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ABSTRACT

Background and aims Several conditions that delay gastrointestinal transit are associated with unconjugated hyperbilirubinaemia. We hypothesised 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 hyperbilirubinaemia in the Gunn rat model.

Methods Gunn rats received, for various time periods, oral polyethylene glycol (PEG) with or without conventional phototherapy treatment to accelerate, or oral loperamide to delay gastrointestinal transit. Gastrointestinal transit time and UCB concentrations in plasma, faeces, intestinal content and bile were determined.

Results Within 36 h, PEG administration accelerated 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 faecal UCB excretion (+153%, $p<0.05$). After 2 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 ($r=0.87$, $p<0.001$).

Conclusion 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 hyperbilirubinaemia. Present results support the feasibility of PEG treatment, either solitary or combined with phototherapy, in patients with severe unconjugated hyperbilirubinaemia.

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 hyperbilirubinaemia.[1] Severe unconjugated hyperbilirubinaemia 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 hyperbilirubinaemia, 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 patients with Crigler–Najjar disease, 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 to more than 12 h per day, many patients with Crigler–Najjar disease will eventually develop irreversible brain damage.[4]

The disadvantages of phototherapy have prompted investigation into alternative treatment strategies for unconjugated hyperbilirubinaemia. One of these strategies involves stimulation of transmucosal UCB diffusion from the blood into the intestinal lumen.[6–10] In hyperbilirubinaemic 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 hyperbilirubinaemic 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 hyperbilirubinaemic 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 hyperbilirubinaemia that enhances transmucosal diffusion, and the subsequent faecal 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 (supplementary 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 hyperbilirubinaemia.

We presently show that acceleration of the gastrointestinal transit by the laxative polyethylene glycol (PEG) decreases plasma UCB concentrations in hyperbilirubinaemic 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 hyperbilirubinaemia in patients. Current results indicate that pharmacological acceleration of the gastrointestinal transit time could be a feasible strategy to treat patients with severe unconjugated hyperbilirubinaemia.

MATERIALS AND METHODS

Animals

Homozygous male Gunn rats (RHA/jj; 268–362 g) from our breeding colony were housed individually, fed ad libitum and had free access to water. Food intake, fluid intake and body weight were determined daily during experiments.

Materials

Hope Farms (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 (Hoofddorp, The Netherlands). Orlistat (Xenical) was obtained from Roche Nederland (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 (St. Louis, Missouri, USA). Xanthobilirubin methyl ester was a gift from Dr J. Fevery (Leuven, Belgium). Urobilin was obtained from Frontier Scientific (Logan, Utah, USA). Carmine red dye was obtained from Macro-imPulse Saveur (Stadtoldendorf, Germany). Phototherapy (17 $\mu W/cm^2/nm$), was administered as described previously.[17]

Methods

Short-term treatment

Gunn rats were randomly assigned to receive no treatment (n=6) or PEG (n=6) via drinking water and intragastric gavage (5.0 ml, every 12 h). Heparinised samples of tail vein blood were obtained under isoflurane anaesthesia at 12, 0, 12, 24 and 36 h for determination of plasma UCB concentrations in the intervention group. Gastrointestinal transit time was determined 3 days before, and immediately upon starting PEG administration, by measuring the interval between oral gavage and faecal appearance of the carmine red marker. Faeces were collected before (baseline period) and after the start of PEG treatment for 36 h to determine faecal UCB excretion. After 36 h 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 36 h. Blood samples were obtained at 0 and 36 h 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 intragastric gavage (2.5 ml, every 24 h). Heparinised samples of tail vein blood were obtained under isoflurane anaesthesia at day 0, 2, 7 and 14 for determination of plasma UCB, sodium, potassium, urea and creatinine concentrations. The gastrointestinal transit time was determined after 2 weeks of treatment, as described above. Faeces were collected during a 3-day period prior to bile canulation to determine faecal 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 total small intestinal length) of carmine red 15 min 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 intragastric gavage (1 ml, every 24 h). Heparinised samples of tail vein blood were obtained under isoflurane anaesthesia at days 0, 2 and 7 for determination of plasma UCB concentrations. Gastrointestinal transit time was determined 1 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 intragastric gavage (2.5 ml and 5 ml, each for 9 days). Heparinised samples of tail vein blood were obtained under isoflurane anaesthesia at day 0 and at the end of each 9-day period. The gastrointestinal transit time was determined simultaneously, as described above. Faeces 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 faecal excretion of UCB, urobilinoids, bile acids, calcium and fat.

Plasma analysis

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

Bile analysis

Bile samples were protected from light, stored at −80 °C under argon directly after collection and processed within 24 h. 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]

Analysis of faeces and intestinal content

Faeces and intestinal content were immediately frozen under argon, freeze-dried for 24 h, mechanically homogenised, and thereafter promptly analysed for UCB and urobilinoid concentrations as described above. Faecal bile acid concentration and bile acid composition were determined as described before.[10 27] Fatty acid concentrations in faeces were determined by gas chromatography on a HP-Ultra-1 column from Hewlett-Packard (Palo Alto, California, USA) after extraction, hydrolysis and methylation.[29] Faecal calcium concentrations were determined as described previously.[10]

Statistical analysis

Normally distributed data (displaying homogeneity of variance) were analysed with Student t tests, and expressed as mean ± SD, or as individual data points with the mean. Non-normally distributed data were analysed 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.

RESULTS

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 36 h of treatment, compared with baseline values ($p<0.001$). PEG administration simultaneously accelerated the gastrointestinal transit (ie, it decreased gastrointestinal transit time) by 45% within the first day of treatment ($p<0.001$; figure 1B). The hypobilirubinaemic effect of PEG was apparent within 12 h of treatment (figure 1C). We compared the hypobilirubinaemic effect of PEG with that of orlistat, a well-known experimental oral treatment for severe unconjugated hyperbilirubinaemia. Orlistat decreased plasma bilirubin by only 10% after 36 h ($p<0.05$; supplementary figure 1A), which was significantly less compared with PEG ($p<0.01$).

Rapid increase in faecal UCB excretion

Figure 1D shows that the PEG-induced decrease of plasma UCB concentrations was accompanied by a 153% increase in faecal UCB excretion during the 36 h treatment period ($p<0.05$). Faecal 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.

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

Table 1 shows that 36 h 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 1). UCB enters the intestinal lumen via transmucosal diffusion or via biliary excretion. Figure 2 shows that the biliary excretion of UCB was not increased after 36 h of PEG treatment. Also, PEG administration did not influence biliary urobilinoid excretion or bile flow.

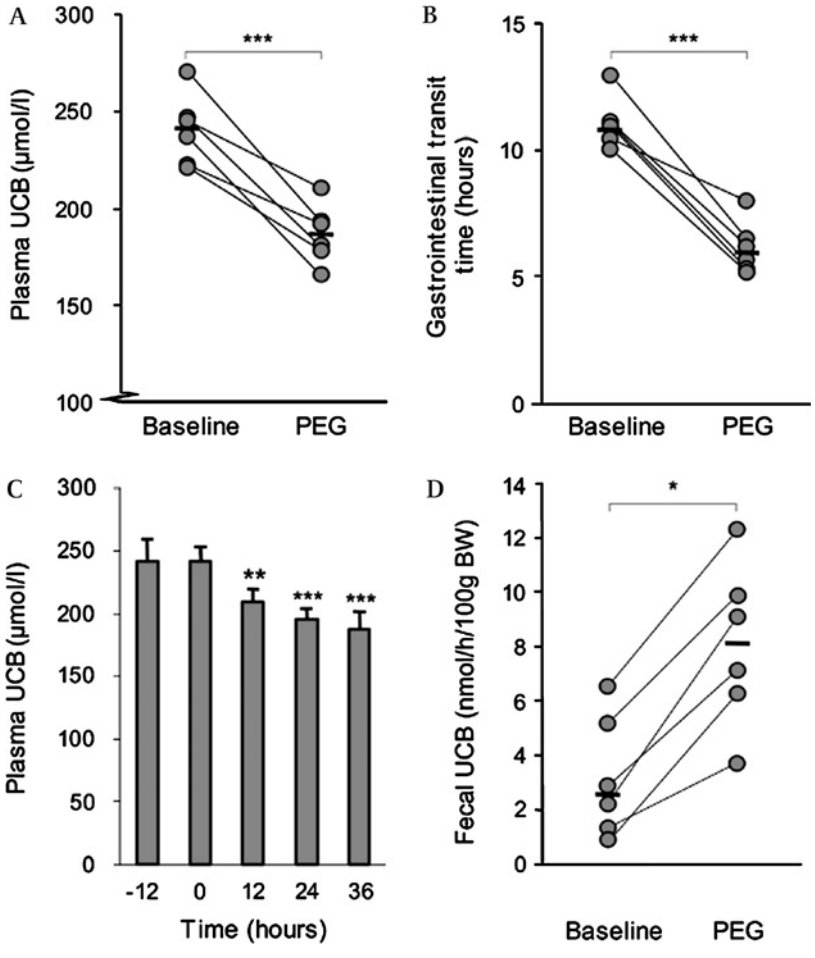

Figure 1 Short-term polyethylene glycol (PEG) administration to Gunn rats decreases plasma unconjugated bilirubin (UCB) concentrations by 23% after 36 h of treatment (A); accelerates gastrointestinal transit within the first day of treatment (B); decreases plasma UCB concentrations within 12 h after the start of treatment (C); and increases faecal UCB excretion during the 36 h of treatment (D). Gunn rats (n=6) were fed the control diet for 6 weeks, followed by PEG administration (via drinking water and via intragastric gavage) for a total period of 36 h. Gastrointestinal transit time was determined 3 days before, and directly upon starting PEG administration. Faeces were collected before (baseline period) and after starting PEG administration (treatment period) for 36 h. Data represent: individual data points with mean (A and B), mean±SD (C), or individual data points with median *(panel D)*; *$p<0.05$; **$p<0.01$; ***$p<0.001$, compared with baseline values.

Long-term treatment

Sustained decrease in plasma UCB concentrations

Next, we investigated the long-term efficacy of PEG treatment with or without phototherapy. Figure 3A–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 3B–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 ($p<0.001$; supplementary figure 1B), 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 (supplementary table 1). PEG administration increased water intake by 120%, compared with controls ($p<0.001$; supplementary table 2).

No effect on faecal UCB and urobilinoid excretion

Long-term hypobilirubinaemic treatment, for example with phototherapy or intestinal capture agents, will eventually result in a new steady-state situation in which the transiently increased faecal 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 4).[10 15] Table 2 shows that after 2 weeks of treatment the faecal UCB and urobilinoid excretion was indeed similar in controls and PEG-treated animals.

An increased faecal excretion of calcium, fatty acids or bile acids has been associated with decreased plasma UCB concentrations in Gunn rats.[10 15 17] Table 2 shows that 2 weeks of PEG administration did not influence the faecal excretion of these compounds, nor did it influence biliary bile acid excretion. Supplementary figure 2 shows that PEG treatment did decrease the amount of secondary bile salts in the faeces and, to a lesser extent, in the bile.

Table 1 Intestinal content composition after 36 h of PEG administration

	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)
Caecum		
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)

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 intragastric gavage) for a total period of 36 h. At 36 h, the intestine was removed and divided into five segments (three equal parts of small intestine, the caecum, 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.
*$p<0.05$; **$p<0.01$.
PEG, polyethylene glycol; UCB, unconjugated bilirubin.

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

Table 3 shows that PEG administration for 2 weeks decreased the total intestinal UCB content by 36%, compared with controls ($p<0.05$). PEG administration decreased the UCB content in the caecum 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 or urobilinoids, or the bile flow (table 2).

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 1 week with loperamide. Figure 5A shows that loperamide increased plasma UCB concentrations by 30% after 1 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 1 week ($p<0.001$, figure 5B), 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).

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 6 shows the strong, positive correlation between gastrointestinal transit time and plasma UCB concentrations in Gunn rats during the dose–response experiment ($y = 15x + 80$; $r=0.75$; $p<0.001$). Upon inclusion of the data obtained in the loperamide experiment, this relationship remained essentially unaffected ($y = 13x + 91$; $r=0.87$, $p<0.001$). After 27 days of PEG administration, the faecal excretion of UCB, urobilinoids, calcium, or fat, did not increase whereas the faecal excretion of bile acids decreased (−30%; $p<0.01$) compared with baseline values (supplementary table 3). PEG treatment did not affect body weight or growth rate (data not shown).

DISCUSSION

In this study we demonstrate that acceleration of the gastrointestinal transit by PEG administration effectively decreases unconjugated hyperbilirubinaemia in Gunn rats. The effect occurred within 12 h, was maximal within 1 week, and was sustained during long-term treatment. Delaying the

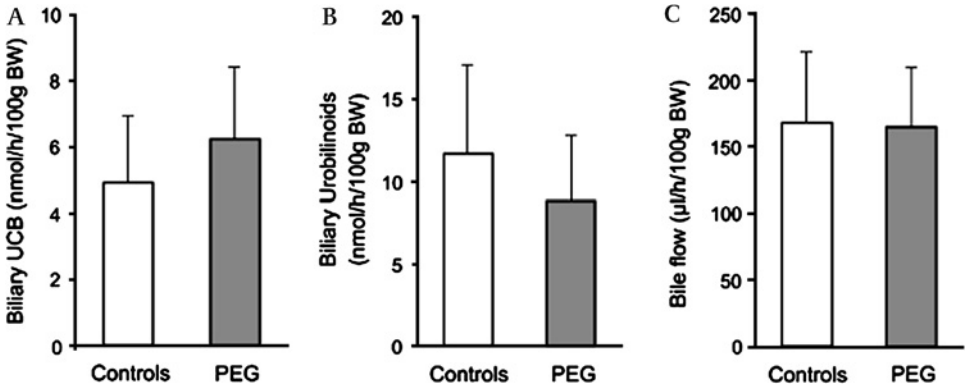

Figure 2 Short-term polyethylene glycol (PEG) administration to Gunn rats does not affect: biliary unconjugated bilirubin (UCB) excretion (A); biliary urobilinoid excretion (B); and bile flow (C). Gunn rats (n=6 per group) were fed control diet for 6 weeks, followed by: control diet (controls) or control diet with PEG administration via drinking water and via intragastric gavage (5.0 ml, every 12 h) for a total period of 36 h. At 36 h, bile was collected for 30 min. Data represent mean±SD.

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. First, it suggests that pharmacological manipulation of the gastrointestinal transit time is a feasible strategy to treat severe unconjugated hyperbilirubinaemia. Second, 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 hyperbilirubinaemia, 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 sulfate) normalised 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 hyperbilirubinaemia. 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 oligosaccharides, 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,

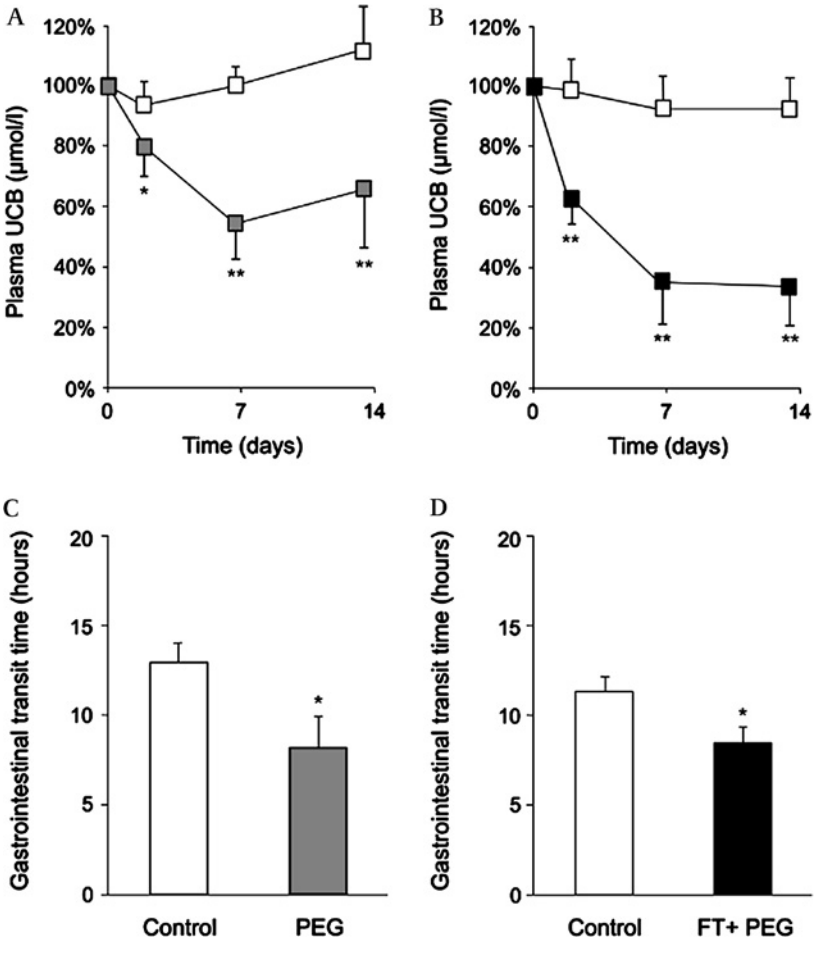

Figure 3 Long-term polyethylene glycol (PEG) administration to Gunn rats decreases plasma unconjugated bilirubin (UCB) concentrations by 41% (A); and accelerates gastrointestinal transit (B) after 2 weeks of treatment. Long-term PEG administration combined with continuous phototherapy decreases plasma UCB concentrations by 62% (C) and decreases gastrointestinal transit time (D) after 2 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 intragastric gavage), or control diet with PEG administration combined with continuous phototherapy (17 µW/cm^2/nm) for a total period of 2 weeks. Data represent mean±SD. *p<0.05; **p<0.001, compared with controls.

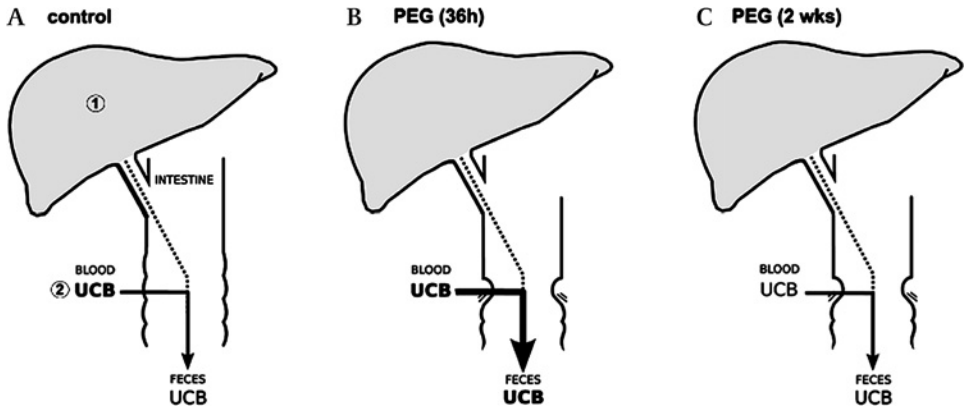

Figure 4 Proposed mechanism: according to previous ^{3}H-unconjugated bilirubin (UCB) kinetic studies, ~95% of the total UCB disposal occurs via the faeces in untreated Gunn rats.[10 37] Approximately 20% of this amount enters the intestinal lumen via the bile (1), whereas ~80% enters the lumen via transmucosal diffusion from the plasma (2) (A).[10 37] Upon starting polyethylene glycol (PEG) administration the intestinal UCB concentration decreases. This results in a translocation of UCB from the plasma to the intestinal lumen (via transmucosal diffusion) from where it is excreted with the faeces. Consequently, the intestinal UCB content and the faecal UCB excretion will transiently increase upon starting PEG administration (B). However, the plasma UCB concentration decreases during treatment, which results in a simultaneous decrease in transmucosal UCB diffusion and, consequently, in faecal UCB excretion. As soon as the transmucosal diffusion and the faecal excretion of UCB have reached pre-treatment values, the plasma UCB concentrations will not decrease any further (ie, remain stable) and a new steady-state situation is reached. The new steady-state is characterised by a stable decrease in plasma UCB concentrations and a relative increase in UCB turnover, since the PEG-treated animals have a similar faecal excretion (indicating a similar transmucosal diffusion rate) in the presence of a lower plasma UCB concentration, compared with untreated animals (C).

but is not very likely since oligosaccharides have no clear effect on bilirubin converting bacteria.[24 35] These observations (supplementary table 1) clearly suggest that manoeuvres 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 hypobilirubinaemic effect of PEG could be mediated by mechanisms other than the acceleration of transit time. It has been demonstrated that an increased faecal 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 faecal excretion of any of these compounds. The decreased amount of secondary biliary and faecal 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 faeces, 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

Table 2 Excretion of several faecal and biliary components after 2 weeks of PEG administration

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

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 intragastric gavage) for a total period of 2 weeks. Faeces were collected during a 3-day period before bile canulation. At 2 weeks bile was collected for 30 min. Data represent mean±SD. No effect on faecal calcium, fat, and bile acid excretion.
PEG, polyethylene glycol; UCB, unconjugated bilirubin.

Table 3 Intestinal content composition after 2 weeks of PEG administration

	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)
Caecum		
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)

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 intragastric gavage) for a total period of 2 weeks. At 2 weeks, the intestine was removed and divided into five segments (three equal parts of small intestine, the caecum, and the remaining colon) which were flushed with phosphate buffered saline (pH 7.4) for analysis of UCB and urobilinoids. Data represent median and range.
*$p<0.05$.
PEG, polyethylene glycol; UCB, unconjugated bilirubin.

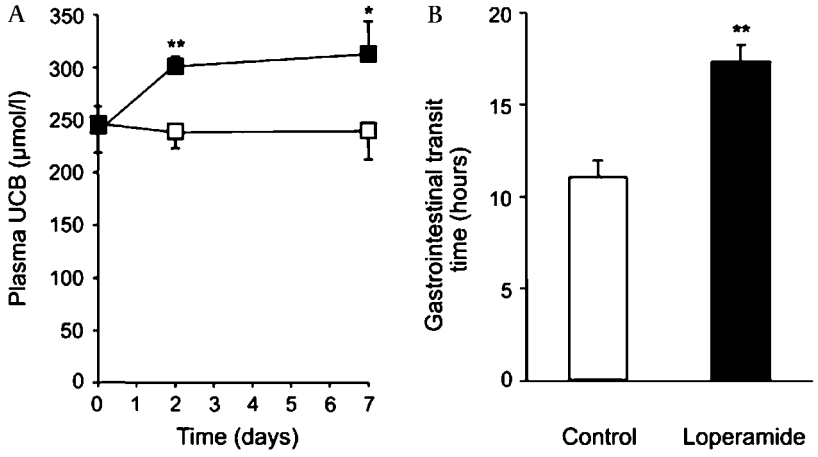

Figure 5 Loperamide administration to Gunn rats increases plasma unconjugated bilirubin (UCB) concentrations (A); and increases the gastrointestinal transit time (B). Gunn rats (n=6 per group) were fed the control diet for 6 weeks, followed by: control diet (controls) or control diet with loperamide administration (7 mg/ml) via intragastric gavage (1 ml, every 24 h) for a total period of 1 week. Gastrointestinal transit time was determined 3 days before and 1 week after the start of loperamide administration in all animals. Data represent mean±SD. *p<0.01; **p<0.001.

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 faecal or biliary bile acid profile, but rather on the increased total faecal 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 hyperbilirubinaemia 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.

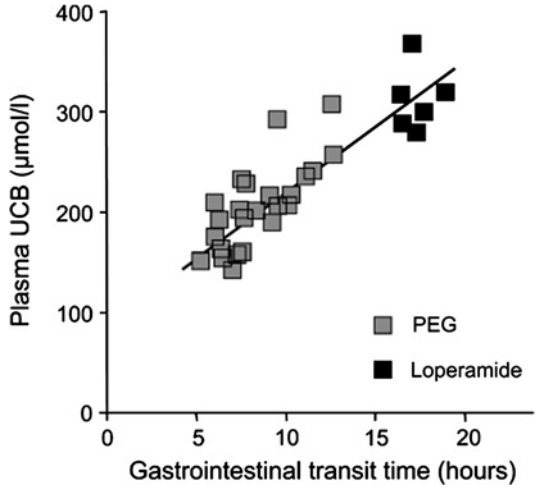

Figure 6 Gastrointestinal transit time and plasma unconjugated bilirubin (UCB) concentrations in Gunn rats were strongly, positively correlated. Gunn rats (n=6) were fed the control diet for 6 weeks, followed by: 9 days of PEG administration via drinking water; 9 days of polyethylene glycol (PEG) administration via drinking water and via intragastric gavage (2.5 ml, every 24 h); and 9 days of PEG administration via drinking water and via intragastric gavage (5.0 ml, every 24 h), 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 set-up in the loperamide experiment, please refer to figure 3. PEG dose–response data points: y=15x+80; r=0.75; p<0.001 (line not shown). PEG dose–response data points combined with loperamide data points: y=13x+91; r=0.87, p<0.001.

PEG seemed to decrease plasma UCB levels by enhancing its disposal with the faeces, based on the increased faecal UCB excretion in the first 36 h of treatment. The enhanced faecal 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.[6–9] The increase in intestinal UCB content after 36 h of PEG administration, however, was not accompanied by an increase in biliary UCB excretion. This finding strongly suggests that the hypobilirubinaemic effect of PEG treatment was due to a selective increase in transmucosal UCB diffusion (figure 4). Transmucosal UCB diffusion occurs bi-directionally (eg, 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 faecal UCB disposal in untreated Gunn rats, as demonstrated by steady-state ^{3}H-UCB kinetic experiments.[10] Its direction is reversed (ie, 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 hyperbilirubinaemia and increased biliary UCB excretion in fasted Gunn and Wistar rats.[18] We hypothesise 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 (eg, biliary UCB excretion) will reach the maximal disposal rate sooner than combining treatments that maximise two distinct routes of UCB disposal (eg, 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.[3 37] The proposed mechanism by which PEG decreases plasma UCB concentrations has been outlined in figure 4.

Our results support the clinical applicability of oral PEG treatment in patients with severe unconjugated hyperbilirubinaemia. PEG treatment decreased plasma UCB more rapidly than did orlistat treatment, which is a well-known experimental oral treatment strategy for unconjugated hyperbilirubinaemia. This rapid decrease supports the clinical feasibility of PEG treatment in hyperbilirubinaemic neonates, in which it could prevent hyperbilirubinaemia due to delayed meconium excretion. The sustainability of the PEG-induced decrease in plasma UCB concentrations clearly supports its clinical use in patients with Crigler–Najjar disease. 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 37] PEG treatment was well tolerated by all animals, and no diarrhoea or dehydration was observed. PEG is presently widely applied for the treatment of constipation and is well tolerated by both adults and children.[38 39] Numerous clinical trials with PEG have shown an absence of serious side effects, and a milder side effect profile compared with other laxatives.[40]

In conclusion, acceleration of the gastrointestinal transit time by PEG effectively treats unconjugated hyperbilirubinaemia in Gunn rats. The underlying mechanism involves stimulation of the transmucosal excretion and the subsequent faecal disposal of UCB. The present results support clinical trials in patients with Crigler–Najjar disease to assess long-term efficacy (eg, prevention of UCB-induced neurological damage) and safety (eg, absorption of water and nutrients) of PEG treatment.[41]

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