Chapter 5

Combined Treatment Strategies for Unconjugated Hyperbilribunemia in Gunn Rats

F.J.C. Cuperus [1]
A.A. Iemhoff [1]
H.J. Verkade [1]

[1] *Pediatric 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.*

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5.1 Abstract

We recently demonstrated that acceleration of the gastrointestinal transit by polyethylene glycol (PEG) treats unconjugated hyperbilirubinemia in jaundiced Gunn rats. It is unclear whether acceleration of gastrointestinal transit also (partly) underlies the therapeutic effects of established hypobilirubinemic treatments, or whether PEG co-treatment might enhance these effects. We treated Gunn rats with phototherapy (17μW/cm^2/nm), orlistat (200mg/kg chow), ursodeoxycholate (5g/kg chow), or calcium phosphate (20g/kg chow) either as single treatment, or in combination with PEG. Three weeks of phototherapy, orlistat, ursodeoxycholic acid or calcium phosphate treatment decreased plasma unconjugated bilirubin (UCB) levels by 47%, 27%, 28%, and 45%, respectively (each $p<0.001$), without a significant impact on gastrointestinal transit time. PEG co-treatment accelerated the gastrointestinal transit in all treatment groups, which resulted in an additive hypobilirubinemic effect of -20% and -26% (final plasma UCB -67% and -53%, respectively) in phototherapy and orlistat-treated animals. PEG co-treatment did not enhance the hypobilirubinemic effect of ursodeoxycholic acid or calcium phosphate. This chapter demonstrates that phototherapy, orlistat, ursodoxycholic acid and calcium phosphate do not exert their hypobilirubinemic effect *via* acceleration of the gastrointestinal transit. PEG co-treatment enhanced the hypobilirubinemic effects of phototherapy, and of orlistat treatment. Current results support the feasibility of PEG co-treatment during phototherapy in hyperbilirubinemic patients.

5.2 Introduction

Neonatal hemolytic jaundice and Crigler-Najjar disease are characterized by severe unconjugated hyperbilirubinemia. In Crigler-Najjar patients, a genetically absent (type I) or decreased (type II) ability to conjugate bilirubin within the liver, results in lifelong jaundice.[1,2] Unconjugated bilirubin (UCB) is neurotoxic, and severe unconjugated hyperbilirubinemia is associated with brain damage.[3] This damage occurs because non-protein bound plasma bilirubin (UCB_{free}) can diffuse across the blood-brain barrier.[4-10] To decrease plasma UCB levels, Crigler-Najjar disease patients rely on lifelong phototherapy, the routine treatment for unconjugated hyperbilirubinemia. Phototherapy, however, becomes less effective with age and eventually fails to prevent bilirubin-induced brain damage in up to 25% of Crigler-Najjar patients.[11,12] This prompted us to develop alternative, and adjunct, treatment strategies for severe unconjugated hyperbilirubinemia.

Severe unconjugated hyperbilirubinemia allows direct transmucosal diffusion of UCB_{free} from the blood into the intestine.[13-15] The efficacy of this pathway is decreased, however, by the intestines' ability to reabsorb UCB_{free} from its lumen.[13,16] We recently hypothesized that acceleration of the gastrointestinal transit might interfere with this ability, and thus enhance the efficacy of transmucosal bilirubin disposal. Indeed, the laxative polyethylene glycol (PEG) proved an effective treatment for unconjugated hyperbilirubinemia in Gunn rats, the animal model for Crigler-Najjars disease.[17] Importantly, our experiments also revealed a strong linear relation between the gastrointestinal transit time and plasma UCB levels in these animals.[14]

Our results raised the question whether other treatments also (partly) rely on acceleration of the gastrointestinal transit for their hypobilirubinemic effects. Interestingly, previous findings have suggested that phototherapy, as well as oral treatments that enhance transmucosal UCB diffusion (*e.g.* orlistat,[18,19] bile salts,[14] or amorphous calcium phosphate (CaP)[20]), might indeed influence transit time.[21-25] We presently show that neither phototherapy, nor oral treatment with orlistat, ursodeoxycholic acid (UDCA), or CaP affects the gastrointestinal transit time in Gunn rats. We also show that addition of PEG to either phototherapy or orlistat treatment enhances their hypobilirubinemic effect.

5.3 Animals, materials, and methods

5.3.1 Animals

Homozygous adult male Gunn rats (RHA/jj; 244-375 g; n=60) were obtained from our breeding colony at the University Medical Center Groningen (UMCG, The Netherlands). Animals, housed individually in an environmentally-controlled facility with a diurnal (12/12 hour) light cycle, were fed *ad libitum* and had free access to water. The Ethics Committee for Animal Experiments of the UMCG approved the experimental protocols for this study.

5.3.2 Materials

Diets

Hope Farms BV (Woerden, The Netherlands) produced all diets. The semi-synthetic control diet (code 4063.02), contained 13 energy % fat.[18] Supplemented diets were identical to the control diet, except for the supplementation of orlistat (200 mg/kg chow), UDCA (5 g/kg chow), or CaP (20 g/kg chow). Gunn rats were fed the semi-synthetic purified control diet (4063.02) during a 6-week run-in period prior to the experiments.[14]

Chemicals

PEG 4000 (Colofort®) was obtained from Ipsen Farmaceutica BV (Hoofddorp, The Netherlands). Colofort® contained per sachet (74 g): 64 g PEG, 5.7 g sodium sulphate (anhydric), 1.68 g sodium bicarbonate, 1.46 g sodium chloride and 0.75 g potassium chloride. We dissolved one sachet of PEG 4000 (Colofort®) in 900 mL water to obtain the PEG solution we used in our experiments (drinking water solution and gavage solution). Orlistat (Xenical®) was obtained from Roche Nederland BV (Woerden, The Netherlands). UDCA was a generous gift from Dr. Falk Pharma GmbH (Freiburg, Germany). Carmine red dye was obtained from Macro-imPulse Saveur Ltd. (Stadtoldendorf, Germany).

Phototherapy lamps

Phototherapy devices were designed according the prototype by Ostrow.[26] Two phototherapy lamps (Philips TL-20W/03T) were suspended in a reflective

framework 20 cm above the bottom of the cage. The light intensity of phototherapy was measured by a Dale 40 phototherapy radiometer (Dale Technologies, Carson City, NV) at 20 cm distance from the light source, and was set to 17 μW/cm²/nm. Gunn rats, shaven on their backs and flanks every 7 days, received continuous phototherapy.[27]

5.3.3 Methods

Long-term experiment

Male Gunn rats were randomized (n=5-6 per group) to receive no treatment (controls) or treatment with phototherapy (17 μW/cm²/nm), orlistat (200 mg/kg chow), UDCA (5 g/kg chow), or CaP (20 g/kg chow). Body weight was determined weekly during the experiments. After a 3-week treatment period, which ensured the presence of steady-state conditions, [14,17-19] we determined the gastrointestinal transit time in untreated (controls) and treated Gunn rats by measuring the interval between intragastrical administration of carmine red and its appearance in the feces.[14] Heparinized samples of tail vein blood were obtained under isoflurane anesthesia before (baseline), and 3 weeks after randomization to determine steady-state plasma bilirubin concentrations. After 3 weeks, these treatments were combined with PEG, both *via* drinking water and *via* intragastrical gavage (2.5 mL, every 12h). The gastrointestinal transit time was determined again 6 weeks after randomization (3 weeks after the addition of PEG). A final blood sample was then obtained, as described above, for the determination of bilirubin, urea (Ur), creatinine (Creat), aspartate aminotransferase (AST), and alanine aminotransferase (ALT).

Short-term experiment

Male Gunn rats were randomized (n=6-7 per group) to receive no treatment (controls), phototherapy (17 μW/cm²/nm), phototherapy (17 μW/cm²/nm) combined with PEG (*via* drinking water and *via* 2.5 mL gavage), orlistat (200 mg/kg chow), or orlistat (200 mg/kg chow) combined with PEG (*via* drinking water and *via* 2.5 mL gavage). Body weight was determined daily during the experiments. Carmine red was administered directly after the start of PEG administration in order to determine the gastrointestinal transit as described above. Heparinized samples of tail vein blood were obtained under isoflurane anesthesia every 12-hour for the first 48 hours for the determination of bilirubin, Ur, Creat, AST, and ALT. A final blood sample was obtained 14 days after randomization.

Plasma analysis

Blood samples were protected from light and processed immediately. Bilirubin, Ur, Creat, AST and ALT were determined by routine spectrophotometry on a P800 unit of a modular analytics serum work area from Roche Diagnostics Ltd. (Basel, Switzerland). We previously found that the total bilirubin concentration in Gunn rat plasma, measured by spectrophotometry, equaled the total UCB concentration, measured by high-liquid performance chromatography after chloroform extraction.[18,27]

Statistical analysis

All data were normally distributed, displayed homogeneity of variance by calculation of Levene's statistic, and were expressed as mean ± SD. Analysis of variance (ANOVA) with post-hoc Bonferroni correction was performed for comparison between groups. Students *t* test was used for comparison of paired data within groups. The level of significance was set at $p<0.05$. Analyses were performed using SPPS 16.0 for Mac (SPSS Inc., Chicago, IL).

5.4 Results

5.4.1 Long-term experiment

Neither phototherapy nor oral treatments affect gastrointestinal transit time

Since acceleration of gastrointestinal transit decreases plasma bilirubin concentrations in Gunn rats, we assessed whether phototherapy and known oral treatments exert their hypobilirubinemic effects, either partly or exclusively, by acceleration of the gastrointestinal transit. Figure 1 shows that 3 weeks of phototherapy, orlistat, UDCA, or CaP decreased plasma bilirubin concentrations by 47%, 27%, 28%, and 45%, respectively, compared with baseline values ($p<0.001$). Figure 2 shows that, after 3 weeks of treatment, neither phototherapy, nor oral treatments with orlistat, UDCA, or CaP significantly affected the gastrointestinal transit time, compared with controls.

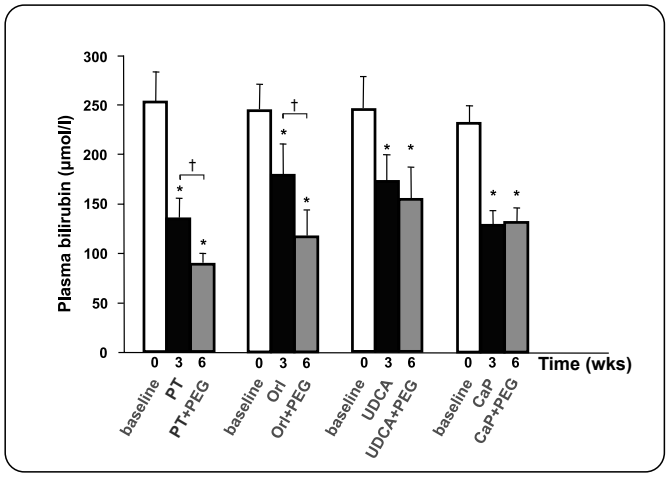

Figure 1. Treatment with continuous phototherapy (PT), or oral treatment with orlistat (Orl), UDCA, or CaP significantly decreased plasma bilirubin concentrations after 3 wks and PEG induced an additive therapeutic effect in phototherapy and orlistat treated animals. Gunn rats (n= 5-6 per group received either no treatment or treatment with phototherapy, orlistat, UDCA, or CaP. After three weeks, these treatments were combined with PEG administration for an additional 3 weeks. *$p<0.001$, compared with baseline values. †$p<0.01$, compared with single treatment.

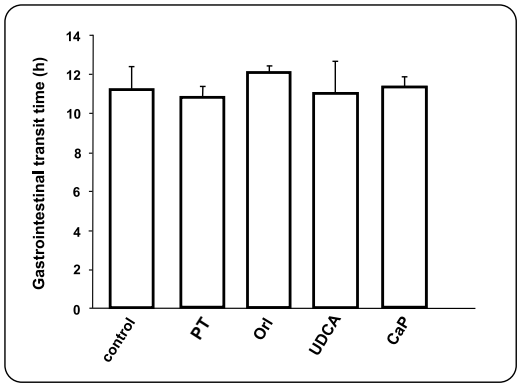

Figure 2. Three weeks of continuous phototherapy (PT), or oral treatment with orlistat (Orl), UDCA, or CaP did not significantly affect the gastrointestinal transit time. Gunn rats (n= 5-6 per group) received either no treatment or treatment with phototherapy, orlistat, UDCA, or CaP. After three weeks we determined the gastrointestinal transit time by measuring the interval between intragastrical administration of carmine red and its appearance in the feces.

Additive therapeutic effect of PEG administration on phototherapy and orlistat treatment

Since neither phototherapy nor any of the oral treatments accelerated the gastrointestinal transit, we assessed whether adjunct PEG treatment could increase their hypobilirubinemic effect. Figure 3 shows that combining phototherapy, orlistat, UDCA, and CaP, with PEG administration for 3 weeks accelerated the gastrointestinal transit by 40%, 33%, 33%, and 28%, respectively, compared with controls ($p<0.001$; each). Figure 1 shows that 3 weeks of combined treatment of PEG with phototherapy, orlistat, UDCA, or CaP decreased plasma bilirubin concentrations by 67%, 53%, 30%, and 45%, respectively, ($p<0.001$). Combining phototherapy or orlistat treatment with PEG thus induced an additive therapeutic effect of 20% and 26%, respectively, compared with single treatment ($p<0.01$). The hypobilirubinemic effect of PEG + phototherapy is in line with our previous results, which showed a 62%-decrease in plasma bilirubin levels after 2 weeks of phototherapy + PEG treatment.(16) Table 1 shows effect of PEG co-treatment on renal function, liver function, and growth rate in the various treatment groups. Addition of PEG to phototherapy, orlistat, UDCA, or CaP did not affect plasma levels of urea, compared with controls, which pleaded against clinically relevant dehydration during PEG co-treatment. PEG + orlistat treatment resulted in a mild increase in plasma creatinine levels compared with the control group, although still within the reference range for Gunn rats.[28] UDCA + PEG treated animals showed a 5%-decrease in growth rate during the entire experiment, compared with a 2.5%-increase in control animals ($p<0.01$; Table 1).

	controls	PT + PEG	Orl + PEG	UDCA + PEG	Ca + PEG
Renal parameters					
Creat (mmol/L)	17 ± 9	23 ± 12	31 ± 11*	11 ± 3	11 ± 4
Urea (mmol/L)	7.7 ± 2.0	6.0 ± 1.3	7.1 ± 0.9	6.1 ± 1.2	6.9 ± 0.9
Liver parameters					
AST (Unit/L)	96 ± 37	74 ± 11	86 ± 17	120 ± 37	98 ± 29
ALT Unit/L)	35 ± 25	21 ± 7	21 ± 10	29 ± 9	28 ± 6
Growth parameters					
Growth rate (%, compared with T=0 wks)	2.5 ± 4.7	-1.9 ± 4.5	-2.0 ± 2.7	-5.3 ± 1.4**	-1.3 ± 2.2

Table 1. Renal and liver parameters at after 6 weeks of treatment. For experimental setup, please refer to Figure 1. Plasma urea, creatinine, AST, ALT concentrations and growth rate were determined after 6 weeks of treatment. Data represent mean ± SD. *$p<0.05$, **$p<0.01$, compared with controls. PT, phototherapy; Orl, Orlistat.

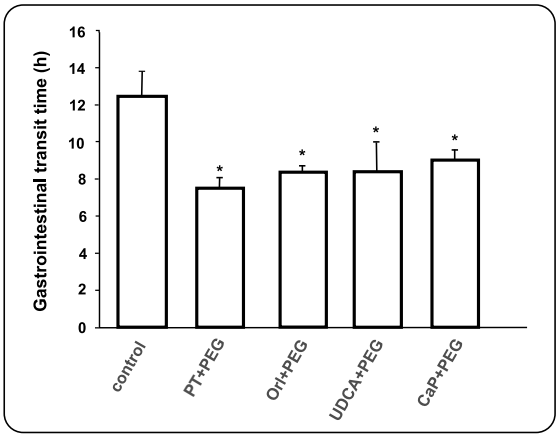

Figure 3. Combining phototherapy (PT), orlistat (Orl), UDCA, and CaP, with PEG administration significantly accelerated the gastrointestinal transit. Gunn rats (n=5-6 per group) received either no treatment or treatment with phototherapy, orlistat, UDCA, or CaP. After three weeks, these treatments were combined with PEG administration for an additional 3 weeks. After six weeks we determined the gastrointestinal transit time as described in Fig 2. *$p<0.001$, compared with controls.

5.4.2 Short-term experiment

Immediate PEG co-treatment with orlistat is effective within days

To further explore its clinical potential, we subsequently determined whether immediate PEG co-treatment could accelerate the hypobilirubinemic effect of phototherapy or orlistat during a short-term period of treatment. Figure 4A shows that phototherapy alone decreased plasma bilirubin concentrations by 35% within 48h ($p<0.001$), compared with control animals. Immediate co-treatment with PEG accelerated the gastrointestinal transit by 28% ($p<0.001$; Fig 4B), and decreased plasma bilirubin concentrations by 37% ($p<0.001$; Fig 4A), compared with controls. Figure 4C shows that orlistat alone decreased plasma bilirubin concentrations by 10% (NS), and that co-treatment with PEG decreased plasma bilirubin by 24% ($p<0.001$), compared with control animals. PEG thus did not enhance the rapid hypobilirubinemic effect of phototherapy, but did increase that of orlistat treatment by 14% ($p<0.05$). Continuation of treatment in the PEG + phototherapy-treated animals for 2 weeks, however, did result in a final decrease of 67%, which was virtually identical to the decrease in the long-term experiment. Table 2 shows that there were no differences renal function, liver function or growth rates between the various treatment groups.

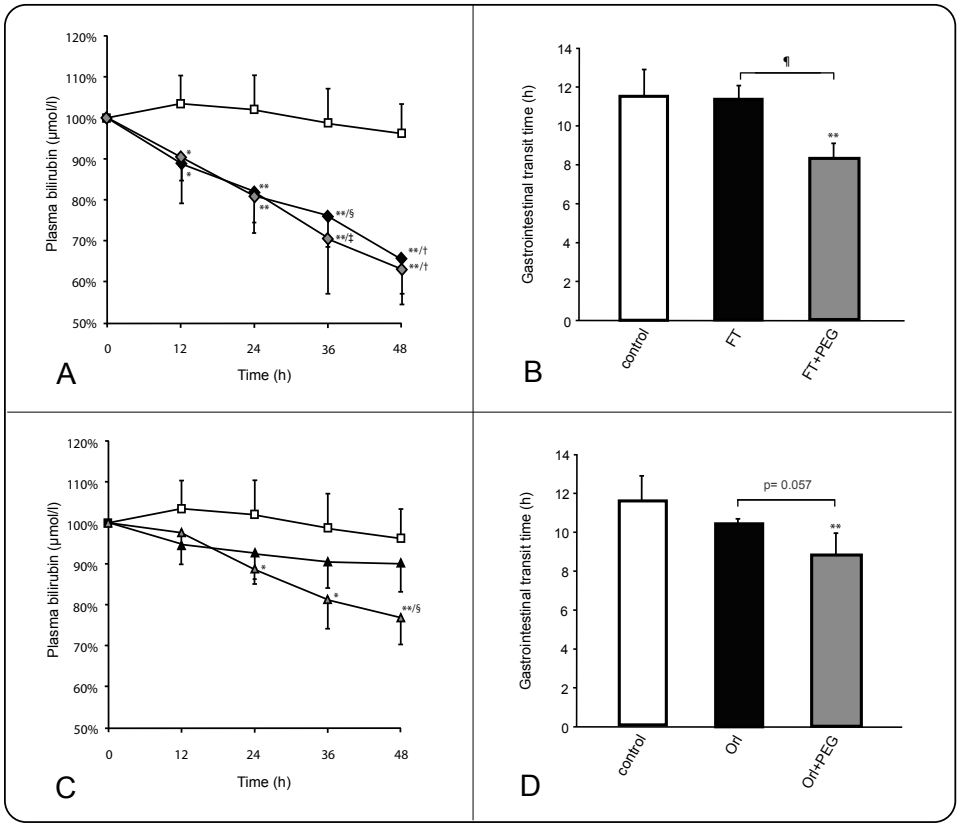

Figure 4. PEG co-treatment induces an additive hypobilirubinemic effect during orlistat (Orl) treatment (panel A/C), and accelerates the gastrointestinal transit in all treatment groups (panel B/D). Gunn rats (n=6-7 per group) received either no treatment or treatment with phototherapy, phototherapy+PEG, orlistat, or orlistat+PEG. The gastrointestinal transit was determined by administration of carmine red directly after the start of treatment as in figure 2. *p<0.05; **p<0.001, compared with controls. ¶p<0.001, compared with single phototherapy treatment. §p<0.05; ‡p<0.01; †p<0.001, compared with single orlistat treatment. □ Controls; ◆ phototherapy (PT); ◈ phototherapy+PEG; ▲ orlistat (Orl); ▲ orlistat+PEG.

	controls	PT	PT + PEG	Orl	Orl + PEG
Renal parameters					
Creat (mmol/l)	16 ± 3	13 ± 2	13 ± 2	19 ± 4	16 ± 4
Urea (mmol/l)	8.6 ± 1.8	5.5 ± 0.7**	6.9 ± 1.5	10 ± 2	5.8 ± 0.9*
Liver parameters					
AST (Unit/L)	95 ± 26	95 ± 17	88 ± 25	125 ± 45	95 ± 13
ALT Unit/L)	55 ± 21	38 ± 7	43 ± 7	73 ± 12	60 ± 12
Growth parameters					
Growth rate (%, compared with T=0 wks)	0.4 ± 2.4	-0.1 ± 0.0	3.7 ± 2.0	4.4 ± 1.4	0.3 ± 2.5

Table 2. Renal and liver parameters at after 2 weeks of treatment. For experimental setup, please refer to Figure 3. Plasma urea, creatinine, AST, and ALT concentrations and growth rate were determined after 2 weeks of treatment. Data represent mean ± SD. *$p<0.05$; **$p<0.01$, compared with controls. PT, phototherapy; Orl, Orlistat.

5.5 Discussion

In this study we demonstrate that neither routine phototherapy nor experimental oral treatments (*e.g.* orlistat, UDCA, or CaP), exert their hypobilirubinemic effect *via* acceleration of the gastrointestinal transit in Gunn rats. We also show that acceleration of the gastrointestinal transit by PEG enhances the therapeutic effects of both phototherapy and orlistat in these animals.

Several studies have suggested a relationship between gastrointestinal transit and plasma bilirubin levels. Conditions that delay the gastrointestinal transit, such as Hirschprungs disease [29] or fasting,[30,31] have been associated with an exaggeration of neonatal jaundice. Conditions that *accelerate* transit, such as prebiotic supplementation of infant formula, seem to mitigate jaundice in neonates.[32,33] We recently demonstrated that acceleration of the gastrointestinal transit by PEG effectively treated unconjugated hyperbilirubinemia in jaundiced Gunn rats.[17] The strong relationship between the gastrointestinal transit and plasma UCB concentrations in that study prompted us to evaluate the mechanistic contribution of the transit time in phototherapy and in experimental oral treatments. The hypobilirubinemic effects of these treatments have traditionally been linked to different underlying mechanisms. Phototherapy, the routine treatment for neonatal jaundice, has been shown to exert its hypobilirubinemic effect by conversion of UCB into photo-isomers.[26] Experimental oral treatments, such as orlistat, UDCA, or CaP, are

thought to bind UCB within the gut lumen, thereby preventing its reabsorption and interrupting its enterohepatic circulation.[14,27,34] Interestingly, both phototherapy and oral treatments have also been suggested to accelerate the gastrointestinal transit.[35,36] Phototherapy, especially intensive phototherapy, can induce diarrhea. Diarrhea is also a well-known potential side effect of orlistat and UDCA treatment. Orlistat has been shown to accelerate gastric emptying,[22] whereas UDCA treatment accelerated the gastrointestinal transit in dogs, and can improve gastrointestinal motility defects in bile stone patients.[21,37] Theoretically, phototherapy or oral treatments could thus well exert (part of) their hypobilirubinemic effect by acceleration of the gastrointestinal transit. Our Gunn rat model, however, allowed us to demonstrate that neither routine phototherapy nor experimental oral treatments exert their hypobilirubinemic effect *via* acceleration of the gastrointestinal transit. These findings prompted us to investigate whether PEG co-treatment might induce an additional decrease in plasma bilirubin levels during these treatments. We started PEG co-treatment only after 3 weeks to ensure steady-state conditions, which prevented any interference of non-steady-state plasma bilirubin fluctuations.[14,17-19]

PEG accelerated the gastrointestinal transit in all treatment groups, and induced an additive therapeutic effect in phototherapy and orlistat treated animals. The most impressive effect was observed in the phototherapy + PEG treatment group, which concurred with the hypobilirubinemic result of adding phototherapy to PEG treatment alone in a previous study by us.[17] The additive therapeutic effect (~20%) thus occurred irrespective of whether phototherapy was added to PEG-treatment (previous study), or *vice-versa* (current study), indicating that both therapies truly complement each other.[17] The efficacy of phototherapy + PEG treatment, which resulted in a final 67% decrease in plasma bilirubin levels, might well result from the distinct mechanisms by which phototherapy and PEG decrease plasma bilirubin levels. Phototherapy predominantly decreases plasma bilirubin levels by enhancing its disposal *via* the bile,[26] whereas oral treatments, including PEG, selectively enhance *net* bilirubin diffusion from the blood into the intestinal lumen.[14,27] We and others have demonstrated in ^{3}H-labelled bilirubin studies that a maximal therapeutic effect often results from combining therapies that enhance distinct pathways for bilirubin disposal.[14,27,38] The complementary effect of PEG and phototherapy might thus well result from the combination of their distinct hypobilirubinemic mechanisms. The additive effect of PEG was not observed during the first days of phototherapy treatment. We speculate that this occurred because short-term phototherapy extracted bilirubin at a maximal rate from the body. This rate could thus not be further increased by PEG co-treatment. Short-term orlistat treatment, on the other hand, was notably less effective compared with phototherapy. In agreement with our postulate above, PEG was a useful adjunct strategy in this treatment group.

PEG co-treatment did not induce an additive therapeutic effect in UDCA or CaP treated animals. This lack of additive effect cannot be attributed to an insufficient acceleration of the gastrointestinal transit, which was comparable between all PEG-treated groups. PEG administration, however, has several effects on the intestinal milieu that might counteract the UDCA or CaP-induced decrease in plasma UCB levels. Firstly, PEG decreases the amount of bile salts in the feces.[14] This, evidently, would counteract the effect of simultaneous UDCA feeding. Secondly, PEG decreases the formation of secondary bile salts, possibly by affecting the microflora that promotes bile salt metabolism. This microflora, however, is also involved in the irreversible degradation of intestinal bilirubin into urobilinoids, which is promoted by UDCA feeding.[14,17] PEG might thus well interfere with the apparent ability of UDCA to promote intestinal bilirubin degradation. Finally, PEG increases the amount of intestinal water and accelerates the gastrointestinal transit. This might well prevent the precipitation of amorphous calcium phosphate with intestinal bilirubin during CaP treatment.[34]

Animals that were treated with UDCA + PEG showed a small (-5%), but significant, decrease in growth rate compared with control animals. Although this was not observed in other groups, it should be emphasized that diarrhea and malabsorption of vitamins (A, D, E, K) have been reported during orlistat, UDCA and CaP treatment.[19,35,36,39] Regular assessments of the nutritional (vitamin) status and/or vitamin supplementation would be indicated if one would combine these treatments with a laxative such as PEG in patient studies. These precautions may not apply for the combination of PEG with phototherapy, which seems safe and deserves further exploration in hyperbilirubinemic patient studies.

In conclusion, our data show that that neither phototherapy, nor orlistat, UDCA, or CaP exerts its hypobilirubinemic effect *via* acceleration of the gastrointestinal transit. Combining PEG-induced acceleration of the gastrointestinal transit with phototherapy decreased plasma bilirubin levels by 67% in Gunn rats. Our results support the evaluation of PEG co-treatment during phototherapy in clinical trials, for example in Crigler-Najjar patients.

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