Chapter 4

Acceleration of the Gastrointestinal Transit by Polyethylene Glycol Effectively Treats Unconjugated Hyperberbilirubinemia in Gunn Rats

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4.1 Abstract

Several conditions that delay the gastrointestinal transit are associated with unconjugated hyperbilirubinemia. We hypothesized that the gastrointestinal transit time is directly related to plasma unconjugated bilirubin (UCB) concentrations, and that this relationship can be used to develop a new therapeutic strategy for severe unconjugated hyperbilirubinemia in the Gunn rat model. Gunn rats received, for various time periods, oral polyethylene glycol (PEG) with or without conventional phototherapy treatment to accelerate, or oral loperamide to delay the gastrointestinal transit. Gastrointestinal transit time and UCB concentrations in plasma, feces, intestinal content, and bile were determined. Within 36 hours, PEG administration accelerated the gastrointestinal transit by 45% and simultaneously decreased plasma UCB concentrations by 23% (each $p<0.001$). The decrease in plasma UCB coincided with an increased small intestinal UCB content (+340%, $p<0.05$) and an increased fecal UCB excretion (+153%, $p<0.05$). After two weeks, PEG decreased plasma UCB by 41% as single treatment, and by 62% if combined with phototherapy (each $p<0.001$). Loperamide delayed gastrointestinal transit by 57% and increased plasma UCB by 30% (each $p<0.001$). Dose-response experiments showed a strong, linear relation between the gastrointestinal transit time and plasma UCB concentrations ($r=0.87$, $p<0.001$). This chapter demonstrates that gastrointestinal transit time and plasma UCB concentrations are linearly related in Gunn rats. This relationship can be exploited by pharmacologically accelerating the gastrointestinal transit, which increases transmucosal UCB diffusion and thereby effectively treats unconjugated hyperbilirubinemia. Present results support the feasibility of PEG treatment, either solitary or combined with phototherapy, in patients with severe unconjugated hyperbilirubinemia.

4.2 Introduction

Patients with Crigler-Najjar disease suffer from a genetically absent (type I) or decreased (type II) capacity to conjugate bilirubin in the liver, resulting in life-long, unconjugated hyperbilirubinemia.[1] Severe unconjugated hyperbilirubinemia can lead to disposition of unconjugated bilirubin (UCB) in the central nervous system, inducing bilirubin-induced neurological damage (BIND), kernicterus, and death.[2] Phototherapy, the conventional treatment for unconjugated hyperbilirubinemia, converts the hydrophobic UCB into polar photo-isomers that can be excreted without conjugation into the bile.[3] Phototherapy, however, does not always lower UCB below toxic levels. Also, long-term phototherapy, as required by Crigler-Najjar patients, has a profound effect on social life and becomes less effective with age.[4,5] In spite of an intensive phototherapy regimen, which may take up more than 12h per day, many Crigler-Najjar patients will eventually develop irreversible brain damage.[4]

The disadvantages of phototherapy have prompted investigation into alternative treatment strategies for unconjugated hyperbilirubinemia. One of these strategies involves stimulation of transmucosal UCB diffusion from the blood into the intestinal lumen.[6-10] In hyperbilirubinemic Gunn rats, the well-established animal model for Crigler-Najjar disease type I, the majority of UCB enters the intestinal lumen via this pathway, rather than via the bile.[10] Transmucosal UCB diffusion thus seems to be a major excretory route for UCB in hyperbilirubinemic conditions. However, the efficiency of transmucosal diffusion with respect to UCB excretion is decreased by reabsorption of UCB from the intestinal lumen.[7,8] Trapping UCB in the lumen can prevent this reabsorption and lowers plasma UCB concentrations in Gunn rats and hyperbilirubinemic patients.[11-16] However, the trapping agents tested so far, including agar,[11] cholestyramine,[12] charcoal,[13] amorphous calcium phosphate,[14] zinc salts,[16] and orlistat[17] have been clinically unsatisfactory due to side-effects and inconsistent results.

We aimed to develop an alternative oral treatment for unconjugated hyperbilirubinemia that enhances transmucosal diffusion, and the subsequent fecal excretion of UCB. Theoretically, this could be achieved by accelerating the gastrointestinal transit, which is expected to decrease the intraluminal UCB concentration. Indeed, several observations in Gunn rats and in human neonates suggest that the gastrointestinal transit is an important regulator for plasma UCB concentrations (Table 1). In Gunn rats, fasting delayed the gastrointestinal transit and simultaneously increased plasma UCB concentrations.[18] In neonates, conditions that delay the gastrointestinal transit, such as fasting,[19,20] pyloric stenosis,[21] and Hirschprung's disease,[22] were associated with increased

plasma bilirubin concentrations. Only recently Bisceglia *et al.* showed that feeding infant formula supplemented with prebiotic galactosaccharides and oligosaccharides, a mixture that increases the daily stool frequency and accelerates the gastrointestinal transit,[23] decreased plasma UCB concentrations in neonates.[24] Because these clinical conditions, as well as prebiotic treatment, affect many physiological processes other than the gastrointestinal transit, it has remained unclear whether the observed changes in plasma UCB concentrations were directly related to the gastrointestinal transit time. If the gastrointestinal transit time would indeed directly influence plasma UCB concentrations, we could exploit this relationship to develop new therapeutic strategies for severe unconjugated hyperbilirubinemia.

We presently show that acceleration of the gastrointestinal transit by the laxative polyethylene glycol (PEG) decreases plasma UCB concentrations in hyperbilirubinemic Gunn rats. We demonstrate a strong, positive correlation between the gastrointestinal transit time and plasma UCB concentrations. Additionally, we compared the therapeutic effect of PEG with that of orlistat treatment, and assessed the use of PEG as adjunct treatment to phototherapy, which is the standard treatment for unconjugated hyperbilirubinemia in patients. Current results indicate that pharmacological acceleration of the gastrointestinal transit time could be a feasible strategy to treat patients with severe unconjugated hyperbilirubinemia.

Delayed gastrointestinal transit and increased plasma UCB	Accelerated gastrointestinal transit and decreased plasma UCB
Fasting unconjugated hyperbilirubinemia [18-20, 30, 31]	Feeding prebiotic oligosaccharides [23, 24]
Pyloric stenosis [21]	Early feeding [31-33]
Hirschprungs' disease [22]	Rectal stimulation [34]

Table 1. Previous studies have indirectly supported a role of the gastrointestinal transit in the regulation of neonatal plasma UCB concentrations. Conditions that are associated with a delayed gastrointestinal transit in neonates, such as fasting, pyloric stenosis, and Hirschprung's disease, have been associated with increased plasma UCB levels. Bisceglia *et al.* showed that feeding infant formula supplemented with prebiotic galactosaccharides and oligosaccharides, a mixture that increases the daily stool frequency and accelerates the gastrointestinal transit, decreased plasma UCB concentrations in neonates. Early feeding and rectal stimulation, both associated with an accelerated meconium passage, are also associated with a decrease in plasma UCB. References are detailed at the end of the chapter (reference list).

4.3 Animals, materials, and methods

4.3.1 Animals

Homozygous adult male Gunn rats (RHA/jj; 268-362 g) from our breeding colony were individually housed, fed *ad libitum* and had free access to water. Food intake, fluid intake and body weight were determined daily during experiments. The Animal Ethics Committee approved all protocols.

4.3.2 Materials

Hope Farms BV (Woerden, The Netherlands) produced the semi-synthetic diet (code 4063.02).[17] Gunn rats were fed this diet during a 6-week run-in period and subsequently during the experimental period.[10,17] PEG 4000 (Colofort®) was obtained from Ipsen Farmaceutica BV (Hoofddorp, The Netherlands). Orlistat (Xenical®) was obtained from Roche Nederland BV (Woerden, The Netherlands). The PEG solution we used (drinking water solution and gavage solution) was obtained by dissolving one sachet (74 g) of PEG 4000 in 900 ml water. Loperamide, bilirubin, and heptadecanoic acid were obtained from Sigma Chemical Co. (St. Louis, MO). Xanthobilirubin-methyl ester was a gift from dr. J. Fevery (Leuven, Belgium). Urobilin was obtained from Frontier Scientific Inc. (Logan, UT). Carmine red dye was obtained from Macro-imPulse Saveur Ltd. (Stadtoldendorf, Germany). Phototherapy (17 μW/cm2/nm), was administered as described previously.[17]

4.3.3 Methods

Short-term treatment

Gunn rats were randomly assigned to receive no treatment (n=6) or PEG (n=6) via drinking water and intragastrical gavage (5.0 ml, every 12h). Heparinized samples of tail vein blood were obtained under isoflurane anesthesia at -12, 0, 12, 24, and 36h for determination of plasma UCB concentrations in the intervention group. Gastrointestinal transit time was determined three days before, and immediately upon starting PEG administration, by measuring the interval between oral gavage and fecal appearance of carmine red marker. Feces were collected before (baseline period) and after the start of PEG treatment for 36h to determine fecal UCB excretion. After 36h bile and intestinal content were collected for analysis of UCB and urobilinoids, as described before.[10] In a separate experiment, Gunn rats (n=7) received orlistat treatment (200 mg/kg

chow) for a period of 36h. Blood samples were obtained at 0 and 36h for determination of plasma UCB.

Long-term treatment

Gunn rats were randomly assigned to receive no treatment (n=6) or PEG (n=6) via drinking water and via intragastrical gavage (2.5ml, every 24h). Heparinized samples of tail vein blood were obtained under isoflurane anesthesia at day 0, 2, 7, and 14 for determination of plasma UCB, sodium (Na), potassium (K), urea (Ur), and creatinine (Creat) concentrations. The gastrointestinal transit time was determined after two weeks of treatment, as described above. Feces were collected during a 3-day period prior to bile canulation to determine fecal UCB, urobilinoids, bile acids, calcium, and fat excretion. After 14 days, bile and intestinal content were collected for analysis of UCB and urobilinoids. In a separate experiment, small intestinal transit was determined by measuring the intestinal progression (as a percentage of the total small intestinal length) of carmine red 15 minutes after its administration in animals that received either no treatment (n=6) or PEG treatment (n=5) during a 2-week period. In two separate experiments, Gunn rats received PEG (as above) with phototherapy (treatment and control group, each n=6) or received orlistat treatment (200 mg/kg chow; treatment group n=7; control group n=11) for 2 weeks. Blood samples and gastrointestinal transit time were determined at identical time points and with identical methods as described above.

Loperamide treatment

Gunn rats were randomly assigned to receive no treatment (n=6) or 7 mg/ml loperamide (n=6) via intragastrical gavage (1 ml, every 24h). Heparinized samples of tail vein blood were obtained under isoflurane anesthesia at day 0, 2, and 7 for determination of plasma UCB concentrations. Gastrointestinal transit time was determined one week after the start of treatment as described above.

Dose-response experiment

Gunn rats were administered PEG via drinking water for 9 days, followed by additional daily PEG administration via intragastrical gavage (2.5 ml and 5 ml, each for 9 days). Heparinized samples of tail vein blood were obtained under isoflurane anesthesia at day 0 and at the end of each 9-day period. The

gastrointestinal transit time was determined simultaneously, as described above. Feces were collected during a 3-day period before starting PEG administration (baseline period), and a 3-day period before termination at 27 days to determine fecal excretion of UCB, urobilinoids, bile acids, calcium, and fat.

Plasma analysis

Blood samples were protected from light and processed immediately. Total UCB, Na, K, Ur, and Creat were determined by routine spectrophotometry on a P800 unit of a modular analytics serum work area from Roche Diagnostics Ltd. (Basel, Switzerland). UCB concentrations were confirmed by reverse-phased high-performance liquid chromatography (HPLC) after chloroform extraction as described before.[17,25,26]

Bile analysis

Bile samples were protected from light, stored at -80°C under argon directly after collection and processed within 24h. UCB concentrations were determined by reverse-phased HPLC after chloroform extraction.[17,25,26] The biliary bile salt concentration was determined with the 3α-hydroxysteroid dehydrogenase method,[27] and bile acid composition was measured by capillary gas chromatography after conversion of bile acids to methyl ester-trimethylsilyl derivatives.[10] Urobilinoid concentrations were determined as zinc complexes of total urobilinoids on a UV-2401PC spectrophotometer (Shimadzu, Duisburg, Germany).[28]

Feces and intestinal content analysis

Feces and intestinal content were immediately frozen (-20 °C) under argon, freeze-dried for 24h, mechanically homogenized, and thereafter promptly analyzed for UCB and urobilinoid concentrations as described above. Fecal bile acid concentration and bile acid composition were determined as described before.[10,27] Fatty acid concentrations in feces were determined by gas chromatography on a HP-Ultra-1 column from Hewlett-Packard (Palo-Alto, CA) after extraction, hydrolysis and methylation.[29] Fecal calcium concentrations were determined as described previously.[10]

Statistical analysis

Normally distributed data (displaying homogeneity of variance) were analyzed with Student t tests, and expressed as mean ± SD, or as individual data points with the mean. Non-normally distributed data were analyzed with Mann Whitney U tests, and expressed as median and range, or as individual data points with the median. The level of significance was set at $p<0.05$. Analyses were performed using SPPS 16.0 for Mac (SPSS Inc., Chicago, IL).

4.4. Results

4.4.1 Short-term treatment

Rapid decrease in plasma UCB concentrations

Figure 1A shows that oral PEG administration in Gunn rats decreased plasma UCB concentrations by 23% after 36h of treatment, compared with baseline values ($p<0.001$). PEG administration simultaneously accelerated the gastrointestinal transit (*i.e.* it decreased gastrointestinal transit time) by 45% within the first day of treatment ($p<0.001$; Figure 1B). The hypobilirubinemic effect of PEG was apparent within 12h of treatment (Figure 1C). We compared the hypobilirubinemic effect of PEG with that of orlistat, a well-known experimental oral treatment for severe unconjugated hyperbilirubinemia. Orlistat decreased plasma bilirubin by only 10% after 36h ($p<0.05$; Fig. 2A), which was significantly less compared with PEG ($p<0.01$).

Rapid increase in fecal UCB excretion

Figure 1D shows that the PEG-induced decrease of plasma UCB concentrations was accompanied by a 153% increase in fecal UCB excretion during the 36h treatment period ($p<0.05$). Fecal urobilinoids, comprising a family of intestinally formed bacterial breakdown products of UCB, were not quantified in this experiment due to spectrophotometric interference by carmine red dye.

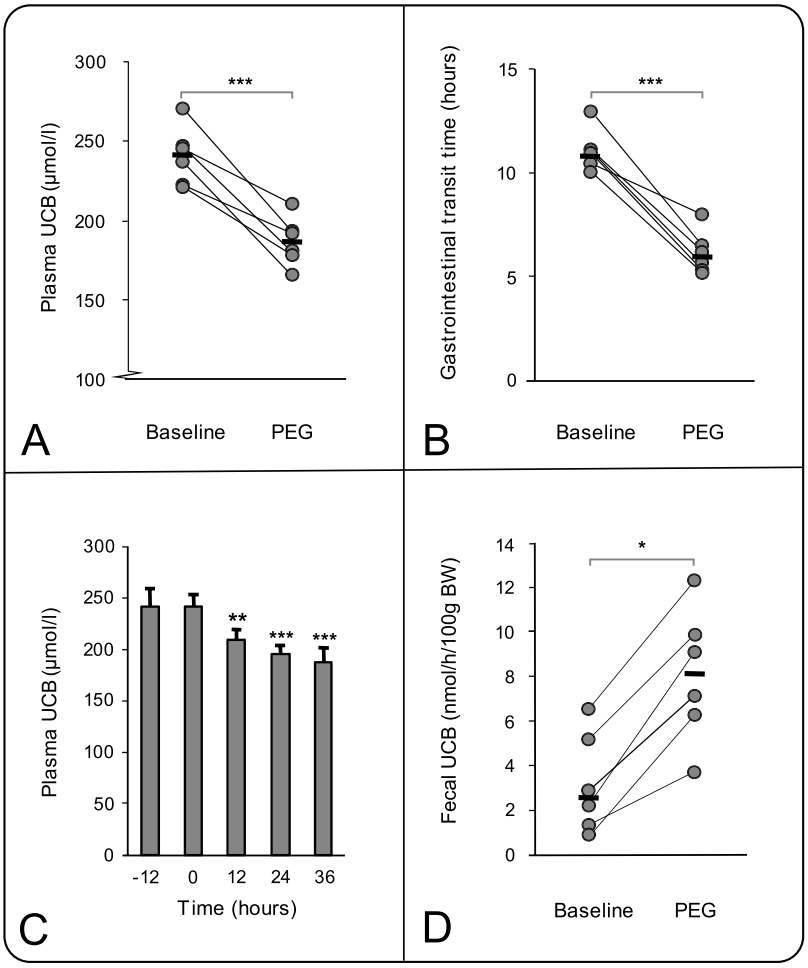

Figure 1. Short-term PEG administration to Gunn rats: decreases plasma UCB concentrations by 23% after 36 hours of treatment (panel A); accelerates gastrointestinal transit within the first day of treatment (panel B); decreases plasma UCB concentrations within 12 hours after the start of treatment (panel C); and increases fecal UCB excretion during the 36 hours of treatment (Panel D). Gunn rats (n=6) were fed the control diet for 6 weeks, followed by PEG administration (via drinking water and via intragastrical gavage) for a total period of 36h. Gastrointestinal transit time was determined three days before, and directly upon starting PEG administration. Feces were collected before (baseline period) and after starting PEG administration (treatment period) for 36h. Data represent: individual data points with mean (panel A and B), mean ± SD (panel C), or individual data points with median (panel D); *p<0.05; **p<0.01; ***p<0.001, compared with baseline values.

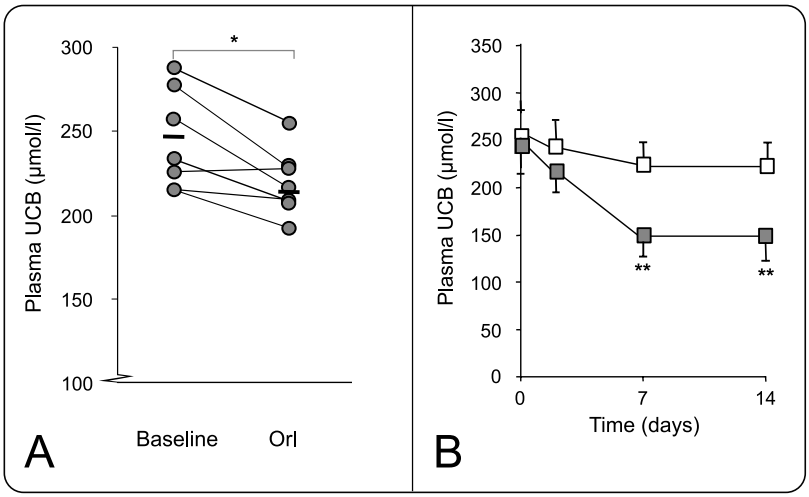

Figure 2. Orlistat administration to Gunn rats decreases plasma UCB concentrations by 10% after 36 hours of treatment (panel A), and by 33% after 2 weeks of treatment (panel B, separate experiments). In the short-term experiment Gunn rats were fed the control diet for 6 weeks followed by the same diet supplemented with orlistat (200 mg/kg chow; n=7) for a period of 36 hours. In the long-term experiment Gunn rats were fed the control diet for 6 weeks followed by the control diet (n=11) or by the control diet supplemented with orlistat (200 mg/kg chow; n=7) for a period of 2 weeks. *p<0.05, compared with baseline; **p<0.001. Data represent mean ± SD

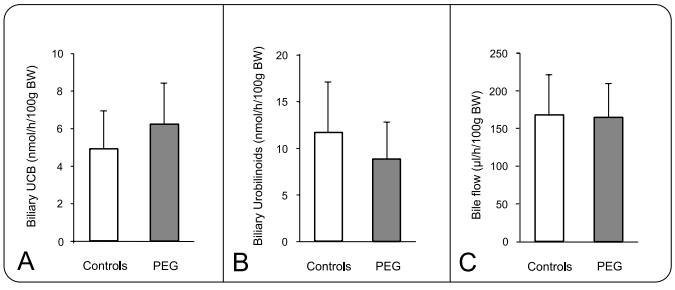

Figure 3. Short-term PEG administration to Gunn rats does not affect: biliary UCB excretion (panel A); biliary urobilinoid excretion (panel B); and bile flow (panel C). Gunn rats (n= 6 per group) were fed control diet for 6 weeks, followed by: control diet (controls) or control diet with PEG administration via drinking water and via intragastrical gavage (5.0 ml, every 12h) for a total period of 36h. At 36h, bile was collected for 30 minutes. Data represent mean ± SD.

Increase in the small intestinal UCB content, but no effect on biliary UCB excretion

Table 2 shows that 36h of PEG administration increased the UCB content in the medial and distal small intestine, compared with controls (+708% and +205%, respectively; each $p<0.05$). In the remainder of the bowel, the PEG-induced increase in intestinal UCB content did not reach statistical significance. PEG administration did not significantly influence the urobilinoid content in the intestinal segments (Table 2).

UCB enters the intestinal lumen via transmucosal diffusion or via biliary excretion. Figure 3 shows that the biliary excretion of UCB was not increased after 36h of PEG treatment. Also, PEG administration did not influence biliary urobilinoid excretion or bile flow.

	controls	PEG
Small intestine (proximal)		
UCB (nmol)	1 (0-3)	1 (1-12)
Urobilinoids (nmol)	0 (0-0)	0 (0-1)
Small intestine (medial)		
UCB (nmol)	6 (4-9)	46 (8-113)**
Urobilinoids (nmol)	10 (4-12)	3 (2-4)*
Small intestine (distal)		
UCB (nmol)	19 (10-37)	59 (27-250)*
Urobilinoids (nmol)	24 (19-34)	31 (11-77)
Cecum		
UCB (nmol)	15 (9-71)	41 (5-76)
Urobilinoids (nmol)	126 (40-463)	305 (106-551)
Large intestine		
UCB (nmol)	24 (23-73)	76 (15-278)
Urobilinoids (nmol)	159 (38-1018)	99 (84-194)
Total intestine		
UCB (nmol)	69 (49-184)	276 (63-719)
Urobilinoids (nmol)	352 (175-1102)	451 (227-703)

Table 2. Intestinal content composition after 36 hours of PEG administration. Gunn rats (n= 6 per group) were fed the control diet for 6 weeks, followed by: control diet (controls), or control diet with PEG administration (via drinking water and via intragastrical gavage) for a total period of 36h. At 36h, the intestine was 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. *$p<0.05$; **$p<0.01$.

4.4.2 Long-term treatment

Sustained decrease in plasma UCB concentrations

Next, we investigated the long-term efficacy of PEG treatment with or without phototherapy. Figure 4A/C shows that 2 weeks of PEG treatment decreased plasma UCB concentrations by 41% and accelerated the gastrointestinal transit by 36% (each $p<0.001$), compared with controls. Additionally, PEG accelerated the small intestinal transit by 17% ($p<0.05$; data not shown). Figure 4B/D shows that 2 weeks of PEG treatment combined with continuous phototherapy decreased plasma UCB concentrations by 62%, and accelerated the gastrointestinal transit by 31% (each $p<0.001$), compared with controls. Combined treatment resulted in an additive therapeutic effect of at least 17% from day 2 onward ($p<0.01$-0.05, for the different time points), compared with single PEG treatment. Orlistat treatment decreased plasma UCB by 33% after 2 weeks ($p<0.001$; Fig. 2B), which was not significantly different compared with single PEG treatment. PEG administration did not affect growth rate or food intake. Renal parameters indicated the absence of dehydration (Table 3). PEG administration increased water intake by 120%, compared with controls ($p<0.001$; Table 3).

	controls	PEG
Diet and growth		
Food intake (g/day)	14 ± 2	14 ± 2
Fluid intake (ml/day)	21 ± 2	47 ± 6**
Body weight (g)	330 ± 34	314 ± 23
Relative growth rate (%)	0%	1%
Renal parameters		
Creat (mmol/l)	14 ± 4	12 ± 3
Urea (mmol/l)	8.1 ± 1.7	6.1 ± 0.6*
Na (mmol/l)	143 ± 1	143 ± 1
K (mmol/l)	6.0± 0.6	6.2 ± 0.6

Table 3. Diet, growth, and renal parameters after 2 weeks of PEG administration. For experimental details, please see the Materials and Methods section and Figure 4. Body weight, food intake, and water intake were determined daily. Plasma sodium, potassium, urea and creatinine concentrations were determined after 2 weeks of PEG treatment. Data represent mean ± SD. *$p<0.05$; **$p<0.001$.

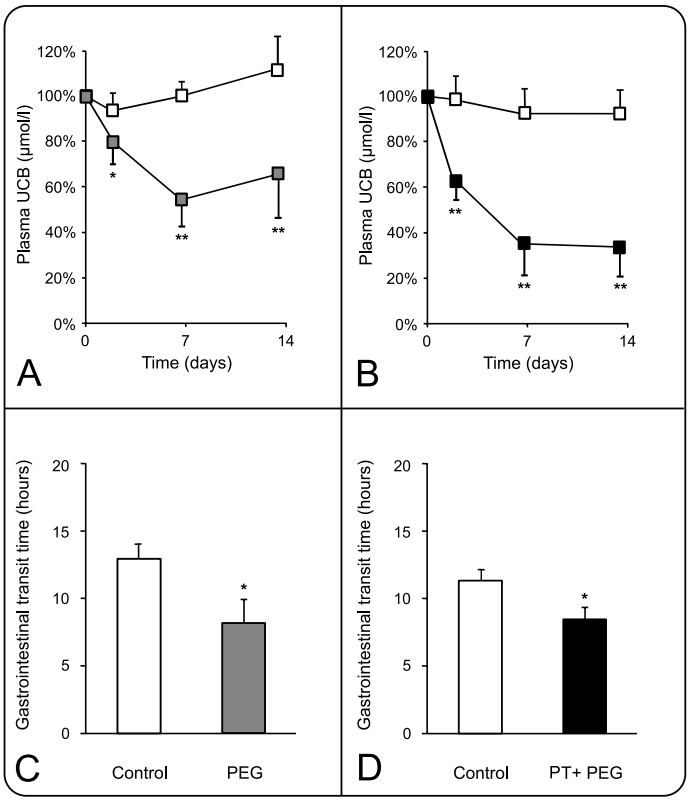

Figure 4. Long-term PEG administration to Gunn rats: decreases plasma UCB concentrations by 41% (panel A); and accelerates gastrointestinal transit (panel C) after two weeks of treatment. Long-term PEG administration combined with continuous phototherapy (PT) decreases plasma UCB concentrations by 62% (panel B) and decreases gastrointestinal transit time (panel D) after two weeks of treatment. Gunn rats (n=6 per group) were fed the control diet for 6 weeks, followed by: control diet (controls), control diet with PEG administration (via drinking water and via intragastrical gavage), or control diet with PEG administration combined with continuous phototherapy (17 µW/cm2/nm) for a total period of 2 weeks. Data represent mean ± SD. *p<0.05; **p<0.001, compared with controls.

No effect on fecal UCB and urobilinoid excretion

Long-term hypobilirubinemic treatment, for example with phototherapy or intestinal capture agents, will eventually result in a new steady-state situation in which the transiently increased fecal UCB disposal has returned to baseline values. The UCB turnover, however, will be increased in these treated animals because the UCB pool has been diminished (Figure 5).[10,15] Table 4 shows that after two weeks of treatment the fecal UCB and urobilinoid excretion was indeed similar in controls and PEG-treated animals.

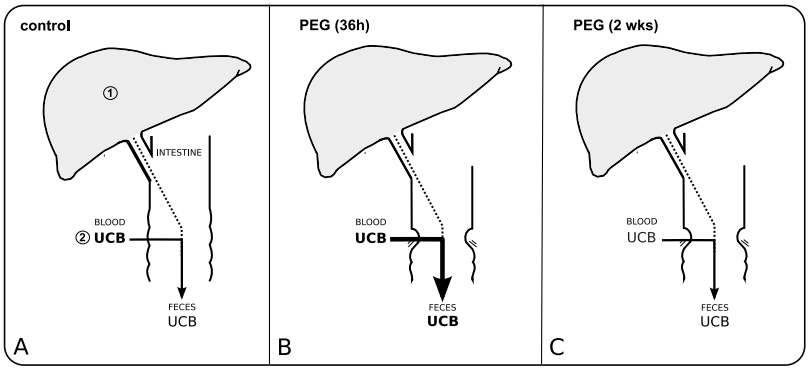

Figure 5. Proposed mechanism: According to previous ^{3}H-UCB kinetic studies, ~95% of the total UCB disposal occurs via the feces in untreated Gunn rats [10, 37]. Approximately 20% of this amount enters the intestinal lumen via the bile (1), whereas ~80% enters the lumen via transmucosal diffusion from the plasma (2) (panel A) [10, 37]. Upon starting PEG administration, the intestinal UCB concentration decreases. This results in a translocation of UCB from the plasma into the intestinal lumen (via transmucosal diffusion), from where it is excreted with the feces. Consequently the intestinal UCB content and the fecal UCB excretion will transiently increase upon starting PEG administration (panel B). However, the plasma UCB concentration decreases during treatment, which results in a simultaneous decrease in transmucosal UCB diffusion and, consequently, in fecal UCB excretion. As soon as the transmucosal diffusion and the fecal excretion of UCB have reached pre-treatment values, the plasma UCB concentrations will not decrease any further (i.e. remain stable) and a new steady-state situation is reached. The new steady-state is characterized by a stable decrease in plasma UCB concentrations and a relative increase in UCB turnover, since the PEG-treated animals have a similar fecal excretion (indicating a similar transmucosal diffusion rate) in the presence of a lower plasma UCB concentration (panel C), compared with untreated animals

	controls	PEG
Feces		
UCB (nmol/h/100g BW)	4.5 ± 1.6	3.1 ± 1.0
Urobilinoids (nmol/h/100g BW)	3.0 ± 1.0	2.5 ± 1.2
Calcium (mmol/h/100g BW)	19 ± 5	15 ± 5
Fat (μmol/h/100g BW)	0.5 ± 0.2	1.0 ± 0.5
Bile acids (nmol/h/100g BW)	224 ± 89	199 ± 70
Bile		
UCB (nmol/h/100g BW)	20 ± 8	15 ± 4
Urobilinoids (nmol/h/100g BW)	16 ± 15	10 ± 4
Bile acids (nmol/h/100g BW)	5066 ± 1331	7290 ± 2441
Bile flow (μl/h/100g BW)	211 ± 29	232 ± 52

Table 4. Excretion of several fecal and biliary components after 2 weeks of PEG administration. Gunn rats (n= 6 per group) were fed the control diet for 6 weeks, followed by: control diet (controls), or control diet with PEG administration (via drinking water and via intragastrical gavage) for a total period of 2 weeks. Feces were collected during a 3 day-period before bile canulation. At 2 weeks bile was collected for 30 minutes. Data represent mean ± SD.

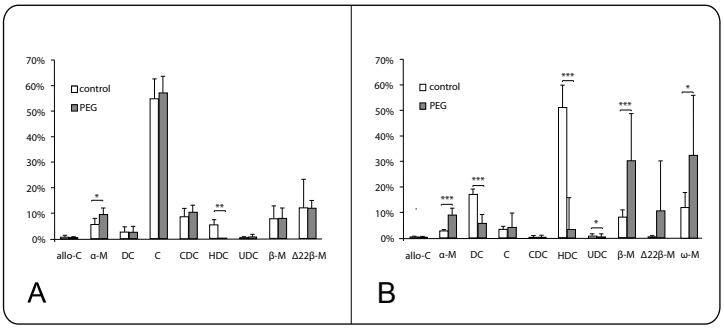

Figure 6. Long-term PEG administration to Gunn rats results in: increased α-M as well as decreased HDC in the bile (panel A), and increased α-M, β-M, ω-M as well as decreased HDC and DC in the feces (panel B). C, cholic acid; M, muricholic acid; DC, deoxycholic acid; CDC, chenodeoxycholic acid; HDC, hyodeoxycholic acid; UDC, ursodeoxycholic acid. For experimental setup, kindly refer to Figure 4 and Table 4 and 5. *p<0.05; **p<0.01, ***p<0.001. Data represent mean ± SD.

	controls	PEG
Small intestine (proximal)		
UCB (nmol)	5 (2 - 9)	3 (1 - 8)
Urobilinoids (nmol)	1 (0 - 4)	1 (0 - 5)
Small intestine (medial)		
UCB (nmol)	14 (6 - 36)	17 (8 - 33)
Urobilinoids (nmol)	8 (0 - 23)	2 (0 - 5)
Small intestine (distal)		
UCB (nmol)	37 (15 - 43)	31 (25 - 56)
Urobilinoids (nmol)	42 (21 - 54)	37 (13 - 50)
Cecum		
UCB (nmol)	53 (26 - 247)	20 (11 - 58)*
Urobilinoids (nmol)	187 (87 - 349)	146 (18 - 416)
Large intestine		
UCB (nmol)	54 (35 - 132)	24 (6 - 43)*
Urobilinoid (nmol)	134 (46 - 344)	186 (114 - 422)
Total intestine		
UCB (nmol)	183 (130 - 330)	118 (67 - 160)*
Urobilinoids (nmol)	411 (193 - 640)	455 (180 - 596)

Table 5. Intestinal content composition after 2 weeks of PEG administration. Gunn rats (n= 6 per group) were fed the control diet for 6 weeks, followed by: control diet (controls), or control diet with PEG administration (via drinking water and via intragastrical gavage) for a total period of 2 weeks. At 2 weeks, the intestine was 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.

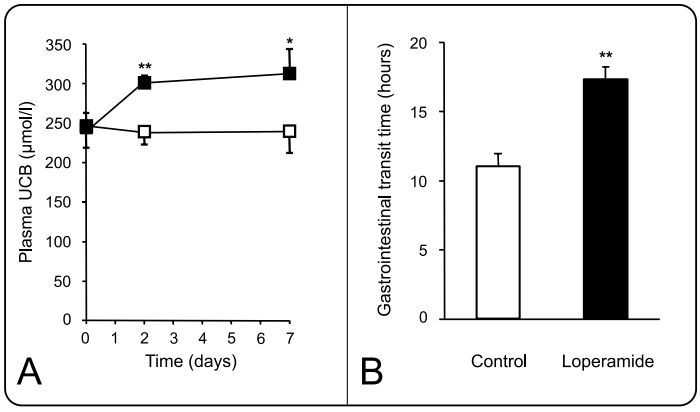

Figure 7. Loperamide administration to Gunn rats: increases plasma UCB concentrations (panel A); and increases the gastrointestinal transit time (panel B). Gunn rats (n=6 per group) were fed the control diet for 6 weeks, followed by: control diet (controls) or control diet with loperamide administration (7 mg/ml) via intragastrical gavage (1 ml, every 24h) for a total period of 1 week. Gastrointestinal transit time was determined three days before and 1 week after the start of loperamide administration in all animals. Data represent mean ± SD. *p<0.01; **p<0.001.

No effect on fecal calcium, fat, and bile acid excretion

An increased fecal excretion of calcium, fatty acids, or bile acids has been associated with decreased plasma UCB concentrations in Gunn rats.[10,15,17] Table 4 shows that two weeks of PEG administration did not influence the fecal excretion of these compounds, nor did it influence biliary bile acid excretion. Figure 6 shows that PEG treatment did decrease the amount of secondary bile salts in the feces and, to a lesser extent, in the bile.

Decrease in the intestinal UCB content, but no effect on biliary UCB excretion

Table 5 shows that PEG administration for two weeks decreased the total intestinal UCB content by 36%, compared with controls ($p<0.05$). PEG administration decreased the UCB content in the cecum and in the large intestine (-63% and -56%, respectively; each $p<0.05$), but did not affect the UCB content in the small intestinal segments. As in the short-term experiment, PEG administration did not affect the amount of urobilinoids in the intestinal segments and did not influence the biliary excretion of UCB, urobilinoids, or the bile flow (Table 4).

4.4.3 Loperamide treatment

Sustained increase in plasma UCB concentrations

To study the effect of a delayed gastrointestinal transit on plasma UCB concentrations, Gunn rats were treated daily for one week with loperamide. Figure 7A shows that loperamide increased plasma UCB concentrations by 30% after one week, compared with controls ($p<0.001$). Loperamide treatment showed a statistically significant effect within 2 days. Loperamide increased the gastrointestinal transit time by 57% after one week ($p<0.001$, Figure 7B), compared with stable values in controls. Mean body weight and water intake did not differ significantly between the loperamide-treated animals and controls. Loperamide administration decreased the food intake by approximately 40%, compared with the controls ($p<0.001$; data not shown).

4.4.4 Dose-response experiment

Strong, positive correlation between plasma UCB concentration and the gastrointestinal transit time

To determine the relationship between the plasma UCB concentrations and the gastrointestinal transit time in more detail, we performed a dose-response experiment. We administered PEG in increasing dosages to Gunn rats in three consecutive 9-day periods. Already at the lowest dose (PEG in drinking water) the plasma UCB concentration was decreased by 22% ($p<0.01$), compared with baseline values. At the highest used dose (PEG in drinking water + daily 5.0 ml gavage), the plasma UCB concentration was decreased by 32% ($p<0.001$), compared with baseline values. Figure 8 shows the strong, positive correlation between gastrointestinal transit time and plasma UCB concentrations in Gunn rats during the dose-response experiment ($y=15*x+80$; $r=0.75$; $p<0.001$). Upon inclusion of the data obtained in the loperamide experiment, this relationship remained essentially unaffected ($y=13*x+91$; $r=0.87$, $p<0.001$). After 27 days of PEG administration, the fecal excretion of UCB, urobilinoids, calcium, or fat, did not increase whereas the fecal excretion of bile acids decreased (-30%; $p<0.01$) compared with baseline values (Table 6). PEG treatment did not affect body weight or growth rate (data not shown).

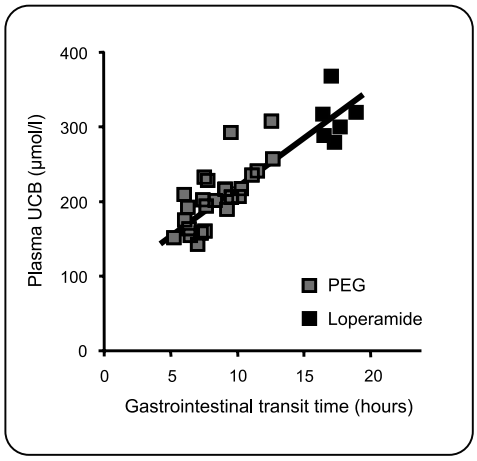

Figure 8. Gastrointestinal transit time and plasma UCB concentrations in Gunn rats were strongly, positively correlated. Gunn rats (n=6) were fed the control diet for 6 weeks, followed by: 9 days of PEG administration via drinking water; 9 days of PEG administration via drinking water and via intragastrical gavage (2.5 ml, every 24h); and 9 days of PEG administration via drinking water and via intragastrical gavage (5.0 ml, every 24h), for a total period of 27 days (three consecutive 9 day-periods). The gastrointestinal transit time was determined at the end of every 9-day period. For experimental setup in the loperamide experiment, please refer to Figure 7. PEG dose-response data points: $y=15*x+80$; $r=0.75$; $p<0.001$ (line not shown). PEG dose-response data points combined with loperamide data points: $y=13*x+91$; $r=0.87$, $p<0.001$.

	baseline	treatment
Feces		
UCB (nmol/h/100g BW)	7.2 ± 6.4	5.4 ± 5.8
Urobilinoids (nmol/h/100g BW)	3.2 ± 0.8	3.3 ± 0.9
Calcium (mmol/h/100g BW)	30 ± 5	26 ± 2
Fat (μmol/h/100g BW)	1.4 ± 0.6	0.9 ± 0.3
Bile salts (nmol/h/100g BW)	233 ± 43	164 ± 20*

Table 6. Excretion of several fecal components after 27 days of administration of increasing amounts of PEG. For experimental details, please see the Materials and Methods section and Figure 8. Feces were collected during a 3 day-period before starting PEG administration (baseline period), and a 3 day-period before termination at 27 days (treatment period). Data represent mean ± SD. *$p<0.01$.

4.5 Discussion

In this study we demonstrate that acceleration of the gastrointestinal transit by PEG administration effectively decreases unconjugated hyperbilirubinemia in Gunn rats. The effect occurred within 12h, was maximal within one week, and was sustained during long-term treatment. Delaying the gastrointesintal transit with loperamide increased plasma UCB levels in Gunn rats, confirming the important role of transit time in bilirubin metabolism.

Our results show that the gastrointestinal transit time and plasma UCB concentrations are linearly related in Gunn rats. This relationship has two important implications. Firstly, it suggests that pharmacological manipulation of the gastrointestinal transit time is a feasible strategy to treat severe unconjugated hyperbilirubinemia. Secondly, it indicates that the gastrointestinal transit time is an important regulator for plasma UCB concentrations under (patho)physiological conditions. Interestingly, our findings corroborate previous observations in animals and humans. Kotal *et al.* showed in Gunn and Wistar rats that fasting-induced hyperbilirubinemia, a well-known condition in humans,[20,30] was associated with a delayed gastrointestinal transit.[18] Feeding fasted Wistar rats non-absorbable bulk (kaolin dissolved in water with magnesium sulphate) normalized the gastrointestinal transit and prevented the fasting-induced increase in plasma UCB.[18] Human conditions that delay the gastrointestinal passage of meconium, such as fasting,[19,20] pyloric stenosis,[21] and Hirschprung's disease,[22] are associated with exaggeration of unconjugated

hyperbilirubinemia. Conditions that *accelerate* the passage of meconium, such as frequent and early feedings,[31-33] or rectal stimulation,[34] seem to lower plasma bilirubin concentrations. Importantly, a recent study showed that supplementing infant formula with prebiotic oligosaccherides, which has been reported to accelerate the gastrointestinal transit,[23] decreased plasma UCB concentrations in neonates.[24] Any specific prebiotic effect on bilirubin metabolism cannot be excluded *a priori* in this study, but is not very likely since oligosaccharides have no clear effect on bilirubin converting bacteria.[24,35] These observations (Table 1) clearly suggest that maneuvers that modify the gastrointestinal transit influence plasma UCB levels in humans. However, these studies do not show a direct influence of the gastrointestinal transit time on plasma UCB concentrations. In the animal study by Kotal, changes in gastrointestinal transit were secondary to fasting, which increases the possibility of unknown confounders.[18] In the human studies, the gastrointestinal transit time was not quantified. Our data are the first to demonstrate a causal relationship between the gastrointestinal transit time and plasma UCB concentrations, using both pharmacological acceleration and pharmacological inhibition.

Theoretically, the hypobilirubinemic effect of PEG could be mediated by mechanisms other than the acceleration of transit time. It has been demonstrated that an increased fecal excretion of calcium,[15] fat,[17] or bile acids,[10] coincides with a decrease in plasma UCB concentrations, presumably because these agents trap UCB in the intestinal lumen. PEG treatment, however, did not increase the fecal excretion of any of these compounds. The decreased amount of secondary biliary and fecal bile acids could indicate an altered activity of microflora in the colon, which might affect the bacterial degradation of UCB to urobilinoids.[36] PEG treatment, however, did not influence the amount of urobilinoids in the intestinal lumen, the feces, or the bile in any of the experiments, which does not support the mechanistic relevance of this possibility. The altered bile salt composition could, theoretically, also affect the transmucosal UCB diffusion from the blood into the intestinal lumen. However, we previously showed in Gunn rats that the increase in transmucosal UCB diffusion during bile acid feeding does not depend on an altered fecal or biliary bile acid profile, but rather on the increased total fecal bile acid excretion, which remained unaffected in this experiment.[10] Finally, and most importantly, we treated Gunn rats with both PEG and loperamide. By doing so, we could demonstrate a strong, linear relationship between transit time and plasma UCB concentrations. This relationship remained stable during pharmacological manipulation of the gastrointestinal transit that was not only bi-directional, but also occurred via two pharmacologically distinct mechanisms. The contribution of fasting appeared limited in loperamide treatment, since the delay in gastrointestinal transit and increased unconjugated hyperbilirubinemia became apparent (at day 2) prior to any decrease in food intake. Taken together, the data suggest that the

gastrointestinal transit time in the individual rats may also underlie their inter-individual variation in basal plasma UCB concentrations. It is tempting to speculate that this could be extrapolated to the human conditions, for example in Gilbert's syndrome or neonatal jaundice.

PEG seemed to decrease plasma UCB levels by enhancing its disposal with the feces, based on the increased fecal UCB excretion in the first 36h of treatment. The enhanced fecal disposal of UCB can only originate from an increase in biliary UCB excretion and/or from an increase in transmucosal UCB diffusion, since UCB exclusively enters the intestinal lumen via these two pathways.[6-10] The increase in intestinal UCB content after 36h of PEG administration, however, was not accompanied by an increase in biliary UCB excretion. This finding strongly suggests that the hypobilirubinemic effect of PEG treatment was due to a selective increase in transmucosal UCB diffusion (Figure 5). Transmucosal UCB diffusion occurs bi-directionally (*e.g.* from blood to gut lumen and *vice-versa*) in the small and large intestine of Gunn rats.[6] Normally, the *net* UCB flux is directed from the blood into the intestinal lumen, thus constituting for ~80% of the total fecal UCB disposal in untreated Gunn rats, as demonstrated by steady-state ^{3}H-UCB kinetic experiments.[10] Its direction is reversed (*i.e.* from the lumen into the blood) in fasting conditions, which delay the intestinal transit and thereby increase the intestinal UCB concentration. This results in a *net* reabsorption of UCB into the enterohepatic circulation, as reflected by the marked hyperbilirubinemia and increased biliary UCB excretion in fasted Gunn and Wistar rats.[18] We hypothesize that PEG treatment decreases the intraluminal UCB concentration, by flushing the intestine, which results in enhanced *net* UCB diffusion into the intestinal lumen. We could not directly validate that PEG decreased the intraluminal UCB concentration, since we needed to rinse the intestine with phosphate buffered saline in order to collect its content. However, the impressive additive therapeutic effect of phototherapy on PEG treatment is in concordance with our hypothesis. The reason for this is that combining two treatments that enhance the same route of disposal (*e.g.* biliary UCB excretion) will reach the maximal disposal rate sooner than combining treatments that maximize two distinct routes of UCB disposal (*e.g.* biliary and transmucosal UCB disposal). Previous studies using ^{3}H-labelled UCB have indeed shown that the therapeutic effect of phototherapy, which exclusively increases biliary excretion, is greatly enhanced by treatments that exclusively increase transmucosal UCB excretion.[37,38] The proposed mechanism by which PEG decreases plasma UCB concentrations has been outlined in Figure 5.

Our results support the clinical applicability of oral PEG treatment in patients with severe unconjugated hyperbilirubinemia. PEG treatment decreased plasma UCB more rapidly than did orlistat treatment, which is a well-known experimental oral treatment strategy for unconjugated hyperbilirubinemia. This

rapid decrease supports the clinical feasibility of PEG treatment in hyperbilirubinemic neonates, in which it could prevent hyperbilirubinemia due to delayed meconium excretion. The sustainability of the PEG-induced decrease in plasma UCB concentrations clearly supports its clinical use in Crigler-Najjar patients. Importantly, the combination of phototherapy with PEG resulted in a therapeutic efficacy that was not only superior to single PEG treatment, but also to treatment combinations that were explored in comparable Gunn rat experiments.[10,12,13,15-17,38] PEG treatment was well-tolerated by all animals, and no diarrhea or dehydration was observed. PEG is presently widely applied for the treatment of constipation and is well-tolerated by both adults and children.[39,40] Numerous clinical trials with PEG have shown an absence of serious side effects, and a milder side effect profile compared with other laxatives.[41]

In conclusion, acceleration of the gastrointestinal transit time by PEG effectively treats unconjugated hyperbilirubinemia in Gunn rats. The underlying mechanism involves stimulation of the transmucosal excretion and the subsequent fecal disposal of UCB. Present results support the feasibility of PEG treatment in hyperbilirubinemic patients. The long-term efficacy (*e.g.* prevention of UCB-induced neurological damage) and safety (*e.g.* absorption of water and nutrients) of PEG treatment will need to be evaluated in future clinical trials.[42]

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