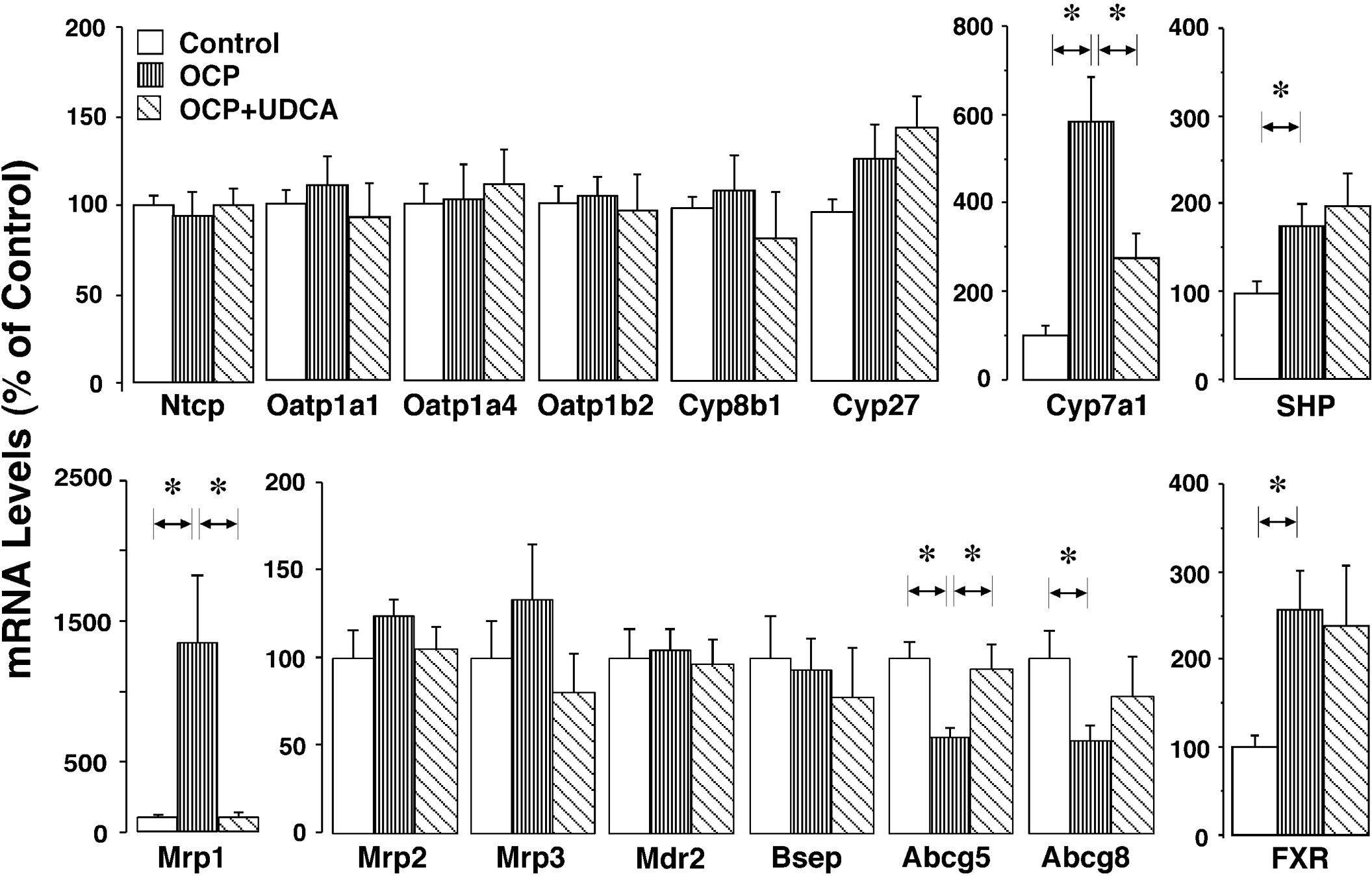

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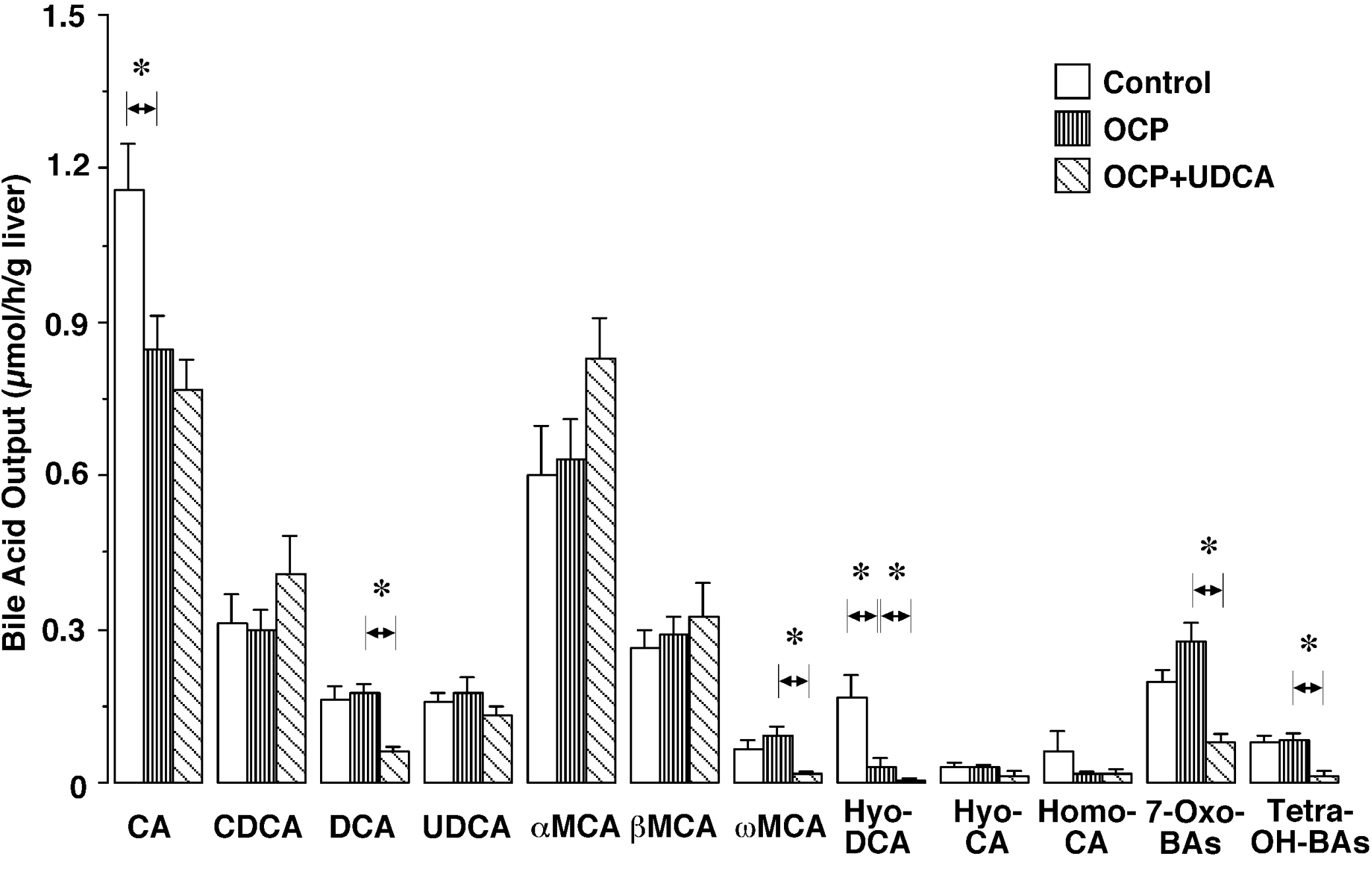

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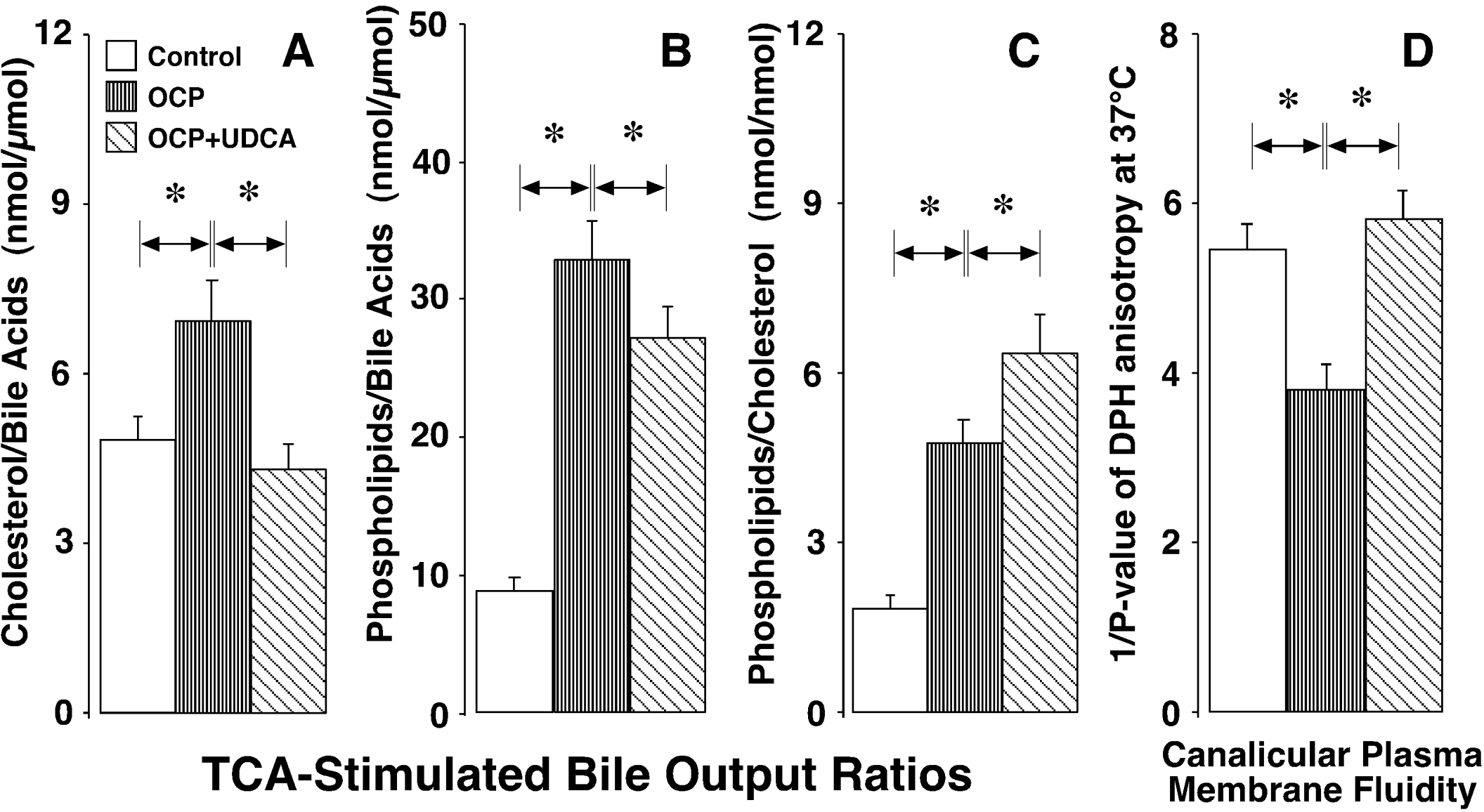

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