Novel treatment strategies for unconjugated hyperbilirubinemia
Cuperus, Frans

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Chapter 3

Effective Treatment of Unconjugated Hyperbilirubinemia with Oral Bile Salts in Gunn Rats

F.J.C. Cuperus 1
A.M. Hafkamp 1
R.Havinga 1
L.Vitek 2,3
J. Zelenka 3
C. Tiribelli 4
J.D. Ostrow 5
H.J. Verkade 1

1Pediatric Gastroenterology and Hepatology, Department of Pediatrics, Center for Liver, Digestive, and Metabolic Diseases, Beatrix Children’s Hospital - University Medical Center Groningen, University of Groningen, The Netherlands.
24th Department of Internal Medicine, 1st Faculty of Medicine, Charles University, Prague, Czech Republic.
3Institute of Clinical Biochemistry and Laboratory Diagnostics, 1st Faculty of Medicine, Charles University, Prague, Czech Republic.
4Centro Studi Fegato, AREA Science Park and Department of Life Sciences, University of Trieste, Trieste, Italy.
5Gastroenterology/Hepatology Division, University of Washington School of Medicine, Seattle, WA, USA.

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3.1 Abstract

We tested the hypothesis that oral administration of bile salts, which are known to increase the biliary excretion of unconjugated bilirubin (UCB), decreases unconjugated hyperbilirubinemia in the Gunn rat model. Adult Gunn rats were fed a standard diet or the same diet supplemented with 0.5 wt% ursodeoxycholic acid (UDCA) or cholic acid (CA) for 1 or 6 weeks. UCB and urobilinoids, a family of intestinal UCB breakdown products, were determined in plasma and/or feces. After 6 weeks of treatment, tracer $^3$H-UCB was administered i.v. to determine steady-state UCB kinetics over the next 60h. One-week treatment with UDCA or CA decreased plasma UCB concentrations by 21% and 30%, respectively (each $p<0.01$). During the first four days of treatment, both UDCA and CA increased the combined fecal excretion of UCB and urobilinoids (+52% and +32%, respectively; each $p<0.01$). Prolongation of treatment to 6 weeks caused a persistent decrease in plasma UCB concentrations to $\sim$40% below baseline (each bile salt $p<0.001$). $^3$H-UCB kinetic studies showed that UDCA and CA administration decreased UCB pool size (-33% and -32%, respectively; each $p<0.05$) and increased UCB fractional turnover (+33% and +25%, respectively; each $p<0.05$). This chapter demonstrates that dietary bile salt administration induces a large, persistent decrease in plasma UCB concentrations in Gunn rats. Both UDCA and CA enhance UCB turnover by increasing its fecal disposal. These results support the application of oral bile salt treatment in patients with unconjugated hyperbilirubinemia.
3.2 Introduction

Unconjugated hyperbilirubinemia occurs in conditions such as neonatal hemolytic jaundice and Crigler-Najjar disease. Crigler-Najjar disease is characterized by a genetically absent (type I) or decreased (type II) capacity to conjugate bilirubin in the liver,[1] which is essential for efficient biliary excretion of the pigment. Impaired conjugation results in unconjugated hyperbilirubinemia, due to retention of unconjugated bilirubin (UCB) in the body. Severe unconjugated hyperbilirubinemia can lead to deposition of UCB in the central nervous system, causing bilirubin-induced neurological dysfunction (BIND), kernicterus, and death.[2] Unconjugated hyperbilirubinemia is conventionally treated by phototherapy, which induces photo-isomerisation of the hydrophobic UCB to polar isomers that can readily be excreted into the bile.[3] Although generally effective, phototherapy does not always decrease plasma UCB to non-toxic levels. Most importantly, long-term phototherapy, such as needed for patients with Crigler-Najjar disease type I, becomes less effective with age and has a profound impact on social life.[4,5] These considerations favor the development of effective alternative treatments for unconjugated hyperbilirubinemia.

During severe unconjugated hyperbilirubinemia, most UCB does not enter the intestinal lumen via biliary excretion, but rather via direct diffusion across the intestinal mucosa.[6,7] The efficiency of this pathway is decreased, however, by the ability of the intestine to reabsorb UCB from its lumen.[8,9] Several experimental therapies have aimed to prevent reabsorption by oral administration of agents that trap UCB in the intestinal lumen. However, trapping agents tested so far, including agar,[10] cholestyramine,[11] charcoal,[12] amorphous calcium phosphate,[13] zinc salts,[14] and orlistat,[15] have been clinically unsatisfactory, due to side-effects and inconsistent results.

Since bile salts can stimulate biliary excretion of organic anions,[16] including bilirubin in rats,[17] we reasoned that bile salt administration could be relevant for treatment of unconjugated hyperbilirubinemia. Ursodeoxycholic acid (UDCA) treatment in healthy volunteers decreased the expiration of $^{14}$CO$_2$ from triolein, suggesting that UDCA also mildly decreases the absorption of fat.[18] Mild fat malabsorption induced by orlistat decreased plasma bilirubin levels in a subset of Crigler-Najjar patients and in homozygous Gunn rats, their well-established animal model.[15,19] Finally, bile salts associate with UCB in vitro, and bile salt administration could therefore also lower plasma UCB concentrations via enhancement of fecal excretion of UCB-bile salt complexes.[20,21]

In the present study we show that dietary administration of UDCA indeed reduces unconjugated hyperbilirubinemia in homozygous Gunn rats. We studied
several dosages and administration periods to evaluate the clinical applicability of this potential treatment. We compared the UDCA effects in Gunn rats with those obtained after administration of cholic acid (CA) to evaluate whether effects were bile salt specific. Finally, we studied steady-state kinetics of i.v. administered $^3$H-UCB, to gain insight in the underlying mechanisms of both UDCA and CA administration.

3.3 Animals, materials, and methods

3.3.1 Animals

Homozygous adult male Gunn Rats (RHA/jj, 240-360g), obtained from our breeding colony (University Medical Center Groningen, the Netherlands), were housed in an environmentally controlled facility and fed ad libitum. The Ethics Committee for Animal Experiments of the University of Groningen approved all experimental protocols.

3.3.2 Materials

Chemicals

UDCA was a generous gift from Dr. Falk Pharma GmbH (Freiburg, Germany). CA and heptadecanoic acid (C17:0) were obtained from Sigma-Aldrich Co. (St. Louis, MO). Xanthobilirubin-methylester was a generous gift from Dr. J. Fevery (Leuven, Belgium). Urobilin was obtained from Frontier Scientific Inc. (Logan, UT). $^3$H-labeled UCB (specific activity 6.02 $\mu$Ci/$\mu$mol) was prepared by biosynthetic labeling of 2,3-$^3$H-labeled 5-aminolevulinic acid (specific activity 13 mCi/mmol; Amersham Biosciences, Piscataway, NJ).[22-24] $^3$H-labeled UCB solution was prepared immediately before injection into Gunn rats as described before.[23]

Diets

The semi-synthetic, purified control diet (code 4063.02) was produced by Hope Farms BV (Woerden, the Netherlands) and contained 13 energy% fat and 5.2 wt% long-chain fatty acids. Diets containing bile salts were identical except for supplementation with UDCA or CA (0.05% - 1.5% by chow weight).
3.3.3 Methods

**Preliminary dose-response experiment**

After a 6-week run-in period on the control diet, Gunn rats were randomly assigned to receive the control diet supplemented with either UDCA or CA (n=6 per group). All animals were housed and fed by dietary group for a period of 10 weeks, during which the dosage of UDCA and CA was increased every 2 weeks. Used dosages: 0.05%; 0.1%; 0.5%; 1%; and 1.5 wt% (by chow weight). Heparinized samples of tail vein blood were obtained under isoflurane anesthesia before and 2, 4, 6, 8, and 10 weeks after dietary randomization for determination of plasma UCB concentrations and, in the last plasma sample, aspartate aminotransferase (AST), alanine aminotransferase (ALT).

**Short-term experiment**

After a 6-week period on the control diet, individually housed Gunn rats were randomly assigned to receive the control diet or the same diet supplemented with UDCA or CA (each 0.5 wt%; n=6 per group). Food intake and animal weights were determined daily. Heparinized samples of tail vein blood were obtained under isoflurane anesthesia at day 0, 1, 3, 5, and 8 for determination of plasma UCB concentrations. Feces were collected every 24h for 4 days before and for 4 days after dietary randomization to determine fecal excretion of UCB, urobilinoids, and bile acids. Eight days after dietary randomization, the common bile duct was cannulated under pentobarbital anesthesia and bile was collected for 30 minutes under light-protected conditions. Bile flow was determined gravimetrically, assuming a density of 1 g/ml. A 1 ml blood sample was then obtained by puncture of the inferior vena cava to determine AST and ALT.

**Long-term experiment**

After 6 weeks on the control diet, individually housed Gunn rats were randomly assigned to receive either the control diet or the same diet supplemented with UDCA or CA (each 0.5 wt %; n=6 per group). Food intake and animal weights were determined weekly. Heparinized samples of tail vein blood were obtained under isoflurane anesthesia before and at 2, 4, and 6 weeks after dietary randomization to determine plasma UCB concentrations. At 5 weeks the rats were gavaged with 1 ml (20 mg/ml) carmine red and stools were examined for red staining to assess intestinal transit time. At 6 weeks, the $^3$H-labeled UCB solution (~0.29 μCi/100g BW) was administered via the penile vein.
Subsequently, heparinized samples of tail vein blood were collected every 12 h for 60 h for determination of plasma UCB concentrations and feces were collected to determine fecal excretion of urobilinoids, $^3$H-label, bile salts, calcium, phosphate, and fat. At ~60 h after the $^3$H-UCB-injection, bile was collected for 30 min, followed by vena cava inferior puncture as described above. The intestine was then removed and divided into 5 segments (three equal parts of small intestine, the cecum, and the remaining colon) that were flushed with phosphate buffered saline (pH 7.4) for analysis of UCB and urobilinoids.

**Plasma analysis**

Blood samples were protected from light and processed immediately. Bilirubin, AST, and ALT levels were determined by routine clinico-chemical spectrophotometry on a P800 unit of a modular analytics serum work area from Roche Diagnostics Ltd. (Basel, Switzerland). Hemoglobin, hematocrit, and reticulocyte counts were determined on a Sysmex-XE-2100 hematology analyzer (Goffin Meyvis, Etten-Leur, The Netherlands). UCB levels were confirmed by reversed-phase HPLC after chloroform extraction as described before.[19] $^3$H content was determined by liquid scintillation as described previously.[19]

**Bile analysis**

Bile samples were immediately frozen under argon and processed within 24 h. UCB levels were determined by HPLC after chloroform extraction as described above. Urobilinoid levels were determined as zinc complexes of total urobilinoids on a UV-2401PC spectrophotometer (Shimadzu, Duisburg, Germany).[25] Bile salt concentration was determined using the 3α-hydroxy steroid dehydrogenase method[26] and bile salt composition was measured by capillary gas chromatography after conversion of bile salts to methyl ester-trimethylsilyl derivatives.[27] $^3$H content was determined by liquid scintillation as described previously.[23]

**Analysis of feces and intestinal content**

Feces and intestinal content were immediately frozen under argon, freeze-dried for 24 h, mechanically homogenized, and promptly analyzed for UCB and urobilinoid levels as described previously.[19,25] Bile acid concentration and composition were determined as described before.[26,27] Fatty acid levels in feces
were determined by gas chromatography on a HP-Ultra-1 column from Hewlett-Packard (Palo-Alto, CA) after extraction, hydrolysis, and methylation of aliquots of feces.[19,28] Fecal calcium levels were determined in duplicate 2.5 g aliquots of dried feces. HCl (2.2 M; 25 ml) was added and the mixture was refluxed for 10 minutes at 200°C. After cooling, ammonia (10% w/v) was added to adjust the pH to 4 and the mixture was filtered through grade-1 filter paper from Whatman Ltd. (Kent, England). The filtrate was analyzed spectrophotometrically on a P800 unit of a modular analytics serum work area from Roche Diagnostics Ltd. (Basel, Switzerland). Fecal phosphate levels were determined in duplicate 1.0 g aliquots of dried feces. H₂SO₄ (96% w/v; 7.5 ml) was added and the mixture was heated at 200°C for 10 minutes and subsequently at 330°C for 60 minutes. After cooling, 5 ml of hydrogen peroxide (33% w/w) was added and the mixture was heated at 330°C for 15 minutes and filtered through grade-1 filter paper (Whatman). The filtrate was analyzed as described above for calcium. ³H content was determined by liquid scintillation as described before.[23]

**Calculation of fluxes based on steady-state ³H-UCB kinetics**

The natural logarithm of plasma ³H-UCB specific activity (dpm/µmol) was plotted against time and the best-fit linear regression curves were calculated. Fractional turnover of ³H-UCB (%/h) was obtained from the slope of the regression line and bilirubin pool size was calculated by dividing the specific activity at T₀ h (Y-axis intercept) by the administered dose (dpm) of ³H-UCB. Total turnover was calculated as the product of ³H-UCB fractional turnover and pool size.[29] Fractional biliary and fractional net transmucosal fluxes of UCB and of UCB derivatives were calculated as described previously.[23]

**Statistical analysis**

Normally distributed data that displayed homogeneity of variance (by calculation of Levene's statistic), were expressed as mean ± SD, and parametric statistical analysis was used. Analysis of variance (ANOVA) with post-hoc Bonferroni correction was performed for comparisons between groups, and Student t test for comparison of paired data within groups. If the data were not normally distributed, non-parametric Kruskal-Wallis and Mann Whitney U tests (with corrected p-values) were performed for comparison between groups and the data were expressed as median and range. The level of significance was set at p<0.05. Analyses were performed using SPPS 14.0 for Windows (SPSS Inc., Chicago, IL).
3.4 Results

3.4.1 Effects of UDCA and CA treatment: dose dependency

Figure 1A shows the effect of increasing dosages of UDCA or CA on plasma bilirubin levels in Gunn rats. The lowest used UDCA or CA dosage of 0.05 wt% (by chow weight) resulted in a mean daily bile salt intake of 32±2 mg/kg bodyweight (BW). This dosage already effectively decreased plasma bilirubin concentrations (-17% and -25%, respectively; each p<0.05). At the highest dose used (1.5 wt%), UDCA and CA decreased plasma bilirubin concentrations by 42% and 50%, respectively (each p<0.001). The rats in both groups did not differ significantly in body weight before the experiments and none of the doses of UDCA or CA affected growth rate (data not shown). We selected a dose of 0.5 wt%, corresponding to a daily bile salt intake of 317±23 mg/kg BW, for further studies to have a substantial effect yet minimize possible bile salt toxicity.

3.4.2 Effects of short-term administration of UDCA and CA

Rapid decrease in plasma bilirubin concentrations

Figure 1B shows that 8 days of dietary UDCA or CA administration (each 0.5 wt%) decreased plasma UCB concentrations in Gunn rats by 21% and by 30%, respectively, compared with controls (each p<0.01). Administration of UDCA or CA induced a statistically significant hypobilirubinemic effect within 3 and 5 days, respectively. Mean body weight and growth rate did not differ significantly among rats in the control, UDCA, or CA group either before or during the 8-day experimental period (data not shown).

Rapid increase in fecal and biliary bile salt excretion

Figure 2A shows that dietary UDCA or CA increased fecal bile salt excretion in the first four days of administration (+663% and +466%, respectively; each p<0.001) as compared with the 4-day pre-treatment period. UDCA administration increased fecal excretion of UDCA, lithocholate, and muricholates, while CA administration increased fecal excretion of CA and deoxycholate (Fig. 2B). Figure 2C shows that the increase in fecal bile salt excretion was accompanied by an increase in biliary bile salt secretion (UDCA
Figure 1. UDCA or CA administration decreases plasma UCB concentration in Gunn rats. Gunn rats (n=6 per group) were fed the control diet for 6 weeks, followed by: dietary UDCA or CA supplementation in doses that were increased every 2 weeks for 10 weeks (panel A); dietary UDCA or CA supplementation (0.5 wt%, each) or no supplementation for 8 days (panel B); or dietary UDCA or CA supplementation (0.5 wt%, each) or no supplementation for 6 weeks (panel C). Plasma UCB values at T0 (µmol/l) in panel B: controls, 241 ± 22; UDCA, 260 ± 25; CA, 251 ± 26 (NS). Data represent mean ± SD. *p<0.05; **p<0.01; ***p<0.001, compared with plasma bilirubin at T0 (panel A), or compared with controls (panels B-C). The used dosages of 0.05 wt%; 0.1 wt%; 0.5 wt%; 1.0 wt%; and 1.5 wt%, corresponded with a dietary bile salt intake (mg/24h/kg BW) of respectively 32 ± 2; 62 ± 5; 317 ± 23; and 633 ± 50.
+127%, CA +128%; each p<0.01), measured after 8 days of treatment. Changes in fecal bile salt composition reflected changes in biliary bile salt composition (Fig. 2D).

Rapid increases in fecal, but not biliary, urobilinoid, and UCB excretion

If bile salt administration enhances bilirubin disposal, the fecal excretion of UCB and urobilinoids, a family of intestinally formed bacterial breakdown products of UCB, would be expected to increase upon starting treatment. Figure 3A & B show that UDCA and CA indeed increased the fecal excretion of both urobilinoids (+42% and +48%, respectively; each p<0.01) and UCB (+56%; p<0.05; and +25%; p=0.06; respectively) in the first four days of treatment. The combined fecal excretion of urobilinoids and UCB was increased by 52% with UDCA and 32% with CA treatment (Fig. 3C; each p<0.01). In contrast, UDCA and CA did not influence the biliary excretion of urobilinoids or UCB (data not shown), or the combined biliary excretion of urobilinoids+UCB, after 8 days of treatment (Fig. 3D).

3.4.3 Effects of long-term administration of UDCA and CA

Sustained decrease in plasma bilirubin concentrations

Figure 1C shows that 6 weeks of dietary UDCA or CA (each 0.5 wt%) decreased plasma bilirubin concentrations by ~40% in Gunn rats from week 2 onwards, compared with stable values in controls (each p<0.001). Mean body weight and growth rate did not differ significantly among rats in the control, UDCA, or CA group either before or during the 6-week period (data not shown).

Changes in biliary and fecal excretion of bile salts, urobilinoids, and UCB

Table 1 shows that, mimicking the short-term experiment, 6-weeks of UDCA or CA administration increased fecal bile salt (+566% and +652%, respectively; each p<0.001) and fecal urobilinoid excretion (+98% and +103%, respectively; p<0.01, each) compared with controls. UDCA, but not CA, increased the fecal excretion of UCB (+256%; p<0.001; Table 1). Interestingly, only administration of CA, but not UDCA, increased biliary bile salt secretion after 6 weeks of treatment (+306%; p<0.001; Table 1). Changes in fecal urobilinoid and UCB
Figure 2. Short-term UDCA or CA administration to Gunn rats: increases fecal bile salt excretion (panel A); changes the composition of bile salts excreted via the feces (panel B); increases biliary bile salt excretion (panel C); and changes the composition of bile salts excreted via the bile (panel D). Gunn rats (n=6 per group) were fed the control diet for 6 weeks, followed by dietary UDCA or CA supplementation (0.5 wt%, each), or no supplementation for 8 days. Feces were collected during a 4-day period before (pre-treatment period) and after (treatment period) dietary randomization. At 8 days, bile was collected during 30min. Data represent mean ± SD. *p<0.01; **p<0.001; †p<0.05–p<0.001. [Statistical analysis in feces: 4-day pre-treatment period (area under the curve) vs. 4-day treatment period (area under the curve). Statistical analysis in bile: UDCA or CA vs. controls.] LC, lithocholic acid; M, muricholic acid; DC, deoxycholic acid; C, cholic acid; CDC, chenodeoxycholic acid; HDC, hyodeoxycholic acid; UDC, ursodeoxycholic acid; HC, hyocholic acid.
Figure 3. Short-term UDCA or CA administration to Gunn rats: increases fecal urobilinoid excretion (panel A); increases fecal UCB excretion (panel B); increases fecal urobilinoid + UCB excretion (panel C); and does not affect biliary urobilinoid + UCB excretion (panel D). For experimental setup, please refer to Figure 2. Data represent mean ± SD. †p=0.06; *p<0.05; **p<0.01. [Statistical analysis in feces: 4-day pre-treatment period (area under the curve) vs. 4-day treatment period (area under the curve). Statistical analysis in bile: UDCA or CA vs. controls.]
Table 1. Steady-state excretion of several fecal and biliary components and intestinal transit time 6 weeks after dietary randomisation. For experimental setup, please refer to Figure 4. After 5 weeks of treatment intestinal transit time was determined. Data represent mean ± SD. *p<0.01; **p<0.001, compared with controls.

<table>
<thead>
<tr>
<th>Component</th>
<th>controls</th>
<th>UDCA 0.5%</th>
<th>CA 0.5%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Feces</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bile salts (µmol/h/100g BW)</td>
<td>0.3 ± 0.0</td>
<td>2.1 ± 0.1**</td>
<td>2.4 ± 0.4**</td>
</tr>
<tr>
<td>UCB (nmol/h/100g BW)</td>
<td>1.6 ± 0.5</td>
<td>5.8 ± 3.8**</td>
<td>2.0 ± 0.6</td>
</tr>
<tr>
<td>Urobilinoids (nmol/h/100g BW)</td>
<td>1.8 ± 0.2</td>
<td>3.6 ± 0.7*</td>
<td>3.7 ± 0.5*</td>
</tr>
<tr>
<td>UCB + urobilinoids (nmol/h/100g BW)</td>
<td>3.4 ± 0.5</td>
<td>9.4 ± 3.5**</td>
<td>5.7 ± 0.9*</td>
</tr>
<tr>
<td>Fat (µmol/h/100g BW)</td>
<td>1.2 ± 0.5</td>
<td>1.1 ± 0.4</td>
<td>0.3 ± 0.0**</td>
</tr>
<tr>
<td>Calcium (mmol/h/100g BW)</td>
<td>33 ± 5</td>
<td>34 ± 9</td>
<td>33 ± 4</td>
</tr>
<tr>
<td>Phosphate (mmol/h/100g BW)</td>
<td>26 ± 5</td>
<td>24 ± 7</td>
<td>20 ± 4</td>
</tr>
<tr>
<td>Transit time (h)</td>
<td>11 ± 1</td>
<td>11 ± 1</td>
<td>9.0 ± 1**</td>
</tr>
</tbody>
</table>

| **Bile**                   |           |           |          |
| Bile salts (µmol/h/100g BW) | 7.1 ± 1   | 10 ± 4    | 29 ± 5** |
| UCB (nmol/h/100g BW)       | 11 ± 3    | 8.2 ± 3   | 11 ± 2   |
| Urobilinoids (nmol/h/100g BW) | 5.9 ± 4   | 8.7 ± 8   | 5.1 ± 3  |
| UCB + urobilinoids (nmol/h/100g BW) | 17 ± 2   | 17 ± 10   | 17 ± 4  |

excretion were not reflected in the bile, since their biliary excretion was similar among all three groups (Table 1).

No increases in fecal excretion of fatty acids, calcium, or phosphate

An increased fecal excretion of fatty acids, calcium, or phosphate has been associated with decreased plasma UCB concentrations in Gunn rats.[13,19] Table 1 shows that UDCA administration for 6 weeks did not affect fecal fat excretion, whereas CA administration even decreased fecal fat excretion (~75%; p<0.001). Table 1 also shows that UDCA and CA administration did not influence fecal calcium or phosphate excretion.
CA administration decreases intestinal transit time

A decrease in intestinal transit time may decrease plasma UCB concentrations in Gunn rats.[30] CA administration for 6 weeks moderately decreased the intestinal transit time (-18%; p<0.001; Table 1), whereas UDCA administration at the same dosage showed no effect.

3.4.4 $^3$H-Bilirubin turnover studies

Decreased pool size and increased fractional turnover of bilirubin

To obtain more detailed mechanistic insights, we determined steady-state $^3$H-UCB-kinetics in the Gunn rats over 60h, after 6 weeks of treatment with control, UDCA, or CA diet. During the kinetic experiment hematocrit, hemoglobin, reticulocytes, AST, and ALT were unaltered (Table 2). Also, plasma bilirubin levels remained stable and the plasma $^3$H-UCB specific activity declined in a semi-logarithmic manner in all groups (data not shown). These findings are in accordance with the absence of significant hemolysis and with the presence of first-order steady-state conditions. Analysis of the semi-logarithmic specific activity curves showed that UDCA and CA treatment decreased bilirubin pool sizes by 33% (p<0.01) and 32% (p<0.05), respectively, compared with controls (Table 3). As shown in Figure 4, the pool sizes were strongly, positively correlated with plasma UCB concentrations ($y=50*x+40$; $r=0.91$; p<0.001). The fractional turnover of $^3$H-UCB increased by 33% and 25% in UDCA and CA treated animals respectively, when compared with controls (each p<0.05; Table 3). Fractional turnover was negatively correlated with both plasma UCB

<table>
<thead>
<tr>
<th></th>
<th>controls</th>
<th>UDCA 0.5%</th>
<th>CA 0.5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin (mmol/l)</td>
<td>7.2 ± 0.7</td>
<td>7.3 ± 0.6</td>
<td>7.7 ± 0.4</td>
</tr>
<tr>
<td>Hematocrit (v/v)</td>
<td>0.36 ± 0.03</td>
<td>0.37 ± 0.03</td>
<td>0.38 ± 0.03</td>
</tr>
<tr>
<td>Reticulocytes (%)</td>
<td>58 ± 17</td>
<td>49 ± 15</td>
<td>42 ± 8</td>
</tr>
<tr>
<td>AST (UI)</td>
<td>90 ± 23</td>
<td>81 ± 20</td>
<td>75 ± 14</td>
</tr>
<tr>
<td>ALT (UI)</td>
<td>70 ± 22</td>
<td>49 ± 6</td>
<td>50 ± 7</td>
</tr>
</tbody>
</table>

Table 2. Hematological and liver function parameters 6 weeks after dietary randomisation. For experimental setup, please refer to Figure 4. Data represent mean ± SD.
Bile salt treatment for unconjugated hyperbilirubinemia

controls | UDCA 0.5% | CA 0.5%
---|---|---
Plasma bilirubin at T=0 h (µmol/l) | 287 ± 40 | 187 ± 25 *** | 181 ± 14 ***
³H-UCB fractional turnover (%/h) | 1.4 ± 0.2 | 1.9 ± 0.2 ** | 1.8 ± 0.1 *
Bilirubin pool size (µmol/100 g BW) | 4.2 ± 0.9 | 2.8 ± 0.7 * | 2.9 ± 0.0 *
Total bilirubin turnover (nmol/100 g BW) | 59 ± 4 | 52 ± 8 | 51 ± 2
Biliary ³H excretion T₆₀h (10⁴ dpm/h) | 10 ± 1 | 12 ± 4 | 11 ± 3
Fecal ³H excretion T₀₆₀h (10⁴ dpm/h) | 5.0 ± 0.5 | 5.4 ± 0.5 | 5.8 ± 0.4

Table 3. Steady-state ³H-bilirubin kinetics 6 weeks after dietary randomisation. For experimental setup, please refer to Figure 4. Data represent mean ± SD. *p<0.05; **p<0.01; ***p<0.001, compared with controls.

concentrations (y=-177*x+506; r=-0.89; p<0.001), and the calculated bilirubin pool sizes (y=-3.2*x+8.8; r=-0.92; p<0.001). Neither UDCA nor CA administration significantly affected total bilirubin turnover, in accordance with similar UCB production rates in the 3 groups (Table 3).

Increased efficiency of biliary and intestinal transmucosal UCB excretion

In each experimental group, the excretion of UCB into the bile, measured by HPLC, comprised ~20% of its total turnover. Because the quantitative excretion of bilirubin occurs almost exclusively via the feces, this implies that the remaining

Figure 4. Bilirubin pool sizes and plasma UCB concentrations in Gunn rats were positively correlated. Gunn rats (n=6 per group) were fed the control diet for 6 weeks, followed by dietary UDCA or CA supplementation (0.5 wt%, each), or no supplementation for 6 weeks. After 6 weeks of treatment, tracer ³H-UCB was i.v.-administered to determine UCB kinetics. Plasma was collected every 12h over 60h. Specific activity of plasma bilirubin declined semi-logarithmically with time in each group.
∼80% of the UCB disposal occurs via net transmucosal excretion. The treated groups thus excreted similar amounts of UCB via either excretory pathway compared with controls. However, due to smaller bilirubin pool sizes, all fractional fluxes (flux per hour as fraction of the UCB pool size) are increased in the treated groups, compared with controls. Figure 5 shows that UDCA mainly increased the fractional transmucosal UCB flux and the fractional biliary flux of derivatives, whereas CA predominantly increased the fractional biliary flux of UCB.

**UDCA, but not CA, administration increases intestinal UCB and urobilinoid content**

Table 4 shows that, throughout the bowel, UCB and urobilinoid content tended to be higher in the UDCA-treated animals (+143% and +106%, respectively; p<0.01), compared with controls. By contrast, CA administration did not significantly alter total intestinal content of UCB and urobilinoids.

**Figure 5.** Fractional biliary and transmucosal fluxes of UCB and UCB-derivatives in Gunn rats, 6 weeks after randomization. Fractional UCB fluxes are expressed as % and fractional UCB-derivative fluxes are expressed as equivalent% of the bilirubin pool size that is excreted per hour. [a] fractional turnover of UCB; [b] fractional biliary UCB excretion; [c] fractional biliary UCB-derivative excretion; [d] fractional fecal excretion of UCB + UCB-derivatives; [e] estimated net transmucosal flux of UCB from the blood into the intestinal lumen, calculated as [a]-[b]. The magnitude of flux [d] equals that of [a] in a steady-state, assuming that UCB turnover equals the fecal excretion of UCB + UCB-derivatives. EHC = enterohepatic circulation. Ovals in upper left of each panel show bilirubin pool size (µmol/100g BW; mean ± SD). For calculation of fractional fluxes, please refer to Methods.
Table 4. Intestinal UCB and urobilinoid content 6 weeks after dietary randomization. For the experimental setup, please refer to Figure 4. After the 60h-period of the $^3$H-UCB kinetic study animals were terminated. The intestine was then removed and divided into 5 segments (three equal parts of small intestine, the cecum, and the remaining colon) that were flushed with phosphate buffered saline (pH 7.4) for analysis of UCB and urobilinoids. Data represent median and range. *p<0.05; **p<0.01, compared with controls. †p<0.05, compared with UDCA-treated animals. Non-parametric tests were used.
3.5 Discussion

In this study we demonstrate that dietary administration of either UDCA or CA significantly decreases plasma UCB concentrations in Gunn rats. The decrease occurs within 3 days after starting administration, is maximal within 2 weeks and is sustained thereafter.

The conclusion that the administration of UDCA or CA enhances fecal bilirubin disposal is based on two independent analytic moieties, namely biochemical and $^3$H-kinetic measurements. First, the fecal excretion of UCB and urobilinoids increased promptly upon starting bile salt treatment. Previous studies have shown that biochemical measurements of UCB and urobilinoids in the feces only account for 25-50% of the expected fecal bilirubin disposal.[31] To address this and to investigate possible alterations in UCB metabolism by the treatments, we performed a $^3$H-UCB kinetic experiment in a steady-state condition.[23] This experiment demonstrated that either bile salt decreased the bilirubin pool size by one-third, mirroring similar decreases in plasma UCB concentrations. Combined, these methodologies indicate that UDCA or CA enhance UCB disposal from the body and that the hypobilirubinemic effects are not due to redistribution among different body compartments.[32]

During steady-state conditions, bilirubin production equals its excretion and both are synonymous with the total turnover of bilirubin (expressed as nmol/h/100g BW; Table 3). If bile salt treatment has no effect on bilirubin production, the total excretory flux of bilirubin is thus similar in the treated groups and the control group during steady-state. To describe differences in the efficiency of bilirubin disposal, the fractional turnover of bilirubin, i.e. the total turnover as percent of the bilirubin pool size (expressed as %/h; Table 3), is preferentially used. The kinetic study showed that the hypobilirubinemic effect of bile salt treatment was not due to a decrease in UCB production, as reflected by the similar total bilirubin turnover, but rather to an increased efficiency of UCB disposal from the body, as reflected by the increased fractional turnover compared with controls.

Fractional turnover, pool size, and total turnover of $^3$H-UCB of the control animals in this study were reassuringly similar to the values obtained in previous studies of radiolabeled UCB kinetics in Gunn rats.[3,23,32] We calculated steady-state fluxes of UCB and its derivatives using a previously used mathematical model, based on the assumption that the quantitative disposal of bilirubin and its derivatives occurs exclusively via the feces.[23] This assumption seems reasonable since only $\sim$6% of total bilirubin turnover in Gunn rats is disposed of via the urine.[7] Our calculations showed that only $\sim$20% of the total bilirubin turnover in each group was disposed of via the bile and $\sim$80% of the total bilirubin turnover was disposed of via net transmucosal excretion. This result underlines the
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previously described importance of the transmucosal excretory route during unconjugated hyperbilirubinemia.[6,7,23]

Analysis of the fractional excretory fluxes (Fig. 5), or flux as a proportion of the bilirubin pool size, suggests that the principal mechanisms differ by which UDCA and CA lower plasma UCB levels. The hypobilirubinemic effect of UDCA is mainly due to an increased fractional transmucosal excretion of UCB, whereas CA increases the fractional biliary excretion of UCB. Unlike the control and CA group, UDCA-fed animals showed a marked increase in UCB content in the colon, i.e. rather distal from the biliary excretion of UCB into the duodenum. Based on this finding we hypothesize that UDCA mainly exerts its hypobilirubinemic effect via intestinal trapping of UCB. This hypothesis is supported by the increased colonic content and the increased fractional biliary flux of urobilinoids in the UDCA group, reflecting enhanced formation and enterohepatic circulation of these bacterial metabolites, probably due to the increased supply of UCB in the intestinal lumen. The mechanism by which CA treatment lowers plasma bilirubin concentrations in our Gunn rats is less clear. Intravenous taurocholic acid administration enhanced biliary UCB excretion in Gunn rats.[17] We observed no increase in biliary excretion of UCB and urobilinoids after 8 days or 6 weeks of CA treatment, although we did not measure directly after starting bile salt treatment. The steady-state $^3$H-UCB kinetic study and the analysis of intestinal bile pigment content favor greater enhancement by CA of the fractional biliary excretion of UCB, but less effective trapping of UCB in the intestine compared with UDCA. In a binary system, UCB binds less avidly to UDCA than to CA, but in the presence of lecithin, UDCA favors the formation of vesicles over micelles, which enhances solubilization of cholesterol and might conceivably do so for UCB.[33]

We acknowledge that other mechanisms may also contribute to the increase in UCB disposal during bile salt administration. The intestinal transit time was decreased by 18% in the 0.5 wt% CA-treated group, which would decrease contact time for mucosal reabsorption.[30] Increased amounts of fat and amorphous calcium phosphate could lower plasma bilirubin via intestinal trapping.[13,19] However, their fecal excretion was not increased during bile salt treatment. Also, UDCA treatment could affect the expression of relevant transporters.[34]

Among all experimental groups, we observed a strong, positive, linear correlation between bilirubin pool size and plasma UCB concentrations, similar to our findings when treating Gunn rats with orlistat and phototherapy.[23] During treatment of Gunn rats with phototherapy, orlistat, or bile salts, changes in UCB pool sizes are thus well predicted by changes in plasma UCB concentrations. However, $^3$H-UCB kinetic studies, using our mathematical model (Fig. 5), remain
necessary to estimate the biliary and transmucosal UCB fluxes, and their relationship to UCB pool size.

Mendez-Sanchez et al.[35] showed that in non-jaundiced mice and rats dietary UDCA supplementation increased the enterohepatic UCB circulation. This opposite finding to our study, in which UDCA decreased net UCB intestinal reabsorption, might result from strain-induced differences in bilirubin metabolism. Homozygous Gunn rats cannot conjugate bilirubin and the accumulated UCB diffuses from the plasma into the intestinal lumen.[6,8] This diffusion is inversely directed in non-jaundiced rodents, as used in the study of Mendez-Sanchez, due to lower plasma UCB levels.[8,9] UDCA-induced changes in microfloral hydrolysis of bilirubin conjugates could further enhance this diffusion. The observed differences between rat strains illustrate that therapeutic application of UDCA in patients with an etiology that is incompatible to our animal model, such as chronic liver disease, should be approached with caution.

The rapid and persistent hypobilirubinemic effect of bile salt administration, however, does support the potential clinical applicability of oral UDCA therapy for unconjugated hyperbilirubinemia in humans. The major therapeutic effect was already present in a low dosage (0.05 wt%), corresponding to a clinically applicable dose of approximately 30 mg/kg/day. Bile salt therapy decreased plasma UCB concentrations in Gunn rats as effectively as did phototherapy in our previous experiments.[15,23] All treated Gunn rats ingested comparable amounts of either UDCA or CA, and treatment with neither bile salt induced diarrhea or impaired growth rates. This corresponds with the fact that UDCA treatment is well-established and well-tolerated in pediatric patients.[36]

In conclusion, dietary administration with UDCA or CA induces a rapid and sustained decrease in plasma UCB concentrations in Gunn rats. The mechanism involves stimulation of UCB turnover and its fecal disposal. Present results support the feasibility of oral bile salt treatment in patients with unconjugated hyperbilirubinemia.

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