Chapter 5

Vitamin A Deficiency Severely Aggravates Obstructive Cholestasis Which Is Effectively Treated by Acute Vitamin A Supplementation

Martijn O. Hoeke, Mark Hoekstra, Janette Heegsma, Han Moshage and Klaas Nico Faber

Department of Gastroenterology and Hepatology, University Medical Center Groningen, Groningen, University of Groningen, The Netherlands

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Chapter 5

Abstract

Cholestasis is accompanied by malabsorption of fat-soluble vitamins, including vitamin A. Moreover, the stellate cells in the liver lose their vitamin A stores upon cholestatic liver injury and start producing excessive amounts of extracellular matrix leading to liver fibrosis. As a consequence, chronic cholestasis is associated with hypovitaminosis A. Vitamin A is an antioxidant and its derivatives are crucial signaling molecules for the retinoid X receptor alpha (RXRα) that regulates bile salt homeostasis in conjunction with the farnesoid X receptor (FXR). Supplementing cholestatic patients with vitamin A is, however, a controversial issue.

We studied the effect of vitamin A deficiency and retinyl palmitate (RP) therapy in an animal model of cholestasis. Rats were made vitamin A deficient (VAD) by omitting vitamin A from their diet for 16 weeks, followed by ligation of the common bile duct (BDL) or a sham operation. 7 Days later the animals were sacrificed. During the final 7 days, half of the animals received daily IP injections with RP.

BDL induced a dramatic weight loss in VAD rats (-15 to -20% in 7 days, compared to -5% in vitamin A sufficient (VAS)-BDL rats) and serum hepatic damage markers (AST/ALT) were approximately 10-fold increased compared to BDL rats receiving an VAS diet. Markers for oxidative stress (Ho-1), inflammation (iNos), fibrosis (α-Sma and Col1a1) were strongly increased. Large regions of necrotic hepatocytes were observed in VAD-BDL rats together with strong proliferation of bile ductular cells. Importantly, all these liver disease markers were efficiently reversed by RP supplementation to the level observed in VAS-BDL rats. Only minor changes in hepatic transcriptional regulation of bile salt homeostasis were detected.

We conclude that vitamin A deficiency dramatically aggravates liver damage caused by obstructive cholestasis, but is efficiently prevented by vitamin A supplementation. These data warrant a detailed analysis of the hepatoprotective effect of vitamin A in patients with chronic cholestasis.

Introduction

Vitamin A is an important nutrient during all stages of mammalian life and is essential in embryogenesis, differentiation of tissues and fertility. Moreover, it is required for eyesight, immune competence and has anti-inflammatory properties. Most of its actions are performed through activation of the nuclear receptors retinoic acid receptor (RAR) and retinoid X receptor (RXR) that are ligand-activated transcription factors responding to vitamin A-derivatives, including all-trans and 9-cis retinoic acid (1, 2). In addition, vitamin A may act as an antioxidant (3, 4). Mammals cannot synthesize vitamin A themselves, so they need to obtain it from their diet, either as pro-vitamin A carotenoids from fruits or vegetables or as retinyl esters from animal sources.
Vitamin A is a fat-soluble vitamin and its efficient absorption in the intestine depends on the action of bile salts (5, 6). Bile salts are synthesized in the hepatocytes in the liver and secreted via the common bile duct and the gallbladder into the duodenum. They form mixed-micelles with phospholipids and those act as carriers for a great variety of fat-soluble compounds in the small intestine. This allows efficient absorption, as for the fat-soluble vitamins A, D, E and K, as well as the effective excretion of waste products from the body, including cholesterol and toxins. At the terminal ileum, bile salts are effectively absorbed and via the circulation transported back to the liver to be reused. This enterohepatic circulation of bile salts is highly efficient. Per cycle only approximately 5% escapes intestinal reabsorption and is excreted through the feces. De novo synthesis of bile salts in the liver compensates for the fecal loss of bile salts (7). Most of the vitamin A that is absorbed in the intestine is also transported to the liver and stored in lipid droplets in hepatic stellate cells. Over 80% of the total vitamin A pool in the healthy body is present in these liver cells (8).

Many liver diseases, both acute and chronic, affect the production and/or secretion of bile salts resulting in cholestasis, e.g. accumulation of bile salts in blood and liver caused by an impaired bile flow. This leads to malabsorption of fat-soluble compounds in the intestine, including the vitamins A, D, E and K. Liver injury, at the same time, leads to the activation, proliferation and differentiation of stellate cells that overproduce extracellular matrix proteins, like collagens and fibronectins, giving rise to fibrosis. The hepatic stellate cells lose their vitamin A content in this transdifferentiation process (8). As a result, chronic liver diseases are associated with vitamin A deficiency/hypovitaminosis A, as described for biliary atresia (9), primary biliary cirrhosis (PBC) (10) and cholelithiasis (gallstone disease) (11). Vitamin A supplementation in the treatment of these patients remains a controversial issue though (12). Vitamin A supplementation has been shown to reduce cholestasis and liver fibrosis in experimental animal models of liver disease including bile duct ligation and chronic CCl₄ administration (13-18). On the other hand, vitamin A overdosing can also cause jaundice (19), cholestasis (20, 21) and fibrosis (22). Previously, we found that vitamin A (-derivatives) also affect bile salt synthesis and transport directly. They act via RXRα (NR2B1), which is the obligatory heterodimer partner of the farnesoid X receptor (FXR/NR1H4) (23), the mammalian bile salt sensor (24-26). FXR/RXRα regulates (directly or indirectly) transcription of key genes involved in bile salt synthesis and transport, including cholesterol 7-alpha-hydroxylase (CYP7A1) and the bile salt export pump (BSEP/ABCB11). Both genes are maximally expressed in the absence of 9-cis retinoic acid (27, 28). Moreover, we found that vitamin A deficiency alone leads to mild cholestasis in rats, most likely due to post-translational mechanisms. Thus, hypovitaminosis A affects bile homeostasis and may directly or indirectly affect the course of liver disease in obstructive cholestasis.

Here, we studied the effect of vitamin A deficiency on liver injury in an animal model for obstructive cholestasis. In addition, we determined the acute effect of vitamin A supplementation on putative excessive liver damage in vitamin A deficient rats with obstructive cholestasis.
Material and methods

Animals
All animal experiments were approved by the ethics committee for animal testing of the University of Groningen, The Netherlands. Weaning male Wistar rats (35-50 grams) (Harlan, Horst, The Netherlands) were fed either a vitamin A deficient (VAD) diet TD.86143 (Harlan Teklad, Madison, USA) (n=22) or a vitamin A sufficient (VAS) diet TD.91280 (Harlan Teklad, Madison, USA) (n=18). The VAS diet was identical to the VAD diet, but supplemented with 20,000 U/kg vitamin A. Animals were housed in a temperature-controlled environment with alternating 12 hours light and dark cycles. Food and water were available *ad libitum*. Blood samples were taken at different time points during the diet phase to monitor the plasma retinol levels.

Experimental design: After 16 weeks on the VAD/VAS diet, animals were bile duct-ligated (BDL) (VAD n=14; VAS n=10) or were given a sham treatment (VAD n=8; VAS n=8). After surgery animals continued on the same diet as before. Starting from the day of surgery, rats were IP injected daily with 50 U/g body weight retinyl palmitate (RP) (Sigma Aldrich, St. Louis, USA) diluted in corn oil (Sigma Aldrich, St. Louis MO, USA), or vehicle. RP or vehicle was given to VAD-BDL (n=7), VAD-sham (n=4), VAS-BDL (n=5) and VAS-sham (n=4) animals. Rats were sacrificed 7 days after BDL or sham surgery.

Biochemical Procedures
Retinol (29) and total bile salt concentrations (30, 31) were determined in blood plasma and liver tissue as described. Concentrations of hepatic and bile ductular enzymes aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (AP) and gamma-glutamyl transferase (γGT) levels were determined in blood plasma.

mRNA was isolated from hepatic, ileal and renal tissue using the Nucleospin II RNA kit (Macherey-Nagel, Düren, Germany) according to the manufacturer’s recommendations. RT-qPCR was performed as described before (32). Taqman primer-probe sets used are displayed in Table 5.1.

Protein expression was determined by western blot analysis, expression was quantified using the Quantity One software package (Biorad, Hercules CA, USA). BSEP and HO-1 were detected using K12 (anti-BSEP; (33)) and OSA-111 (anti-HO-1; Stressgen, Victoria BC, Canada) respectively followed by goat anti-rabbit immunoglobulins/HRP (P0448, Dako, Glostrup, Denmark). α-SMA and GAPDH were detected using A-2547 (anti-α-Sma; Sigma Aldrich, St. Louis MO, USA) and 6C5 (anti-GAPDH, CB1001, Calbiochem, Darmstadt, Germany), respectively, followed by rabbit anti-mouse immunoglobulins/HRP, (P0260, Dako Glostrup, Denmark). Sodium potassium ATPase was detected using anti-Na⁺K⁺-ATPase (kind gift from Dr. W. Peters, St. Radboud University Medical Center, Nijmegen, The Netherlands) followed by rabbit anti-goat immunoglobulins/HRP (P0160, Dako).
Immunohistochemistry on liver slides was performed as described before using K12 (α-BSEP) followed by Alexa Fluor 488, goat anti-rabbit IgG (H+L) (A11008, Invitrogen, Paisley, United Kingdom) and M7249 (α-Ki67; Dako) followed by goat anti-rabbit immunoglobulins/biotinylated, (E0432, Dako) followed by peroxidase-conjugated streptavidin (P0397, Dako) (27).

### Table 5.1. TaqMan primer-probe sets used for RT-qPCR

<table>
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<th>Gene</th>
<th>Oligo sequences</th>
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<tr>
<td>α-Sma</td>
<td>Fwd: 5'-GCCAGTCGCCCATCAGGAAC-3'&lt;br&gt;Rev: 5'-CACACCAGAGCTGTGCTGTTT-3'&lt;br&gt;Probe: 5'-CTTCACACATAGCTGGAGCAGCTTCTCGA-3'</td>
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<tr>
<td>Bsep</td>
<td>Fwd: 5'-CCAAGCTGCCAAGGATGCTC-3'&lt;br&gt;Rev: 5'-CCTTCTCCAACAGGGTGTC-3'&lt;br&gt;Probe: 5'-CATTATGCGCCCTGCAGGCA-3'</td>
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<tr>
<td>Krt19</td>
<td>Fwd: 5'-GAGGACTTTGCGCGCAAGA-3'&lt;br&gt;Rev: 5'-GCCGGGATCTGCTCAGCTG-3'&lt;br&gt;Probe: 5'-CAGGCCATTGAGAACTCCAAGATAGGCT-3'</td>
</tr>
<tr>
<td>Col1a1</td>
<td>Fwd: 5'-TGGTGAACGCTGGTGTCAC-3'&lt;br&gt;Rev: 5'-CAGGTACCACTTGGGACTG-3'&lt;br&gt;Probe: 5'-TCCTGCTGCTGGCCAGGGAATA-3'</td>
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<tr>
<td>Cyp7a1</td>
<td>Fwd: 5'-CAGGGAGATGCTCTGTGCTT-3'&lt;br&gt;Rev: 5'-AGGCTACATCCTCCGTGA-3'&lt;br&gt;Probe: 5'-TGCAAAACCTCCAATCTGAGACCTCC-3'</td>
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<td>Ho-1</td>
<td>Fwd: 5'-CACAGGGTGACAGAAGGCTAA-3'&lt;br&gt;Rev: 5'-CTGGTCTCTTGGTCATGCT-3'&lt;br&gt;Probe: 5'-CTGCAAAACCTCCAATCTGAGACCT-3'</td>
</tr>
<tr>
<td>iNos</td>
<td>Fwd: 5'-GTGCTAATGCGGAGGCTG TG-3'&lt;br&gt;Rev: 5'-CGACTTTTCTGTCTCAGTGA A-3'&lt;br&gt;Probe: 5'-CCCAGGCTGCAAGGCAACGTC-3'</td>
</tr>
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<td>18S</td>
<td>Fwd: 5'-CGGCTACCACATCGGCA-3'&lt;br&gt;Rev: 5'-CAGATTACAGGGCTCGA AA-3'&lt;br&gt;Probe: 5'-CAGGCAAATTCACCACCTCCGA-3'</td>
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Probes used for RT-qPCR were FAM TAMRA labeled.

### Statistical Analysis
Relative mRNA levels were corrected for 18S and presented as a box plot with medians represented by the horizontal lines with the 75th percentiles at the top and the 25th percentiles at the bottom of the boxes. Ranges are represented as whiskers. Other data are presented as mean ± SD. Differences between the animal groups were determined with SPSS 17.0 (IBM, Armonk NY, USA) by Kruskal-Wallis followed by pairwise comparison of groups by Mann-Whitney U with p<0.05.
Chapter 5

RESULTS

Vitamin A Depletion by Diet Does Not Affect Animal Growth
To establish vitamin A deficiency, weaning male Wistar rats were fed a diet lacking this vitamin. No significant differences were detected in bodyweight of the animals receiving a control diet containing standard amounts (20,000 IU/kg) of vitamin A (VAS; vitamin A sufficient) (n=18) or a vitamin A deficient (VAD) (n=22) diet after 16 weeks on these diets (Figure 5.1A). After 16 weeks VAD diet, the plasma retinol levels had dropped 77% to 0.025 ± 0.012 mg/ml (Figure 5.1B).

![Figure 5.1](image1.png)

Figure 5.1. VAD diet did not affect the growth of the animals
Weaning male Wistar rats were fed either a vitamin A sufficient (VAS) (n=18) or deficient (VAD) (n=22) diet A). No significant differences were detected in bodyweight of the animals receiving a VAS or VAD diet over the whole period of 16 weeks. B) After 16 weeks (prior to BDL/sham surgery) VAD diet, the plasma retinol levels dropped in to on average 23%. Data displayed as mean ± SD. *) significant difference between VAS and VAD, p<0.05.

Vitamin A Deficiency Aggravates the Obstructive Cholestatic Phenotype
After 16 weeks on diet, all animals underwent surgery, either a ligation of the common bile duct (BDL) or a sham operation, and were subdivided into 4 groups; VAS-sham, VAS-BDL, VAD-sham and VAD-BDL (Figure 5.2). The VAS-sham, VAD-sham and VAS-BDL animals lost approximately 5% body weight in the first 2 days following surgery, after which it stabilized until sacrifice at day 7 (Figure 5.2A). In contrast, VAD-BDL rats continued to lose weight almost linearly during the whole 7 day period post-surgery. On average they had lost approximately 70 grams (= 13%) bodyweight at time of sacrifice. Upon retrieval of the livers, clear macroscopic liver damage, e.g. yellow spots at the surface (Figure 5.2C), was observed in livers of the VAD-BDL animals, whereas this was not evident in livers from VAS-BDL (Figure 5.2B) and the sham operated animal (not shown). Moreover, bile accumulation was observed immediately before the site of ligation dilating the common bile duct and containing over 1 ml of bile. No such “bile sacs” were formed in VAS-BDL rats, or were merely the size of a pin-head from which only minor amounts of bile was retrieved (data not shown).
Vitamin A Deficiency Severely Aggravates Obstructive Cholestasis

Figure 5.2. Vitamin A deficiency dramatically aggravates obstructive cholestatic disease course
Vitamin A sufficient (VAS) and deficient (VAD) rats were bile duct ligated (BDL) or sham treated and sacrificed 7 days after surgery. A) VAD-BDL animals dramatically lost weight, while animals in the VAS-BDL group had weights similar to sham treated animals on either diet. Data displayed as percentage of weight prior to BDL (day 0), mean ± SD. VAD-BDL animals showed clear macroscopic liver damage (C) compared to livers of VAS-BDL animals (B). Vitamin A deficiency strongly increased serum levels of hepatic and ductural damage markers aspartate transaminase (AST) (D), alanine transaminase (ALT) (E), alkaline phosphatase (AP) (F) and gamma-glutamyl transferase (γGT) (G) as a consequence of bile duct ligation. Data displayed as a box plot with medians represented by the horizontal lines with the 75th percentiles at the top and the 25th percentiles at the bottom of the boxes. Ranges are represented as whiskers. Significant difference between groups, \( p < 0.05 \), a significantly different from VAS-sham; b significantly different from VAS-BDL; c significantly different from VAD-sham; d significantly different from VAD-BDL.
As documented before (34), 7 days BDL in control (VAS) rats leads to slightly increased AST levels compared to sham-operated VAS rats (Figure 5.2D; 259 ± 71 versus 124 ± 158 U/L). A significant increase was also detected for serum AP levels (Figure 5.2F, 0.7 ± 0.6 versus 52 ± 32 U/L for VAS-sham and VAS-BDL, respectively). No significant increase was detected in serum ALT and γGT levels after 7 days BDL in VAS animals (Figure 5.2E and G). VAD alone did not lead to an increase in any of these markers in the serum of sham operated rats. In sharp contrast, serum levels of AST, ALT and γGT were strongly elevated and respectively 12.2-, 7.7- and 183-fold higher in VAD-BDL rats compared to VAS-BDL rats (Figure 5.2D, E and G). While clearly increased in VAD-BDL animals, AP serum levels were not significantly higher compared to the VAS-BDL group (Figure 5.2F). Remarkably, BDL led to a further reduction of serum retinol levels both in VAS (from 0.289 ± 0.014 U/L to 0.141 ± 0.054 U/L) and VAD (from 0.043 ± 0.022 U/L to 0.006 ± 0.007 U/L) rats compared to their respective control rats (Table 5.2). In contrast, retinol levels in the livers of VAS-BDL rats were significantly increased (10.53 ± 3.93 mg/ml versus 16.98 ± 7.44 in mg/ml VAS-sham and VAS-BDL, respectively). Hepatic retinol levels were low in VAD-sham rats and remained unchanged after BDL, suggesting the absence of alternative retinol stores. Collectively, these data show that vitamin A deficiency severely aggravates liver damage in obstructive cholestasis in rats.

Vitamin A Therapy Prevents Liver Injury in Vitamin A Deficient Cholestatic Rats

In order to determine whether acute supplementation of vitamin A may prevent excessive liver damage, VAD-BDL rats received daily IP injections with retinyl palmitate (RP; 50 U/g bodyweight) on all days following surgery. Half of the animals from the other 3 groups (VAS-sham, VAS-BDL and VAD-sham) also received RP-therapy for control analyses. RP supplementation increased serum retinol concentrations in VAD-BDL rats to levels comparable to VAS-BDL rats (Table 5.2; 0.145 ± 0.046 mg/ml versus 0.141 ± 0.054 mg/ml, respectively). Moreover, RP supplementation increased the hepatic retinol levels both in the VAS-BDL and the VAD-BDL group, indicating BDL-induced hepatic recruitment of retinol(s). RP supplementation significantly suppressed the excessive weight loss of the VAD-BDL animals, which stabilized at approximately -7% three days after BDL until sacrifice at day 7 (Figure 5.3A). Macroscopically, the livers from VAD-BDL-RP animals were comparably to VAS-BDL rats (compare Figure 5.3C and Figure 5.2B), lacking the yellow spots at the surface as observed for the VAD-BDL animals (Figure 5.2C and included again in 5.3B for comparison). Accordingly, serum levels of hepatocyte and bile duct damage markers AST, ALT, AP and γGT were drastically reduced in VAD-BDL after RP supplementation (Figure 5.3D-G). However, RP supplementation did not prevent the formation of the “bile sac” in the VAD-BDL rats at the site of the ligation (data not shown). An overview of physical and biochemical animal parameters of rats from all 8 treatment groups is given in Table 5.2.
Vitamin A Deficiency Severely Aggravates Obstructive Cholestasis

Table 5.2. Animal characteristics after BDL and vitamin A therapy

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<td></td>
<td>Body weight day 0 (g)</td>
<td>514 ± 39</td>
<td>33 ± 60</td>
<td>538 ± 37</td>
<td>525 ± 40</td>
<td>517 ± 37</td>
<td>483 ± 43</td>
<td>523 ± 40</td>
<td>538 ± 37</td>
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<td>Body weight day 7 (g)</td>
<td>491 ± 36</td>
<td>510 ± 38</td>
<td>487 ± 53</td>
<td>530 ± 30</td>
<td>514 ± 36</td>
<td>514 ± 36</td>
<td>514 ± 36</td>
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<td>Plasma retinol day 7 (mg/L)</td>
<td>0.289 ± 0.014</td>
<td>0.224 ± 0.053</td>
<td>0.141 ± 0.054</td>
<td>0.116 ± 0.031</td>
<td>0.043 ± 0.022</td>
<td>0.183 ± 0.034</td>
<td>0.006 ± 0.007</td>
<td>0.145 ± 0.046</td>
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<td>Liver retinol day 7 (mg/g)</td>
<td>10.53 ± 3.93</td>
<td>10.74 ± 3.58</td>
<td>16.98 ± 7.44</td>
<td>24.55 ± 12.25</td>
<td>0.12 ± 0.24</td>
<td>3.69 ± 1.60</td>
<td>0.08 ± 0.21</td>
<td>7.54 ± 5.17</td>
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<td>Plasma bile salt day 7 (µmol/L)</td>
<td>23 ± 14</td>
<td>9.2 ± 4.3</td>
<td>1187 ± 357</td>
<td>1029 ± 322</td>
<td>39 ± 14</td>
<td>24 ± 16</td>
<td>1206 ± 268</td>
<td>970 ± 387</td>
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<td>Liver bile salt day 7 (µmol/g)</td>
<td>1.18 ± 0.11</td>
<td>1.13 ± 0.11</td>
<td>2.01 ± 0.30</td>
<td>2.05 ± 0.31</td>
<td>1.09 ± 0.18</td>
<td>1.16 ± 0.38</td>
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<td></td>
<td>AST (U/L)</td>
<td>124 ± 58</td>
<td>129 ± 57</td>
<td>259 ± 71</td>
<td>273 ± 97</td>
<td>163 ± 67</td>
<td>75 ± 17</td>
<td>316 ± 57</td>
<td>350 ± 190</td>
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<td></td>
<td>ALT (U/L)</td>
<td>46 ± 22</td>
<td>46 ± 22</td>
<td>970 ± 387</td>
<td>92 ± 61</td>
<td>750 ± 17</td>
<td>92 ± 61</td>
<td>630 ± 595</td>
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<td>γGT (U/L)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>1.3 ± 1.3</td>
<td>4.0 ± 2.2</td>
<td>n.d.</td>
<td>n.d.</td>
<td>22 ± 2.1</td>
<td>24 ± 2.1</td>
</tr>
</tbody>
</table>

Animal characteristics of rats fed a VAD diet, then were bile duct ligated and received vitamin A therapy, including corresponding controls. Data displayed as mean ± SD.

Significantly different from V AS-sham-vehicle; bsignificantly different from V AS-sham-RP; csignificantly different from V AS-BDL-vehicle; dsignificantly different from V AS-BDL-RP; esignificantly different from V AD-sham-vehicle; fsignificantly different from V AD-sham-RP; gsignificantly different from V AD-BDL-vehicle; hsignificantly different from V AD-BDL-RP.
Figure 5.3. Vitamin A therapy rescued VAD BDL animals from dramatic weight loss and a severe cholestatic phenotype

Vitamin A deficient, bile duct ligated (VAD-BDL) or sham treated (VAD-sham) rats received either retinyl palmitate (RP) or vehicle (-) IP on all days following surgery and were sacrificed 7 days after surgery. Vitamin A therapy prevented excessive weight loss of VAD BDL animals. Data displayed as percentage of weight prior to BDL (day 0), means ± SD (A). Vitamin A therapy prevented macroscopic liver damage as observed in VAD-BDL-vehicle treated rats (B). Livers of VAD-BDL rats receiving vitamin A therapy (C) resembled the livers of VAD-BDL rats (compare to Figure 5.2B). Hepatocyte and bile duct damage markers AST (D), ALT (E), AP (F) and γGT (G) were drastically reduced in VAD-BDL after RP supplementation. Data displayed as a box plot with medians represented by the horizontal lines with the 75th percentiles at the top and the 25th percentiles at the bottom of the boxes. Ranges are represented as whiskers. Significant difference between groups, p<0.05, a significantly different from VAD-sham-vehicle; b significantly different from VAD-sham-RP; c significantly different from VAD-BDL-vehicle; d significantly different from VAD-BDL-RP.
Hematoxylin and eosin (H&E) stainings confirmed the macroscopic damage in the livers of VAD-BDL rats (Figure 5.4). Large necrotic areas were readily detectable in the livers of VAD-BDL rats (Figure 5.4D). Such necrotic regions were only rarely observed and much smaller in VAS-BDL liver (Figure 5.4C), while they were absent in sham operated VAS and VAD rats (Figure 5.4A and B).

Figure 5.4. Vitamin A deficiency gives rise to necrotic regions in livers of bile duct ligated rats
Hematoxylin and eosin (H&E) stainings were performed to visualize liver damage. Large areas of the liver, both on the surface and inside the liver were necrotic in VAD-BDL rats (D). Such necrotic regions were infrequent and much smaller in VAS-BDL liver (C), while they were absent in sham operated VAS and VAD rats (A and B).
In addition, VAD-BDL livers were characterized by a much more pronounced bile duct proliferation compared to VAS-BDL livers (Figure 5.5 A-D). This was also evident from the significant increase in cytokeratin 19 (Krt19) (Figure 5E), a marker of cholangiocytes, which was 33-fold increased in VAD-BDL and only 12-fold increased in VAS-BDL, both compared to VAS-sham livers. RP supplementation strongly suppressed the bile duct proliferation in VAD-BDL rats, which was accompanied by a significant reduction in Krt19 mRNA levels in VAD-BDL rats to 20-fold increased compared to VAS-sham (Figure 5.5). RP supplementation did not change Krt19 expression nor bile duct proliferation (data not shown) in VAS-BDL rats.

**Figure 5.5. Vitamin A deficiency aggravates BDL-induced bile duct proliferation and is reversed by vitamin A therapy.**

A-D) Immunohistochemical staining for Ki67 on livers from a control rat (A), a VAS-BDL rat (B) a VAD-BDL rat (C) and a VAD-BDL rat treated with retinyl palmitate (D). BDL leads to bile duct proliferation in VAS rats as shown by Ki67 positive bile duct epithelial cells (B). The area of Ki67-positive bile duct cells is strongly increased in VAD-BDL rats (C). Retinyl palmitate treatment strongly reduces bile duct proliferation and the number of Ki67 positive bile duct epithelial cells in VAD-BDL rats (D). Insets show enlarged areas with bile duct proliferation. E) Quantitative RT-qPCR analysis of the cholangiocyte marker Krt19. Hepatic Krt19 expression was significantly higher after 7 days BDL in VAD rats compared to VAS rats. Vitamin A therapy (RP) reduced Krt19 levels in VAD-BDL rats, but not in VAS-BDL rats. mRNA data was corrected for 18S and presented as a box plot with medians represented by the horizontal lines with the 75th percentiles at the top and the 25th percentiles at the bottom of the boxes. Ranges are represented as whiskers. Significant difference between groups, p<0.05, a significantly different from VAS-sham-vehicle; b significantly different from VAS-BDL-vehicle; c significantly different from VAD-BDL-vehicle; d significantly different from VAD-BDL-RP.
Retinyl Palmitate Does Not Change Cyp7a1 and Bsep Expression in VAS-BDL and VAD-BDL Rats

FXR/RXRα-mediated expression of mouse/human Bsep/BSEP is highest in the absence of RXR-ligands (27, 35). Moreover, VAD rats display increased bile acid concentrations in blood compared to control rats (Chapter 4). Thus, we next analyzed whether the excessive liver damage in VAD-BDL rats may be caused by high Bsep and Cyp7a1 expression resulting in bile accumulation in bile ducts.

Figure 5.6. BSEP expression does not correlate with increased Cyp7a1 expression as a result of VAD and VAD-BDL. Cyp7a1 mRNA expression is increased after 7 days of BDL in both VAS and VAD rats (A). This increase in bile salt synthesis in BDL animals in not matched by increased expression of Bsep mRNA (B). Increased levels of Bsep protein in liver homogenates (C) and liver tissue (D) was also not observed in VAD and VAD-BDL animals. The sodium/potassium-(Na⁺K⁺) pump was used as a loading control in western blot analyses. mRNA data was corrected for 18S and presented as a box plot with medians represented by the horizontal lines with the 75th percentiles at the top and the 25th percentiles at the bottom of the boxes. Ranges are represented as whiskers. Significant difference between groups, \( p<0.05 \), *significantly different from VAS-sham-vehicle; †significantly different from VAS-sham-RP; ‡significantly different from VAS-BDL-vehicle; §significantly different from VAS-BDL-RP; ‡significantly different from VAD-sham-vehicle; ‡significantly different from VAD-sham-RP; ‡significantly different from VAD-BDL-vehicle; §significantly different from VAD-BDL-RP.
In line with earlier studies we found that *Cyp7a1* (12.7-fold) and *Bsep* (1.9-fold) expression were increased by BDL in VAS rats (*Figure 5.6A* and *B* and *Table 5.3*). *Cyp7a1* expression is induced to the same extent in VAD-BDL rats (10.1 ± 3.7-fold compared to VAS-sham) and in VAS-BDL (12.7 ± 2.4-fold compared to VAS-sham) rats. Moreover, *Bsep* expression is not increased by VAD in BDL rats and effectively 55% lower compared to VAS-BDL rats. Whereas RP supplementation dramatically reduced liver damage in VAD-BDL rats, it did not change expression of *Cyp7a1* or *Bsep*. To rule out possible post-transcriptional regulation or high local expression of *Bsep* in functional liver tissue in VAD-BDL rats, we also analyzed Bsep protein expression by western blotting and immunohistochemistry. These analyses revealed no general or local increase in BSEP expression in VAD-BDL livers (*Figure 5.6 C and D*).

Serum (1.187 ± 0.357 mmol/L) and liver (2.01 ± 0.30 μmol/g) bile salt concentrations were increased in VAS-BDL, but not significantly different in VAD-BDL animals (1.206 ± 0.268 mmol/L and 2.69 ± 1.39 μmol/g, respectively (*Figure 5.7* and *Table 5.2*). While RP supplementation seems to lower the bile salt concentrations in VAD-BDL a bit, no significant difference with vehicle-treated VAD-BDL rats was detected. Taken together, (de)regulation of bile salt synthesis and transport in VAD-BDL could not explain the excessive liver damage in VAD-BDL rats, nor the therapeutic effect of RP supplementation.

![Figure 5.7. Vitamin A does not change serum and liver bile salt concentrations of BDL rats](image)

Bile salt concentrations in serum (A) and liver tissue (B) of sham- or BDL-treated VAS and VAD animals. BDL increased serum and hepatic bile acid concentrations to a similar level in VAS and VAD rats. Moreover, vitamin A supplementation (RP) did not significantly change serum and hepatic bile salts concentrations in VAD-BDL animals. Data presented as mean ± SD. *) indicates significant difference from the respective control; *p* < 0.05. †) indicates significant difference between RP and vehicle treated animals; *p* < 0.05.
Table 5.3. Relative mRNA expression data

<table>
<thead>
<tr>
<th>Gene</th>
<th>Group</th>
<th>Condition</th>
<th>Mean ± SD</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V AS-sham-vehicle</td>
<td></td>
<td>1.0 ± 0.2</td>
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</tr>
<tr>
<td></td>
<td>V AS-sham-RP</td>
<td></td>
<td>1.2 ± 0.3</td>
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</tr>
<tr>
<td></td>
<td>V AS-BDL-vehicle</td>
<td></td>
<td>1.7 ± 0.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>V AS-BDL-RP</td>
<td></td>
<td>12.3 ± 2.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>V AD-sham-vehicle</td>
<td></td>
<td>1.4 ± 0.3</td>
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<tr>
<td></td>
<td>V AD-sham-RP</td>
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<td>1.8 ± 1.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>V AD-BDL-vehicle</td>
<td></td>
<td>32.7 ± 7.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>V AD-BDL-RP</td>
<td></td>
<td>61.3 ± 17.5</td>
<td></td>
</tr>
</tbody>
</table>

Relative mRNA data determined by RT-qPCR displayed as mean ± SD. 

- a significantly different from V AS-sham-vehicle; 
- b significantly different from V AS-sham-RP; 
- c significantly different from V AS-BDL-vehicle; 
- d significantly different from V AS-BDL-RP; 
- e significantly different from V AD-sham-vehicle; 
- f significantly different from V AD-sham-RP; 
- g significantly different from V AD-BDL-vehicle; 
- h significantly different from V AD-BDL-RP; 
- p > 0.05.
Chapter 5

A

Relative Ho-1 expression (mRNA versus 18S)

B

Relative iNOS expression (mRNA versus 18S)

C

Relative α-Sma expression (mRNA versus 18S)

D

Relative Col1a1 expression (mRNA versus 18S)

E

Relative HO-1 expression (protein versus GAPDH)

F

Relative α-SMA expression (protein versus GAPDH)

All figures show data from sham and BDL groups with and without Ringer's perfusion (RP) and VAD treatment.
Vitamin A Deficiency Severely Aggravates Obstructive Cholestasis

Figure 5.8. Vitamin A therapy lowers oxidative stress, inflammation and fibrosis markers in VAD obstructive cholestatic rats

mRNA levels of markers of oxidative stress hemeoxygenase-1 (Ho-1) (A), inflammation inducible nitric oxide synthase (iNos) (B) and fibrosis alpha-smooth muscle actin (α-Sma) (C) and Collagen type I (Col1a1) (D) were all increased in livers of VAD-BDL animals. Transcription of all these markers was normalized to levels observed in VAS-BDL-treated animals by RP supplementation. Vitamin A therapy prevented the elevated expression of HO-1 and α-SMA in VAD animals as a result of BDL (E and F). Protein expression was determined for all animals individually and corrected for GAPDH. Western blots shown are representative figures. Data is presented as mean ± SD. mRNA data was corrected for 18S and presented as a box plot with medians represented by the horizontal lines with the 75th percentiles at the top and the 25th percentiles at the bottom of the boxes. Ranges are represented as whiskers. Significant difference between groups, $p<0.05$, significantly different from VAS-sham-vehicle; $b$ significantly different from VAS-sham-RP; $c$ significantly different from VAS-BDL-vehicle; $d$ significantly different from VAS-BDL-RP; $e$ significantly different from VAD-sham-vehicle; $f$ significantly different from VAD-sham-RP; $g$ significantly different from VAD-BDL-vehicle; $h$ significantly different from VAD-BDL-RP.

Vitamin A Therapy Reduces, Necrosis, Oxidative Stress, Inflammation and Fibrosis

Besides a putative role in regulating bile salt homeostasis, vitamin A(-derivatives) may also act as anti-oxidant, immune regulator and inhibitor of inflammation. In the liver specifically, vitamin A may control the development of fibrosis as activation of hepatic stellate cells is associated with loss of their retinol stores (13). Bile duct ligation in VAS rats increased the transcription of heme oxygenase-1 (Ho-1; oxidative stress marker), inducible nitric oxide synthase (iNos; inflammation marker), alpha-smooth muscle actin and collagen type I (α-Sma and Col1a1; fibrosis markers) by 5.5-, 8.0-, 2.9- and 12-fold, respectively, compared to sham operated VAS rats (Figure 5.8 and Table 5.3). VAD alone did not increase iNos, α-Sma and Col1a1 mRNA expression and only slightly induced Ho-1 (3.5-fold). However, all these markers were strongly increased in VAD-BDL rats (Ho-1, iNos, α-Sma and Col1a1 to 21-, 167-, 33- and 81-fold, respectively, compared to VAS animals); an increase of respectively 275, 1049 and 563 % compared to VAS-BDL. RP supplementation prevented the increase of all these markers in VAD animals as a result of BDL and the transcript levels of these four genes were similar or close to levels observed in VAS-BDL animals (Figure 5.8 A-D). Western blot analyses confirmed the strong increase in HO-1 and α-SMA protein expression in the livers of VAD-BDL rats and their repression upon RP supplementation (Figure 5.8 E and F).

Discussion

In this study, we show that vitamin A deficiency severely aggravates liver damage in rats with obstructive cholestasis, progressing to a life-threatening condition after 7 days. Hepatocyte necrosis, bile duct proliferation, inflammation, oxidative stress and activation of hepatic stellate cells are dramatically increased when vitamin A is absent in obstructive cholestasis, but
are all strongly repressed when these rats are treated with retinyl palmitate. Vitamin A is therefore essential to prevent excessive liver damage in obstructive cholestatic liver disease. Vitamin A deficiency is a frequent condition in patients with chronic liver disease (10, 36-41). Disturbed bile salt homeostasis impairs intestinal absorption of fat-soluble vitamins and liver injury promotes the depletion of the vitamin A stores from hepatic stellate cells. Especially new-borns with obstructive cholestasis, as in biliary atresia, are at high risk for vitamin A deficiency as they have limited supplies of vitamin A immediately after birth. However, vitamin A deficiency is not restricted to chronic cholestatic liver diseases. Also inflammatory bowel disease (IBD), diabetes, obesity and alcoholic liver diseases are associated with vitamin A deficiency (42-49). Furthermore, treatment with various drugs may lower the hepatic vitamin A content (50). Establishment of vitamin A deficiency as an independent factor that causes liver damage is therefore relevant for a large and variable patient group.

Vitamin A supplementation has been shown before to have beneficial effects in models of chronic liver disease, including long term (5-6 weeks) BDL and CCl₄ administration (13-18). The combination of all-trans retinoic acid and ursodeoxycholic acid (UDCA), a synthetic bile acid used to treat cholestasis in humans, proved more successful in reducing liver fibrosis and necrosis as a result of BDL than UDCA alone (51). Until now, these studies were performed with vitamin A sufficient animals and did not reveal the dramatic effects we detected in VAD rats. We did not observe significant therapeutic effects of RP supplementation in VAS rats after 7 days BDL with respect to liver damage markers AST, ALT, γGT and serum bile salts. In fact, we detected a significant increase in the inflammation marker iNos in RP-treated VAS-BDL rats, whereas oxidative stress and stellate cell activation were not altered. The therapeutic effects detected in other studies are therefore likely to occur at later stages of liver disease. This is in line with our observation that liver retinol levels are actually increased after 7 days BDL, while serum levels are decreased. This suggests that vitamin A is recruited to the liver at the initial stages of bile obstruction and that only after progression of the disease hepatic VAD develops due to chronic intestinal malabsorption and hepatic stellate cell activation.

Vitamin A deficiency and vitamin A therapy did not alter bile salt levels in liver or serum, nor the regulation of hepatic bile salt synthesis and transport at day 7 after bile duct ligation. This is in contrast to our earlier observations in vitro and in mice that FXR-mediated expression of BSEP is maximal in the presence of FXR- and absence of RXRa ligands (Chapter 2, (27)). Moreover, we found earlier that VAD rats develop mild cholestasis (Chapter 4). Both in VAS and VAD animals RP supplementation seems to lower bile salt concentrations in serum, although this decrease did not reach statistical significance (Table 5.2).

We expected that excessive liver damage would arise from increased bile salt accumulation in liver bile ducts leading to bile infarcts. Much of the observed phenotype actually resembles effects of maintained or increased canalicular bile salt transport. In particular the excessive bile duct proliferation and bile duct damage, as indica-
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ted by high γGT levels, suggest increased bile toxicity in the liver. Even though no increased BSEP levels were detected at day 7, it is possible that BSEP-mediated secretion earlier after the start of bile duct ligation is higher in the VAD animals and is the primary cause of excessive liver damage. When secondary inflammation and oxidative stress develop, BSEP expression may become suppressed as described for inflammation models of the liver (52, 53). The combination of VAD and BDL leads to the strong accumulation of bile in front of the ligation, suggesting increased bile flow in these animals. Remarkably, this increased bile flow was not suppressed by RP supplementation, while bile duct proliferation and γGT were strongly decreased. This indicates that RP reduces the levels of (a) toxic compound(s) in bile without reducing bile flow.

In contrast to genes involved in bile salt homeostasis, expression of biomarkers for oxidative stress (Ho-1), inflammation (iNos) and fibrosis (α-Sma and Col1α1) were strongly increased in VAD-BDL rats and strongly suppressed when these animals received RP supplementation. The transcription profiles of these biomarkers follow the occurrence of hepatic and ductular enzymes in blood plasma. Both BDL and VAD have independently been associated with increased oxidative stress, inflammation and fibrosis (54-58). In our study, we did not observe induction of iNos, α-Sma and Col1α1 and only a minor induction of Ho-1 mRNA in VAD rats. The absence of clear changes in these markers in VAD rats may be because the rats in this study were just depleted from vitamin A, whereas in other studies they may have been suffering from vitamin A deficiency for prolonged periods. Our data indicate that vitamin A deficiency per se is not inducing hepatic stellate cell activation and thus does not immediately lead to fibrosis.

However, in combination with bile obstruction, stellate cells become superactivated. Rather than causing fibrosis in such a short time period, this may render the stellate cells highly contractile because of the strong induction of α-SMA expression. As these highly extended cells completely surround the sinusoids, cell contraction may lead to reduced blood flow through the liver. As this may lead to reduced oxygenation of the liver, it could contribute to the observed oxidative stress. Normal levels of vitamin A are required for integrity of epithelial barriers in various tissues and vitamin A supplementation has been reported to be beneficial in various disorders where inflammation is involved (57).

RP supplementation (vitamin A therapy) prevented the formations of necrotic regions and the strong increase in Ho-1, iNos, α-Sma and Col1α1 transcription/expression in VAD-BDL animals and thus has acute therapeutic effect to slow down the process of liver injury by obstructive cholestasis in VAD rats. Vitamin A may directly repress the activation of stellate cells (59) and/or by its anti-oxidant function, reduce oxidative stress in the liver. Moreover, vitamin A may inhibit the influx of macrophages and strongly reduce inflammation (60). Most likely, the strong repression of liver damage by RP supplementation in VAD-BDL rats is a combination of several or all of these processes that may occur at specific time points after the start of the obstruction.
Independent of the exact mechanisms involved, this study reveals the importance of vitamin A in preventing excessive liver damage in chronic liver diseases. Until now, this vitamin is regarded as an important vitamin to monitor in liver diseases, but not as a factor that may actually contribute to liver damage when vitamin A stores are limited.

Contributing to the controversy surrounding vitamin A therapy is the lack of reliable non-invasive biomarkers to assess vitamin A status. As is evident from multiple studies, plasma retinol levels are a poor measure for the total pool of vitamin A in the body. Both in hyper- and hypovitaminosis A, plasma retinol levels may appear within normal range. Only when the liver is overflowing with, or nearly depleted of, vitamin A, this can be measured in plasma. And only when plasma retinol levels drop below 10 mg/L in humans this is correlated to the vitamin A content in the liver (61). In line with this, we found that serum retinol levels drop only after 12 weeks of feeding a VAD diet to rats. Assuming a linear decrease of the liver stores of vitamin A during the diet period, the liver had lost over 90% of its vitamin A content before serum levels dropped significantly.

Taken together, our data show that vitamin A deficiency strongly aggravates liver damage in obstructive cholestasis in rats and retinyl palmitate supplementation is a safe and effective therapeuticum to reduce liver damage. These findings warrant a detailed analysis and monitoring of the vitamin A status in patients with cholestasis, as the condition of cholestatic patients with low vitamin A levels could deteriorate quickly, but might be effectively treated with vitamin A.

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