Received: 23 February 2022 | Revised: 29 June 2022 | Accepted: 9 July 2022

DOI: 10.1002/jat.4364

RESEARCH ARTICLE

Journal of AppliedToxicology WILEY

Disruption of vitamin A homeostasis by the biocide tetrakis(hydroxymethyl) phosphonium sulphate in pregnant rabbits

Natalia Estrada-Ortiz[1] | Viktoriia Starokozhko[1] | Hidde van Steenwijk[1] | Cor van der Heide[1] | Hjalmar Permentier[1] | Lisanne van Heemskerk[1] | Grietje Harmanna Prins[1] | Janette Heegsma[2] | Klaas Nico Faber[2] | Steffi Bressers[3] | Guy Steiblen[4] | Antoinette de Groot[5] | Steve Groome[6] | Erik van Miert[5] | Geny Groothuis[1] | Inge Anne Maria de Graaf[7,8]

[1]Groningen Research Institute of Pharmacy (GRIP), University of Groningen, Groningen, The Netherlands

[2]Department of Gastroenterology and Hepatology, University of Groningen, University Medi-cal Center Groningen, Groningen, The Netherlands

[3]Charles River Laboratories, 's-Hertogenbosch, The Netherlands

[4]Solvay, Toxicological and Environmental Risk Assessment Unit, Genas, France

[5]Solvay, Toxicological and Environmental Risk Assessment Unit, Bruxelles, Belgium

[6]Solvay, Product Stewardship, Oldbury, UK

[7]University Medical Center Groningen, Surgical Research Laboratory, University of Groningen, Groningen, The Netherlands

[8]School of Science and Engineering, University of Groningen, Groningen, The Netherlands

Correspondence
Inge Anne Maria de Graaf, University Medical Center Groningen, Surgical Research Laboratory, University of Groningen, Groningen, The Netherlands.
Email: i.a.m.de.graaf@rug.nl

Abstract

The biocide tetrakis(hydroxymethyl)phosphonium sulphate (THPS) and other members of the tetrakis(hydroxymethyl) phosphonium salts (THPX) family are associated with liver toxicity in several mammalian species and teratogenicity in rabbits. Malformations include skeletal changes and abnormalities in eye development and are very similar to those seen with vitamin A deficiency or excess. For this reason, it was hypothesized that teratogenicity of THPS(X) might be attributed to disturbances in retinol availability and/or metabolism as a result of maternal toxicity, for example, either due to insufficient dietary intake by the mothers or due to liver toxicity. Therefore, in the present study, liver toxicity and vitamin A homeostasis were studied in pregnant rabbits that were exposed to 13.8 or 46.0 mg/kg THPS during organogenesis and in precision-cut liver slices of rats and rabbits exposed to 0–70 μM THPS. Results show that in vivo exposure to THPS leads to a marked reduction of food intake, increased plasma concentrations of γ-glutamytransferase, degenerative changes in the liver and to changes in retinoid content in liver and plasma in the rabbits during organogenesis. In addition, THPS, both in vivo and ex vivo, caused a change in expression of proteins related to vitamin A metabolism and transport. Together, these observations could explain the birth defects observed in earlier teratogenicity studies.

KEYWORDS

cytochrome p45026A1, precision-cut liver slices, retinoid binding proteins, retinoids, teratogenicity, tetrakis(hydroxymethyl) phosphonium sulphate, THPS, VAD, vitamin A homeostasis

1 | INTRODUCTION

The biocide tetrakis(hydroxymethyl) phosphonium sulphate (2/1), CAS number 55566-30-8 (THPS), and other members of the tetrakis(hydroxymethyl) phosphonium salts (THPX) family are associated with liver toxicity in several mammalian species (i.e., dogs, mice, rats, and rabbits) (van Esch, 2000). In addition, in rabbits, exposure to THPS and THP(X) species during pregnancy was shown to lead to severe birth defects in the offspring. These malformations include skeletal changes and abnormalities in eye development (Barker, 1991). Although some of these abnormalities were also observed in rat teratogenicity studies, the frequency was comparable with historical data of control rats so they could not be attributed to the compound. Effects on the offspring in rabbits were only seen at dose levels at which maternal toxicity (reduced food intake, weight loss, and hepatotoxicity) was observed, at 45 mg/kg THPS.

The mechanism of the eye and skeletal effects following THPX exposure is not known. However, vitamin A deficiency (VAD) or excess is associated with clinical findings (microphthalmia, aphakia, retinal dysplasia, digits absence, or shortening; Clagett-Dame & Knutson, 2011; Maden, 2001) similar to those observed with THP(X) species. For this reason, it was hypothesized that teratogenicity of THPS(X) might be attributed to disturbances in retinol availability and/or metabolism as a result of maternal toxicity, for example, either due to insufficient dietary intake by the mothers or due to liver toxicity. The liver plays an important role in vitamin A homeostasis, not only because it is the major location for storage of retinoids but also because it produces proteins, like retinol binding protein 4 (RBP4) and transthyretin (TTR) that play an important role in transport of retinoids to peripheral tissues (e.g., the embryonic yolk sac) and uptake of retinoids from blood to tissue compartments (via the signalling receptor and transporter of retinol, STRA6, the assumed receptor for RBP4) (Clagett-Dame & Knutson, 2011; O'Byrne & Blaner, 2013). Apart from its role in retinoid storage and distribution, some liver functions also depend on vitamin A. Particularly, retinoids play an important role in fat storage in the liver and VAD has been associated with nonalcoholic steatohepatitis (Saeed, Dullaart, et al., 2017).

After uptake in cells, retinol binds to retinol binding protein CRBP (RBP1), and as such can be bio-activated into retinoic acids in two steps. In the first step, retinylaldehyde is formed, which is further metabolized into 9-cis-retinoic acid (9-cis-RA) and all-trans retinoic acid (ATRA), amongst others. These retinoic acids are the actual bioactive components that mediate embryogenesis and many other processes through binding with nuclear receptors, of which RXR (retinoid X receptor) and RAR (retinoic acid receptor) are thought to be the most important in embryogenesis (Clagett-Dame & Knutson, 2011; O'Byrne & Blaner, 2013) (Figure 1).

Human exposure to THPS and other THPX species is low (unpublished research done in the context of registration of the compound). The application of THPS is mainly in oilfields, where it is used by workers that are protected from exposure by personal protection equipment. Also, exposure to wildlife and entrance in the food-chain is negligible; THPS degrades abiotically, is readily biodegradable, and is non-bioaccumulating. Nevertheless, due to the reliance of classification and labelling upon intrinsic hazards, an understanding of the mode of action is important. To investigate our hypothesis and shed light on the effects of THPS on key players in vitamin A homeostasis, both in vivo and ex vivo experiments were performed. For the in vivo experiments, pregnant rabbits were exposed to different doses of THPS by gavage. In the ex vivo experiment, liver slices of rabbits and rats were exposed to various concentrations of THPS. This approach was chosen to separate other maternal effects (such as reduced food

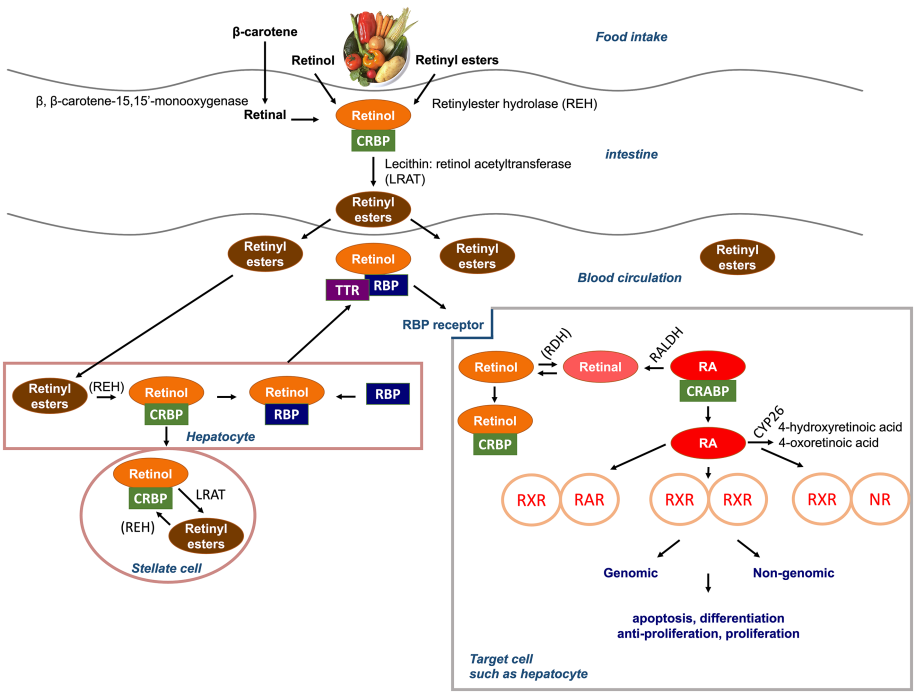

FIGURE 1 Vitamin A metabolism and functions

intake) from the liver effects regarding vitamin A homeostasis. In this paper, we present the results of these studies and discuss potential mechanisms of THPS teratogenicity.

2 | MATERIALS AND METHODS

2.1 | In vivo experiments

Fifteen female New Zealand White rabbits (Charles River, Chatillon sur Chalaronne, France), 17–19 weeks old and weighing between 3074 and 4062 g at the initiation of dosing, were assigned to three dose groups (five rabbits/group) by a computer-generated random algorithm according to body weights. The rabbits arrived on Day 0 post coitum, which was defined as the day of successful mating. The rabbits were housed individually in cages with perforated floors (Ebeco, Germany, dimensions 67 × 62 × 55 cm), equipped with water bottles. Municipal tap water was freely available via these water bottles, and pelleted diet for rabbits (Global Diet 2030 from Harlan Teklad®, Mucedola, Milanese, Italy) was provided ad libitum throughout the study. In addition, pressed hay (Tecnilab-BMI bv, Someren, The Netherlands) was provided during the study period. Target temperatures of 18°C to 24°C and relative target humidity of 40% to 70% and a 12-h light/12-h dark cycle were maintained. The study plan was reviewed and agreed by the Animal Welfare Body of Charles River Laboratories Den Bosch B.V. within the framework of project license AVD2360020172866 approved by the Central Authority for Scientific Procedures on Animals (CCD) as required by the Dutch Act on Animal Experimentation (December 2014).

Animals of group 1 received water (vehicle control, 5 ml daily), whereas groups 2 and 3 received THPS. THPS, as a solution of 76.6% w/w in water, was provided by Solvay Solutions UK Limited. Rabbits received 5 ml of THPS (76.6%) diluted in water to 3.6 or 12 mg/ml to reach dose levels of 18 mg/kg (low dose) and 60 mg/kg (high dose) (13.8 or 46.0 mg/kg of the active ingredient THPS, respectively). Vehicle and THPS were administered once daily by oral gavage from day 7 up till and including day 19 post coitum. Dose and dosing schedule were chosen to be equal to earlier OECD414 teratogenicity studies. Mortality/morbidity checks were done twice daily, whereas clinical observations were done at least once a day beginning on day 7 post coitum and lasting up to the day prior to necropsy. In addition, body weight was assessed on days 3, 7, 9, 12, 15, 18, and 20 post coitum. Food consumption was quantitatively measured for day 3–7, 7–9, 9–12, 12–15, 15–18, and 18–20 post coitum. Water consumption was monitored on a regular basis by visual inspection of the water bottle. For laboratory evaluations, blood was collected every 2 days, starting on day 7 post coitum prior to dosing, and on the day of scheduled necropsy, processed to plasma, snap frozen and stored at −80°C. Faeces was collected every 2 days, starting on day 3 post coitum, and on the day of scheduled necropsy.

On day 20 post coitum, all rabbits were euthanized by intravenous injection of pentobarbital (approx. 1 ml/kg Euthasol® 20%). For necropsy, all animals were subjected to an external, thoracic and abdominal examination, with special attention paid to the liver, intestines, and stomach. Pieces of liver (20–70 mg) were snap frozen for biochemical analysis of triglyceride content, retinoid content, and gene and protein expression. Figure 2 summarizes the dosing and sampling scheme.

2.2 | Liver and intestinal toxicity determination in the in vivo study

Clinical chemistry (using an automated clinical analyser, Olympus AU400) was performed on the samples processed to plasma of day 20 post coitum to determine alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), alkaline phosphatase (ALP), and γ-glutamyl transferase (GGT) content. In addition, liver and intestines of all animals underwent histopathological examination after haematoxylin and eosin staining.

To quantify the extent of hepatic steatosis, the triglyceride (TG) content was determined in liver samples after extraction with a mixture of chloroform/methanol/water (2:2:1.8) according to Bligh and Dyer (1959). After 30 min of vigorous mixing, additional chloroform (500 µl) and water were added to improve the yield of triglycerides. Phase separation was achieved by centrifugation. The lower phase (consisting of chloroform and lipids) was collected. After evaporation of chloroform, extracted lipids were resuspended in 600 µl chloroform containing 2% Triton-X100. After evaporation of chloroform, extracted lipids were resuspended in MilliQ water. Triglyceride content in extracted lipids was measured at 540 nm using a Trig/GB kit (Roche Molecular Biochemicals, Almere, the Netherlands)

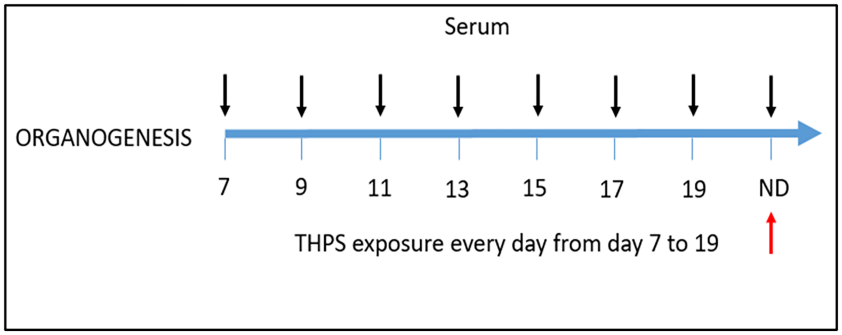

FIGURE 2 Dosing and sampling scheme of the rabbit study in vivo. THPS was given every 24 h, between day 7 and 19 post-coitum (period of organogenesis). Sampling of serum for determination of RBP4 and retinol took place every 48 h starting from day 7, and at the day of necropsy (day 20) (black arrows). At the day of necropsy, in addition, plasma was sampled to evaluate liver enzymes and liver and intestinal samples were taken for determination of key players in vitamin A homeostasis (red arrow) [Colour figure can be viewed at wileyonlinelibrary.com]

according to the protocol provided by the manufacturer. Liver TG content was normalized to protein content measured by the Lowry assay (BioRad DC Protein Assay, Herculus, CA, USA) according to instructions from the manufacturer.

2.3 | Ex vivo experiments with precision-cut liver slices (PCLS)

Male Wistar rats (Charles River, Saint Germain-Nuelles, France) of 250–300 g were housed under a 12 h dark/light cycle at constant humidity and temperature. Animals were permitted ad libitum access to tap water and standard lab chow. To obtain the liver, rats were anaesthetized with isoflurane/O_2. Hereafter, the animals died by exsanguination. All experiments were approved by the committee for care and use of laboratory animals of the University of Groningen and were performed according to strict governmental and international guidelines. Livers from female White New Zealand Rabbits (untreated control animals from other studies) were obtained with approval of the Radboud UMC animal ethical committee. The rabbits were sedated with domitor/ketamin and sacrificed with an overdose pentobarbital. Rabbit livers were perfused in situ with UW to immediately cool down the liver. Livers harvested from the rabbits and rats were immediately placed in University of Wisconsin solution (UW, ViaSpan, 4°C) until further use.

Liver cores were prepared by using a hollow drill bit. PCLS were made as described by de Graaf et al. (2010); the cores were sliced with a Krumdieck tissue slicer (Alabama R&D, Munford, AL, USA) in ice-cold Krebs–Henseleit buffer, pH 7.4, saturated with carbogen (95% O_2 and 5% CO_2).

Liver slices (5 mg, ~250 μm thickness) were incubated individually in 12-well plates (Greiner bio-one GmbH, Frickenhausen, Austria), at 37°C in 1.3 ml Williams' medium E (WME, Gibco by Life Technologies, UK) with glutamax-1, supplemented with 25-mM D-glucose (Gibco) and streptomycin (Gibco) in an incubator (Panasonic biomedical) in an atmosphere of 80% O_2 and 5% CO_2 while shaking horizontally (90 times per minute). Slices were preincubated for 1 h to remove debris and dead cells and then transferred to plates with fresh medium with THPS. Stock solutions of THPS were prepared as 100× concentrated stock solutions and were added to the medium (13 μl), 20 min before starting the incubation. For each liver, three slices received the same treatment. After this, PCLS were incubated for 24 (rat and rabbit slices) or 72 h (rat slices). After 24 h of culturing, medium was refreshed and test compounds were again added. At the end of incubation, slices were snap frozen to determine ATP content and for RNA isolation or preserved in 4% w/v formalin for histomorphological studies. Medium was collected and kept at 4°C for LDH measurement.

2.4 | Liver toxicity determination in PCLS

To assess viability, slices were collected individually after incubation for ATP and protein determination, by snap freezing them in 1 ml of ethanol (70% v/v) containing 2-mM EDTA with pH = 10.9. After thawing, the slices were homogenized using a minibead beater and centrifuged. The supernatant was used for the ATP assay using the ATP Bioluminescence Assay kit CLS II (Roche, Mannheim, Germany) as described previously (de Graaf et al., 2010). The protein content of the PCLS was determined in the remaining pellet by the Bio-Rad DC Protein Assay using a bovine serum albumin (BSA, Sigma-Aldrich, Steinheim, Germany) calibration curve. The TC_{50} value was calculated as the concentration of THPS reducing the viability of the slices by 50%, in terms of ATP content corrected by the protein amount of each slice, and relative to the slices without any treatment using a nonlinear fitting of log (concentration compound) versus response.

Furthermore, a lactate dehydrogenase (LDH) assay was performed to determine the percentage of LDH leakage as a second viability parameter by using the Cyto Tox-ONE Homogenous Membrane Integrity Assay kit (Promega, Madison, USA). After the incubation with the tested compounds, 200-μl medium from every well was collected and kept at 4°C for maximally 1 week. In addition as a control, three slices were collected separately after 1 h preincubation in empty tubes (1.5-ml Eppendorf). The control slices were homogenized for 45 s with glass beads and 1.3 ml of WME in a minibead beater (Biospec Products, Bartlesville, USA) and centrifuged. WME culture medium was used as blank control. A volume of 50 μl of each medium or supernatant of the sample, blank, and control were pipetted into a black 96-well plate (Costar cat# 3915) and then 50 μl of Cytox-one reagent was added to each well, and the plates were covered with aluminium foil and incubated for 10 min. Afterwards, 25 μl of stop solution was added to each well and the fluorescence was read at excitation 590 nm and emission 560 nm using a Spectra Max Gemini XPS fluorescence plate reader (Molecular Devices). The LDH content of the control PCLS after 1-h preincubation was used as a 100% value of LDH content in a slice at the beginning of the incubation.

In addition, liver slice histomorphology was investigated. For this, PCLS were fixated in 4% formalin for 24 h and stored in 70% ethanol at 4°C until processing for morphology studies. After dehydration, the slices were embedded in paraffin and sectioned (sections 4 μm thick) parallel to the surface of the slice and stained with H&E for histopathological evaluation.

Lipid (triglyceride) accumulation in PCLS was examined using Oil Red O (ORO) staining as described previously by Kinkel et al. (2004) with some modifications. First, cryo-blocks of the slices were prepared by using a 1 × 1 cm metal mould mounted with a thin glass cover slip at the bottom. The blocks were assembled by slowly pouring a thin layer of cryogenic gel (Klinipath, the Netherlands); the mould was placed in a small volume isopentane (Sigma-Aldrich, Germany) on dry ice to solidify the gel. Directly after the incubation, slices were placed on a solidified layer of gel and covered with a new layer of gel. This procedure was repeated until the three slices were placed in separate layers. Each block was wrapped in aluminium foil and stored at −80°C until use. After cutting, cryosections were air dried and fixed with 4% formaldehyde/PBS for 10 min at room temperature. After that, the sections were immersed in 60% 2-propanol briefly two times and then stained in ORO solution (0.6-mg Oil Red O powder in 36% 2-propanol) for 10 min. Thereafter, the slides were

again briefly dipped in 60% 2-propanol and rinsed immediately in running tap water. Samples were counterstained in haematoxylin for 1 min followed by a wash step in running tap water for about 3 min. Finally, sections were covered with a cover slip using Aquatex (Merck, Damstadt, Germany) and examined under the light microscope.

2.5 | Retinoid analysis in liver and serum of in vivo studies

Pieces of liver (20–70 mg) and serum of rabbit dams were snap frozen in liquid N_2 and stored at −80°C until use. The pieces of tissue were homogenized with a minibead beater using a metal bead in 1.5-ml antioxidants mixture (400-mg pyrogallol, 50-mg butylated hydroxytoluene in 100-ml ethanol) and 100-µl internal standard acitretin (1.0 ng/µl in acetonitrile). The obtained homogenate was transferred to a nontransparent 15-ml glass tube followed by the addition of 3.0-ml milli-Q water and 1.2 ml of methyl tert-butyl ether (MTBE). The samples were then vigorously mixed with a vortex for 30 s. Afterwards, the tubes were centrifuged at 400 × *g* for 5 min at 4°C. The organic layer was then transferred to a new nontransparent glass tube and dried under a flow of N_2 at room temperature. The obtained product was dissolved in 200-µl acetonitrile. For extraction of serum samples, all the solvents, sample, and internal standard were added to a nontransparent glass tube. The following steps were performed as described for liver homogenates with some modifications on the volumes as follows: 100-µl serum, 0.6-ml antioxidant mixture, 1.2-ml milli-Q water, 100-µl internal standard, and 5-ml MTBE. The extracted samples were kept in the dark at 4°C in the autosampler, and 20 µl were then injected into the HPLC-MS/MS system.

Some of the livers of the dams had a high triglyceride content that has been shown to have a negative influence on the extraction efficiency of fat soluble retinoids (in particular the retinyl esters) (Saeed et al., 2021). For this reason, a more vigorous extraction method using hexane was used for the measurement of retinyl palmitate in the livers of dams. According to this method, 3-ml hexane was added instead of MTBE. After shaking, the hexane layer was separated from the ethanol/water layer. This was repeated once. After the collected hexane had evaporated, the residue was taken up in 200 µl of acetonitrile. Because the use of hexane resulted in ion suppression in the HPLC-MS/MS system (data not shown) using the method described below, the retinylpalmitate content of the livers of dams was determined using HPLC-UV by the protocol originally published by Kim and Quadro (Kim & Quadro, 2010).

Recovery of the liquid–liquid extraction with MTBE was determined by spiking liver homogenate and serum (containing unknown amounts of physiological retinoids) with known amounts of retinol and retinylpalmitate and the internal standard acitretin before extraction (pre-spiked) and after extraction (post-spiked). Extraction efficiency was then calculated as follows: Extraction efficiency = ((pre-spiked − not spiked)/post spiked-not spiked) * 100% and was 108%, 88%, and 38% for retinol in liver, retinol in serum, and retinylpalmitate in serum, respectively. Repeatability of the extraction with MTBE was determined by calculating the variance coefficient of the outcome of five samples of liver/serumhomogenate that were spiked with 1.55 ng/µl retinol and 75 ng/µl retinylpalmitate and was 19.5%, 3.4%, and 19.0% for retinol in liver, retinol in serum, and retinylpalmitate in serum, respectively. For the extraction of retinyl palmitate from liver with hexane a recovery of 75–95% was previously published (Kim & Quadro, 2010), no further in house validation was performed.

The HPLC-UV-MS/MS system consisted of a SIL-20AC HT autosampler, a CTO-20AC column oven, a LC-20 AD liquid chromatograph, a SPD-20A UV/VIS detector (connected in series) (all from Shimadzu, USA), and a API 3000 mass spectrometer from PE Sciex (Canada) with turbo ionspray. For the liver samples, a Phenomenex Aeris 150 × 2.1 mm 3.6 µm C18 Widepore column was used, whereas for serum, a Waters Cortecs C18 + 100 × 2.1 mm 2.7 µm column was used, at an oven temperature of 30°C and a flow rate of 0.3 ml/min. The mobile phases A and B consisted of H_2O + 0.1% formic acid and acetonitrile + 0.1% formic acid, respectively. The LC solvents used were purchased from Biosolve (Valkenswaard, the Netherlands). The gradient for the reverse phase chromatography started at 50% mobile phase B and was increased to 70% in 5 min. Subsequently, the percentage mobile phase B was increased to 100% in 3 min and remained stable for 12 min. Thereafter, the column was flushed for 4 min at 60% mobile phase B.

The analysis of retinoids was performed with a combined LC-UV-MS system, recording LC-UV chromatograms at 350 nm and LC-MS chromatograms in MRM mode. The analytes were quantified with MS using MRM transition m/z 269.3 > 93.2 for both retinol and retinyl palmitate. Both compounds had the same transition because of in source fragmentation of retinylpalmitate into retinol. Unlabelled standards of retinol and retinyl palmitate (Sigma-Aldrich, Merck Life Science NV, Amsterdam, the Netherlands) were used for peak identification and quantification. The retinoid analogue acitretin (Sigma-Aldrich Merck Life Science NV, Amsterdam, the Netherlands) was used as a technical internal standard. Both UV and MS signals were used for quantitation. While the MRM-based LC-MS analyses are very specific and sensitive, they suffer from interferences and ion suppression particularly in complex extracts (like liver and serum). For this reason, the UV signal was used for quantification of retinol in serum. The LC-UV and LC-MS chromatograms were eventually carefully compared to extract the required quantitative information for retinol and retinyl palmitate. For confirmation, the retinol and retinyl palmitate standards were spiked in some extracts to ensure that the correct peaks were selected for quantitation in both UV and MS. For both compounds, no shoulder peaks were apparent in the extracts or the standards, so we concluded that the quantified UV signals can be reliably assigned to retinol and retinyl palmitate. Calibration curves made from the retinol and retinyl palmitate standards were linear in the range of sample concentrations. The lower limit of detection for retinol and retinyl palmitate were 0.05 and 0.007 ng/µl, respectively.

Calibration curves were generally analysed at the start, mid-way, and at the end of sample batch. For quantification, in most cases, the average of the three calibration curves was used.

2.6 | Determination of gene expression in liver samples and PCLS by qRT-PCR

RNA was isolated with the Maxwell® 16 simplyRNA Tissue Kit (Promega, Leiden, the Netherlands) according to the manufacturer's protocol. A piece of liver (10–40 mg) or three slices that had received the same treatment were snap-frozen in RNase free Eppendorf cups and were homogenized in homogenization buffer using a minibead beater and the homogenate was diluted 1:1 with lysis buffer. Afterwards, the mixture was processed using a Maxwell® 16 LEV Instrument. Finally, RNA concentration was quantified on a NanoDrop One UV-Vis Spectrophotometer (Thermoscientific, Wilmington, US) right before conversion to cDNA. For cDNA preparation, RNA samples were diluted to 0.5 μg in 8.5 μl of RNAse free water. cDNA was generated from RNA using random primers with TaqMan Reverse Transcription Reagents Kits (Applied Biosystems, Foster City, CA). To each sample, the following solutions were added: 2.5 μl 5× RT-buffer, 0.25 μl 10-mM dNTPs, 0.25-μl Rnasin (10 units), 0.5-μl M-MLV Reverse Transcriptase (100 units), 0.5-μl random primers. cDNA was generated in the Eppendorf mastercycler (Hamburg, Germany) with a gradient of 20°C for 10 min, 42°C for 30 min, 20°C for 12 min, 99°C for 5 min, and finally, 20°C for 5 min. Real-time quantitative PCR was used to determine relative mRNA levels of a set of specific genes involved in toxicity pathways and vitamin A homeostasis. PCR was performed using SensiMixTM SYBR Low-ROX kit (Bioline, London, UK) with the QuantStudio 7 Flex Real-Time PCR System (Thermoscientific, Wilmington, US) with 1 cycle of 10 min at 95°C, 40 cycles of 15 s at 95°C and 25 s at 60°C, with a final dissociation stage of 15 s at 95°C, 1 min at 60°C and 15 s at 95°C. cDNA for each sample was diluted to 2 ng/μl and measured in triplicate. All primers were purchased from Sigma-Aldrich (Table 1). Fold induction of each gene was calculated using the housekeeping gene GAPDH.

2.7 | Quantification of RBPs and TTR protein in liver samples and PCLS

Pieces of liver or intestine (circa 50–100 mg) were snap frozen in liquid N_2 and stored at -80°C until use. The pieces of tissue were homogenized in ice cold PBS or dilution buffer (RBP1/RBP4) to achieve a concentration up to 100 mg/ml (TTR), 200 mg/ml (RBP4), or 600 mg/ml (RBP1). Afterwards, the samples were centrifuged at 4°C and 6000 × *g* for 5 min. Supernatants were collected and transferred to new tubes and stored at −20°C until further use.

Three PCLS per treatment were stored together in a sample cup at −80°C. After thawing, PCLS were homogenized in 750 μl of RIPA buffer (250-mM Tris/HCl [pH 7.5], 750-mM NaCl, 100-μl Igepal, 1.9-ml 2.5% sodium deoxycholate, 0.5% SDS, Milli-Q water, and protease inhibitor cocktail tablet) and incubated for 1 h on ice with periodic mixing. Afterwards, the tissue was lysed using a cell ultrasonic

TABLE 1 Primer sequences used in qPCR

Function	Gene	Forward (5′-3′)	Reverse (5′-3′)
Rat			
Housekeeping	GAPDH	CGCTGGTGCTGAGTATGTCG	CTGTGGTCATGAGCCCTTCC
Retinoids transport and metabolism	RBP1	TCATCACCCTCAATCCACTG	AGGCATAGATGACCGCAAGT
	RBP4	GTTTTCTCGTGACCCCAATG	ACTGTTTCTTGAGGGTCTGC
	TTR	GGACTGATATTTGCGTCTGAAGC	ACTTTCACGGCCACATCGAC
	CRABP2	TCCGATCTGTTCTGCAAAGGG	TCATCATGTTCACCCCCAGAG
	CRABP1	AAGACATCCACTACGGTGCG	CTCATCGTTGGCCAGCTCTC
	CYP26A1	GTTTCTGCAGATGAAGCGCA	GCGAGGAATCATGCAGGTTG
	LRAT	TTGCCTATGGAGCGGACATC	CTCAGCCTGCGGACTGATAG
RA signalling	RxRα	CCAGACAGCTCCTCCCCAAA	TGCACAGAGCCGTTTGCCAG
	RxRβ	CTCTACCCAGGTGAACTCTT	TGCTGCTCACAGGGTTCATG
Rabbit			
Houskeeping	GAPDH	ATTTGAAGGGCGGAGCCAAA	TCATGAGCCCCTCCACAATG
Retinoids transport and metabolism	RBP1	CACGTCGGGAAGGAGTTTGA	AACACTTGCTTGCACACCAC
	RBP4	GTTTCGCGGGTACCTGGTATG	GGGTCTTCGGTGTCTGTGAA
	TTR	GAAGCATGGCTTCTCAGCAC	AAAGGGCTCCCAGGTCTCAT
	CRABP2	GTCCAAGCCAACAGTGGAGA	TTCAGAAGCCTCTGTTCGCA
	DGAT	TGGCTCTAGTCCCTGCCTAT	CAATCGTGGTTACAGGCCGA
	LRAT	ATCAGAGGAGCGTTCTTGCC	ACAGTGCTCATACTGATTTGTGG
	CYP26A1	ACTTACCTGGGCCTCTACCC	AAAGGTCTTCAGAGCCACCC
RA signalling	RaRα	TGTTCCCAGATGGAACCTCG	CCCCAAATCATGGCAGACCA
	RxRβ	CGCGGTGTAAATGATGTATTTTGTG	AAACAGTTCTTCTGACGAGGACA

disrupter for 30 s and the samples were centrifuged at 4°C and 6000 × *g* for 5 min. Supernatants were collected and transferred to new tubes and stored at −20°C until further use. The medium of the slices was collected as well and stored at −20°C until use.

RBP1, RBP4, and TTR rabbit ELISA kits were purchased from MyBioSource Inc. (San Diego CA, USA). The specific instructions from the providers were followed for each sandwich ELISA kit. In brief, the specific antibodies are immobilized on the wells of the plate, and subsequently, the addition of a secondary antibody with a HRP moiety and its substrate generates a colour reaction that is directly proportional to the amount of protein present in the sample. The concentration of protein was calculated using the calibration curve prepared from standards provided on the kits.

Rat ELISA kits were purchased from XpressBio (Frederick MD, USA), Abcam (Cambridge, UK), and Cloud-Clone Corp. (Houston TX, USA), for RBP1, RBP4, and TTR, respectively.

2.8 | Data analysis

All in vitro (slice) experiments were conducted with at least three livers as biological replicates. Of each liver, at least three slices underwent the same treatment and the average value of those slices was used for further data analysis. In vivo experiments were conducted with four to five rabbits.

Statistical significance of the results was determined by using GraphpadPrism. One-way analysis of variance (ANOVA) with Dunnett's post hoc test was used in case the effect of one variable was tested. In case of two determining variables (e.g., time and THPS concentration), a two-way ANOVA or a mixed-model ANOVA was used, with Dunnett's post hoc test. If *p* values were lower than 0.05, differences were considered statistically significant.

3 | RESULTS

3.1 | Clinical observations

New Zealand White Rabbits were exposed to either vehicle (rabbit 1–5) or THPS at a low (13.8 mg/kg, rabbit 6–10) or a high dose (46.0 mg/kg, rabbit 11–15) from days 7–19 post coitum. Upon termination of the study, one rabbit (N°5) of the control group and one rabbit (N°14) of the high dose group appeared to be nonpregnant.

TABLE 2 Food intake (g/day) and body weight gain (%) in rabbits

Group	Rabbit	Day 3–7 (g/day)	Day 7–9 (g/day)	Day 9–12 (g/day)	Day 12–15 (g/day)	Day 15–18 (g/day)	Day 18–20 (g/day)	Body weight gain (%)[a]
Control	1	143	132	96	129	131	132	0.3
	2	158	149	147	85	117	129	1.4
	3	139	142	135	80	129	118	0.6
	4	144	147	149	136	89	137	2.4
	5 (NP)	134	144	90	89	128	149	3.1
	Average	143.6	142.8	123.4	103.8	118.8	133.0	1.2
	SD	9.0	6.6	28.3	26.5	17.5	11.3	0.9
13.8 mg/kg	6	159	153	153	82	142	135	1.7
	7	162	145	145	94	0	63	−4.1
	8	147	132	150	94	1	82	−5.1
	9	93	131	147	107	15	107	5.8
	10	131	134	138	78	47	125	−2.2
	Average	138.4	139.0	146.6	91.0	41.0*	102.4	−0.8
	SD	28.2	9.6	5.7	11.4	59.6	29.8	4.50
46.0 mg/kg	11	114	52	67	46	83	103	1.1
	12	139	80	54	34	41	25	−6.8
	13	150	48	8	2	1	0	−11.3
	14 (NP)	88	45	27	85	79	111	0.1
	15	100	23	6	27	21	34	−4.1
	Average	118.2	49.6**	32.4**	38.8**	45.0*	54.6**	−5.3
	SD	26.0	20.4	27.3	30.4	35.8	49.5	5.2

Note: */**group average was statistically different from control with $p \leq 0.05$ (*/** Dunnett's test based on pooled variance significant at 5% (*) or 1% (**) level). NP = nonpregnant. Data of nonpregnant rabbits were omitted from the average/SD and statistical analysis.

[a]Body weight corrected for uterus weight as a percentage of the weight at day 7 post coitum.

A decrease of food consumption was observed in both THPS-exposed groups, but most pronounced in the high dose group (statistically significant from controls during the whole treatment period) in comparison to the low dose group (statistically significant only at days 15–18 post coitum) (Table 2). Reduced body weight gain compared with concurrent controls (statistically significant at days 12 and 15 post coitum) was observed in the high dose group. Moreover, mean body weight in the high dose group at day 20 post coitum was lower than of historical controls. In addition, two females of the high dose group had orange discoloration of the urine, and one female also presented itself with piloerection and pale appearance. Finally, reduced faeces production was observed in all groups (including vehicle control) but was increased in severity with increasing dose levels and duration of treatment, which is thought to be related to the reduced food consumption seen in animals treated with THPS (data no shown).

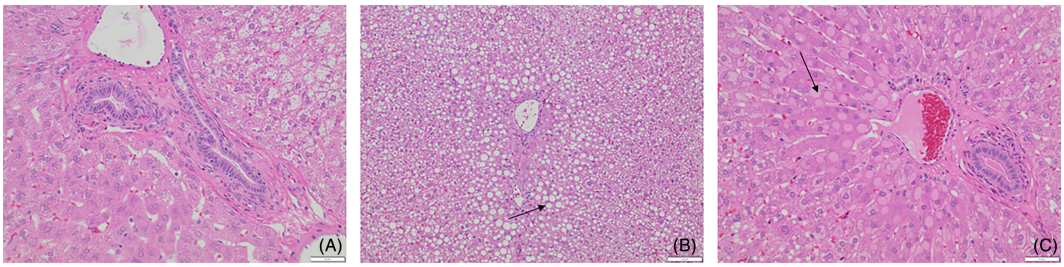

FIGURE 3 (A) Periportal area of rabbit from the control group. (B) Diffuse macrovesicular vacuolization (rabbit 13 of high dose group). (C) Periportal cytoplasmic inclusions (rabbit 15 of high dose group).

TABLE 3 Liver toxicity in rabbits (in vivo study)

Group	Rabbit	Cytoplasmic inclusions	Macrovesicular vacuolization	TG content (mg/mg)	ALAT (U/L)	ASAT (U/L)	ALP (U/L)	GGT (U/L)
Control	1	None	None	0.65	80.0	133.4	37	5.5
	2	None	None	0.63	27.0	22.8	56	5.8
	3	None	None	0.56	32.4	17.6	50	5.6
	4	None	None	1.28	37.0	19.5	123	8.9
	5 (NP)	None	None	0.37	32.1	12.5	66	4.3
	Average			0.78	44.1	48.3	67	6.5
	SD			0.34	23.3	56.8	33.3	1.6
13.8 mg/kg	6	None	None	0.56	38.4	19.9	88	3.6
	7	None	Grade 1	10.1	33.0	16.1	31	8.1
	8	None	None	2.05	74.2	37.1	171	10.3
	9	None	None	1.14	20.1	18.1	63	2.9
	10	None	None	0.34	46.7	15.6	53	5.6
	Average			2.83	42.6	21.4	81	6.1
	SD			4.11	20.2	9.0	54	3.1
46.0 mg/kg	11	Grade 2	None	0.45	101.9	48.4	51	14.9
	12	Grade 1	None	1.35	59.9	32.9	58	11.6
	13	Grade 2	Grade 3	26.3	46.2	36.9	112	20.8
	14 (NP)	Grade 2	None	0.46	23.0	12.8	75	11.8
	15	Grade 4	Grade 2	4.72	28.7	19.6	38	8.9
	Average			8.20	59.2	34.5	65	14.1*
	SD			12.20	31.5	11.9	33	5.1

Note: GRADE 1 = Minimal/very few/very small GRADE 2 = Slight/few/small GRADE 3 = Moderate/ moderate number/moderate size GRADE 4 = Marked/many/large. Observed vacuolization was diffuse, whereas inclusions were observed in the portal area of the liver. */**group average was statistically different from control with $p \leq 0.05$ (*/** Dunnett's test based on pooled variance significant at 5% (*) or 1% (**) level). NP = nonpregnant. Data of nonpregnant rabbits were omitted from the average/SD and statistical analysis.

3.2 | Liver toxicity (in vivo and ex vivo)

There were clear signs of liver toxicity in the rabbits dams of the high dose THPS group. As shown in Table 2, these rabbits had significantly elevated serum γ-glutamyltransferase levels (other liver damage parameters remained unchanged). In addition, degenerative changes in the liver were observed upon microscopical examination; all rabbits of the high dose group had periportal cytoplasmatic inclusions to some extent (Figure 3, panel C). These inclusions were clearly defined and stained pink (eosinophilic) in the H&E staining, which indicates the presence of proteins. Three rabbits that received a low or high dose of THPS (7, 13, and 15) (also) showed diffuse (not clearly zonal) vacuolization (Figure 3, panel B). As indicated by the quantitative triglyceride determination (Table 3), these vacuoles were probably due to fat deposition.

Liver toxicity by THPS in PCLS of rabbits and rats was observed in the low micromolar range (Figure 4). The sensitivity of rabbit slices incubated for 24 h with THPS (TC_{50} = 310.3 μM; 95% confidentiality interval 168.6–571.1 for ATP content) was somewhat lower than of rat slices (TC_{50} = 79.8; 53.6–118.7 μM for ATP content and 84.1; 62.1–114.0 for LDH leakage). Subchronic (72 h) exposure decreased the TC50 in rat liver slices approximately threefold to 31 μM; 95% confidentiality interval 17.9–53.6 μM for ATP and 31.1; 23.2–42.2 μM for LDH leakage. Upon histomorphological examination of the slices incubated with THPS, zonal cell death was observed, although not clearly limited to the periportal area (Figure 5). Interestingly, fat deposition was observed in rat liver slices after prolonged (72 h) culturing, similar to in vivo. In slices, mostly microvesicular steatosis was seen.

3.3 | Vitamin A homeostasis disruption

Both in vitro and in vivo, the gene expression of several proteins involved in vitamin A homeostasis was affected by THPS (Figure 6). In rat slices, the gene expression of the retinoids binding proteins

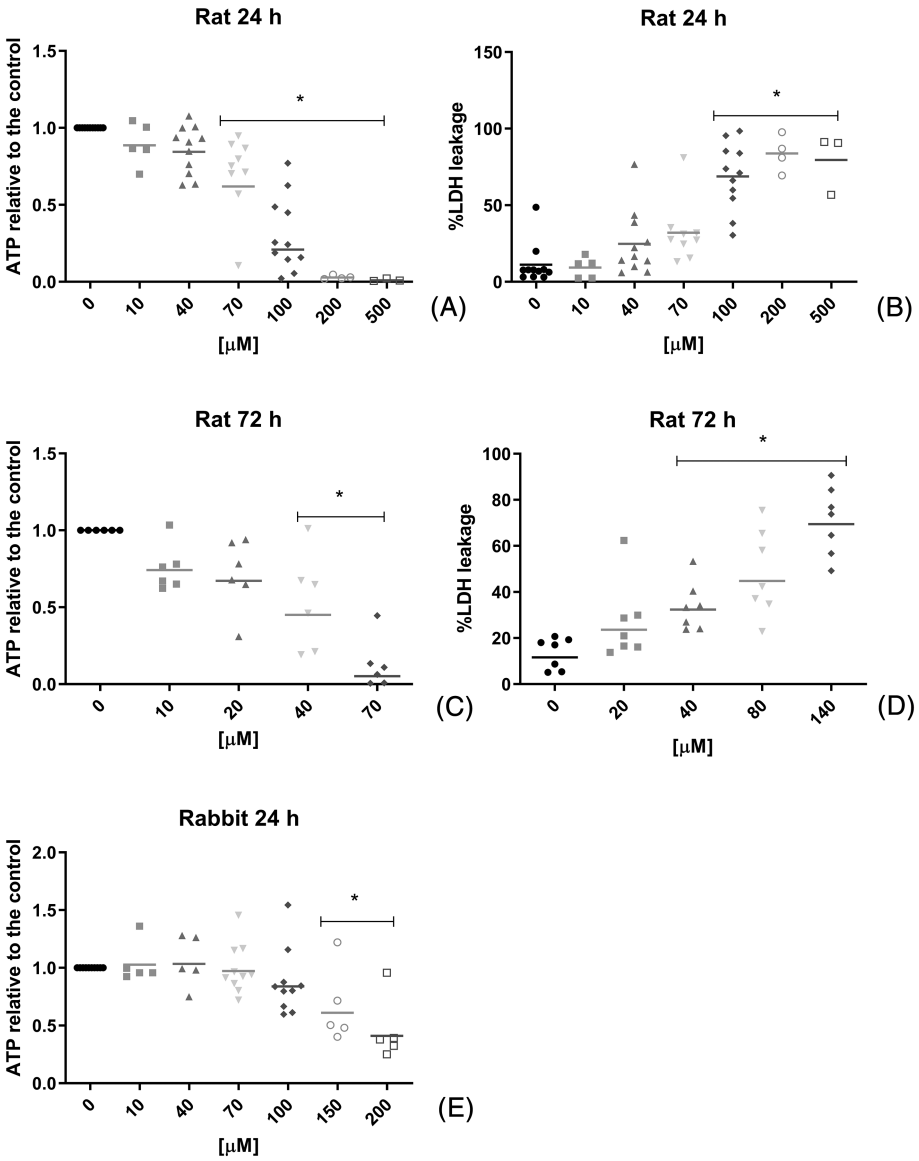

FIGURE 4 The effect of THPS on slice viability determined by ATP content and LDH leakage. (A) ATP content of rat liver slices after 24-h exposure. (B) LDH leakage of rat liver slices after 24-h exposure. (C) ATP content of rat liver slices after 72-h exposure. (D) LDH leakage of rat liver slices after 72-h exposure. (E) ATP content of rabbit liver slices after 24 h exposure. Each data point reflects the average value of three slices of one liver. N = 5–11 livers. Statistical significance was assessed by one-way ANOVA with Dunnett's post hoc test. * indicates $p < 0.05$.

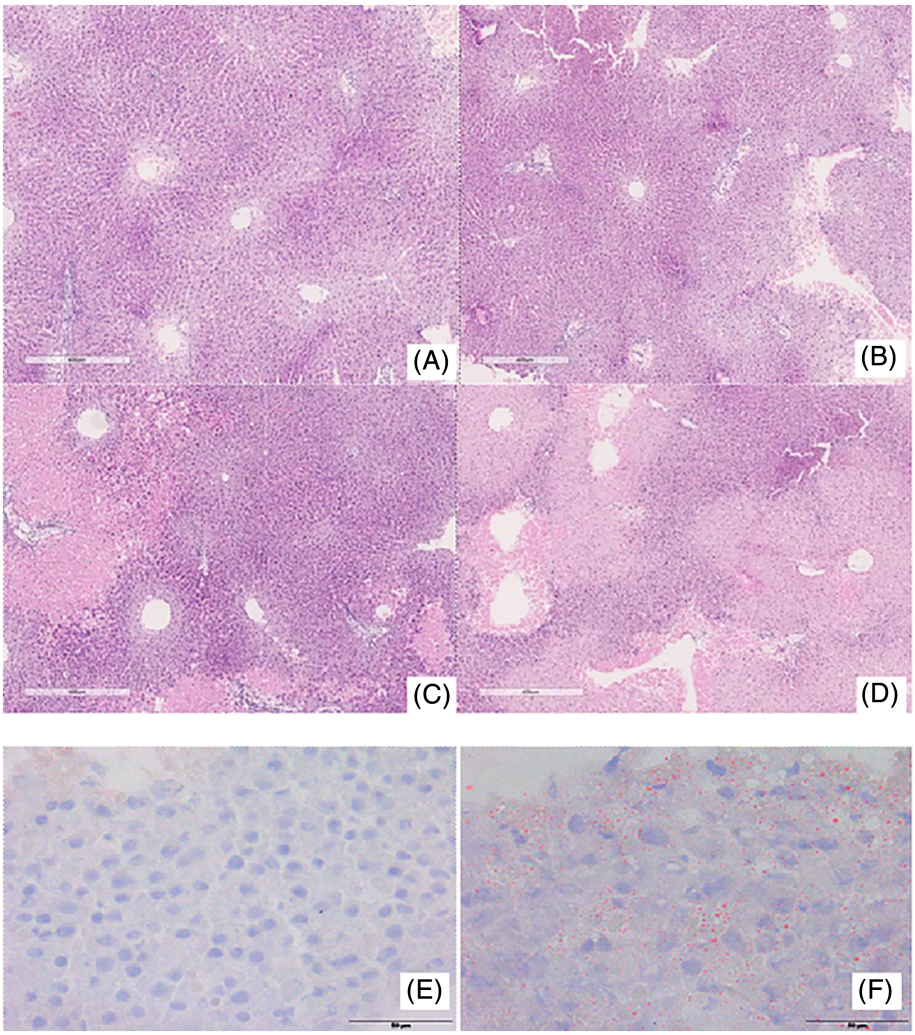

FIGURE 5 Haematoxylin–eosin staining of sections of rat PCLS after 24-h incubation without (A) or with 40, 70, or 100 μM (B–D) THPS. Scale bar: 400 μm. (E) and (F) show results of Oil Red O staining on untreated slices and slices exposed to 10-μM THPS for 72 h.

RBP1, RBP4, and TTR and of CYP26A1 and lecithin retinol acyltransferase (LRAT), involved in the metabolism of retinoids, was statistically significantly decreased already after 24 h of incubation at concentrations below the TC_{50} that was measured by ATP content and LDH leakage. In addition, after 72 h, the gene expression of the intracellular binding proteins of retinoic acid CRABP1 and 2 and of RXRα was decreased. In rabbit liver slices, the decrease of mRNA expression was only statistically significant for RBP1, while a trend towards a decreased expression of LRAT and CYP26A1 was seen at almost all concentrations tested. In addition, a dose-dependent increase of RAR gene expression was observed in rabbit slices after 24-h incubation with THPS. A statistically significant decrease in gene expression of CYP26A1 and LRAT was also observed in the livers derived from the rabbits that were exposed to a high dose of THPS in vivo.

In addition to gene expression, protein expression of RBP1, RBP4, and TTR was measured by ELISA in slices and in the rabbit livers of the in vivo study (Figures 7 and 8). In rat liver slices incubated for 24 h with THPS, a slight but significant decrease of protein expression of RBP1 was seen, but only at a highly toxic THPS concentration. Interestingly, the amount of RBP4 that was excreted by the slices into the culture medium was dramatically and dose-dependently decreased up to fivefold (40-μM THPS) and 20-fold (70-μM THPS). No changes were seen in rabbit slices, although it should be noted that these slices were incubated for only 24 h.

In the liver derived from the in vivo rabbit study, RBP1 and RBP4 protein expression was dose-dependently and statistically significantly decreased, whereas TTR expression remained unchanged (Figure 8). In serum, RBP4 levels on average were lower at every time point in the high dose group in comparison to control, but these results were not statistically significant.

Retinol levels in rabbit liver at the end of the in vivo study decreased dose dependently, but not significantly after exposure to THPS, likely due to the high variation between the treated rabbits (Figure 9A). Retinyl palmitate levels in livers remained unchanged in all groups (Figure 9B). Retinol levels in serum, however, were lower in the low dose and high dose group than in the control group at all time points. This difference was statistically significant at day 17 (low dose) and day 19 and the day of necropsy (high dose) (Figure 8C).

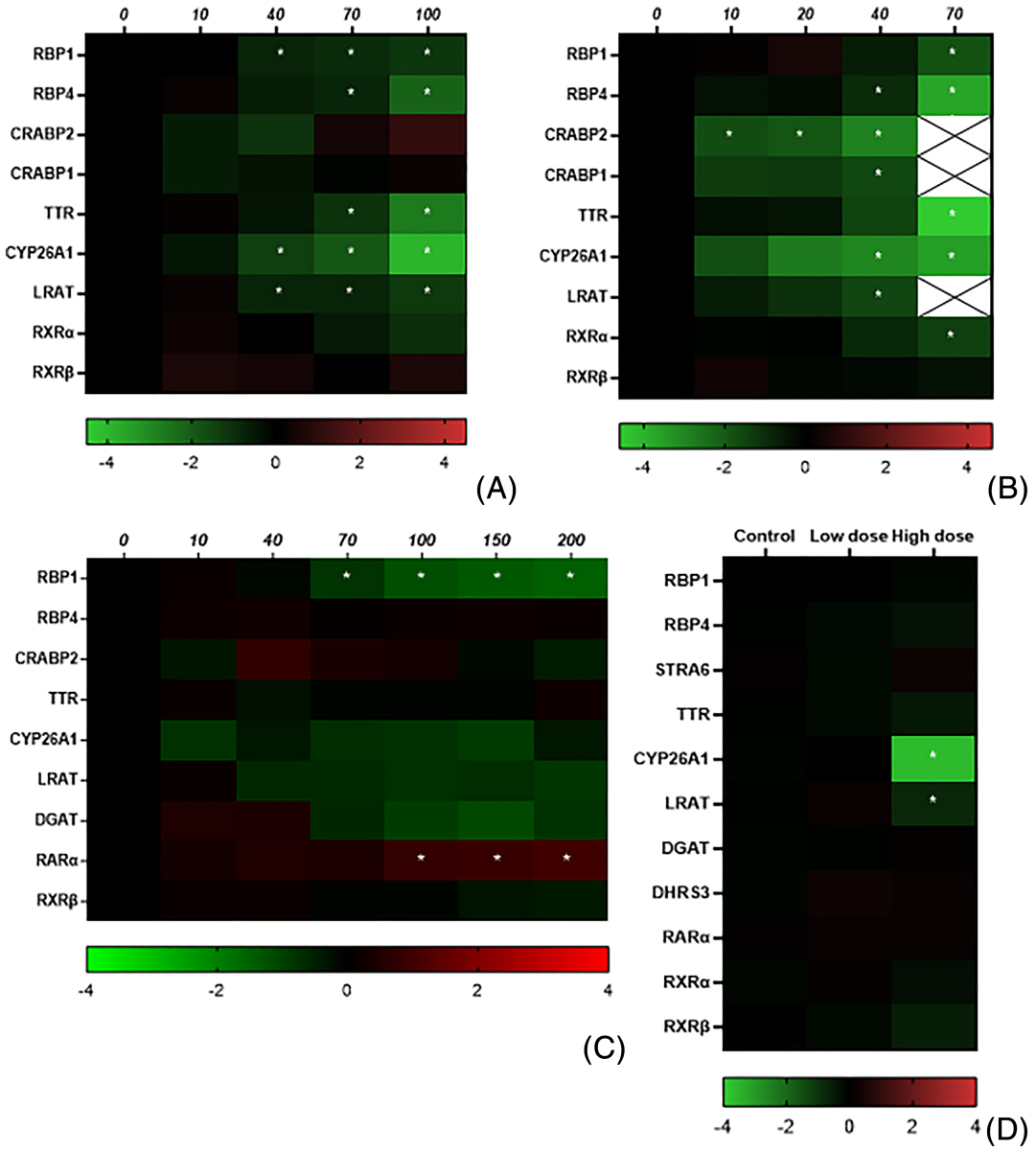

FIGURE 6 The effect of THPS on expression of genes related to vitamin A homeostasis in (A) rat slices incubated for 24 h, (B) rat liver slices cultured for 72 h, (C) rabbit liver slices incubated for 24 h, (D) livers of rabbits derived from the in vivo studies (nonpregnant rabbits excluded). Values reflect log2 of the average fold induction of $n = 3/4$ (rats) or 9 livers (rabbits) for the slice experiments and with four to five rabbits for the in vivo experiments. Statistical significance was assessed by one-way ANOVA with Dunnett's post hoc test. * indicates $p < 0.05$, compared with control. White boxes with cross: not determined

4 | DISCUSSION

In the present study, we investigated the hypothesis that THPS induces teratogenicity as a result of impairment of retinoid homeostasis in pregnant rabbits. Retinoids are crucial compounds during embryogenesis. During development, the fetuses rely on retinoid supply from their mothers by two main sources, RBP4-bound retinol and retinyl esters packed in chylomicrons (Quadro et al., 2005). Due to (prolonged) fasting, plasma retinyl ester content can decrease to very low levels (O'Byrne & Blaner, 2013), and the fetuses become mainly dependent on the supply of RBP4-bound retinol. Similar to earlier in vivo studies with rabbits with THP(X) species (Barker, 1991), exposure of rabbits to THPS in the present study led to reduced intake of food, particularly in the highest dose group. In this group, some rabbits completely ceased eating from day 9 to day 19. Also in the low dose group, there was a significant reduction in food intake, but generally at later time-points (day 15–18) than in the high dose group. Because it can be anticipated that reduced food intake also leads to a reduced uptake of retinoids in these rabbits, it is tempting to attribute the birth defects seen in rabbit pups in the earlier study to the reduced food intake during organogenesis. However, food restriction to 15 g per day from gestation day 7–19 in White New Zealand

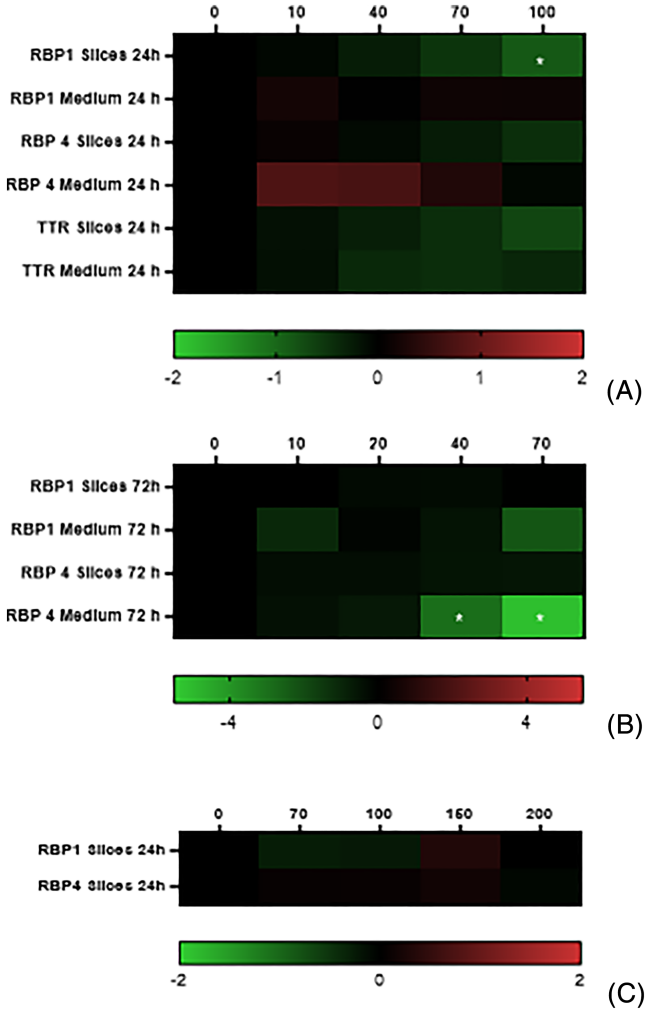

FIGURE 7 The effect of THPS on expression of proteins related to vitamin A homeostasis in (A) rat slices incubated for 24 (B) or 72 h (C) rabbit liver slices incubated for 24 h. Values reflect log2 of the average fold induction of $n = 3–5$ experiments (livers). Statistical significance was assessed by one-way ANOVA with Dunnett's post hoc test. * indicates $p < 0.05$, compared with control.

rabbits, which is comparable with the food intake of some rabbits in this study, was reported to lead to abortion and alterations in ossification, but not to malformations (Cappon et al., 2005).

Usually, the liver ensures a continuous supply of retinol, also after prolonged malnutrition of pregnant mothers due to its enormous storage capacity of retinyl esters. For this reason, only after months of vitamin A deficient diet, serum retinol levels drop (Kubo et al., 2000). Also, in the present study, despite the decrease in food intake following THPS exposure, liver retinyl ester storage seemed to be barely affected in most animals. On the other hand, liver retinol content (trend) was decreased dose dependently in THPS-treated animals and was up to five times lower in individual animals than average values in the control group. This is remarkable, because liver retinol content is thought to remain constant, unless retinyl ester storages are (completely) depleted, which was not the case in this study. This could indicate that THPS, besides reducing the uptake of retinoids via food, also interferes with the mobilization of retinol from the retinyl ester storage.

There are several other findings that indicate that THPS indeed interferes with vitamin A homeostasis in the liver. First of all, in both the ex vivo liver slices of rats and rabbits and in the in vivo study with rabbits, gene expression of CYP26A1 and LRAT was (significantly) decreased after exposure to THPS. In vivo, LRAT expression in the liver of THPS-exposed rabbits was down-regulated by 30% in the highest dose group. LRAT immobilizes retinol by metabolizing it into retinyl esters for storage in the liver. A decrease in expression could indicate that the liver tries to counteract retinoid depletion by preventing conversion of retinol to retinyl esters (Ross & Zolfaghari, 2004). Moreover, a decrease in available LRAT ensures that retinol stays available for target tissues outside the liver. Particularly interesting is the decrease of expression of the gene encoding for CYP26A1. This enzyme is involved in the catabolism of all trans retinoic acid (ATRA) and is highly specific for this reaction (Thatcher & Isoherranen, 2009). The enzyme is under tight regulation by ATRA and is known to be highly and rapidly induced in case of ATRA excess, whereas it is strongly down-regulated in case of ATRA deficiency to keep control of ATRA mediated signalling (Topletz et al., 2015). It was found that CYP26A1 gene expression was strongly down-regulated (five to 100 times) in all rabbits of the highest dosage groups. Interestingly, both LRAT and CYP26A1 were also down-regulated significantly in the liver slices, at concentration levels that caused only slight to moderate toxicity. This indicates that reduced availability of retinoids due to decrease in food intake is not the (only) cause of regulation of these enzymes, because this factor does not play a role for liver slices.

In addition, we observed changes in the expression of binding proteins of retinoids, both in vivo and ex vivo. In slices, we found a decreased gene expression of RBP1 (rats and rabbit), RBP4 and TTR (rats). In vivo, gene expression was unaffected. On the other hand, in vivo protein expression of RBP1 and RBP4 was significantly decreased in the tissue, whereas in the slices those changes were only seen at highly toxic concentrations. Differences between ex vivo and in vivo results could possibly be explained by the different exposure times (12 days in vivo and 3 days ex vivo). RBP1 is the major intracellular binding protein for retinol. RBP1 is under direct transcriptional regulation of ATRA and ATRA deficiency could result in reduced expression of this protein (Smith et al., 1991). Besides, tissue levels of RBP1 have been reported to decrease when rats are fed a retinoid deficient diet (Kato et al., 1985). RBP4 and TTR, both produced by the liver, transport retinol to the target tissues (Saeed, Hoekstra, et al., 2017). The liver excretes RBP4 only when it is "loaded" with retinol (holoprotein) (Bellovino et al., 1999; Dixon & Goodman, 1987; Ronne et al., 1983). Interestingly, we found that in rat slices after prolonged (72 h) culturing, substantially less RBP4 was excreted in the medium, whereas slice levels remained more or less constant. This could indicate that also in slices there is a shortage of available retinol in the tissue, which is another indication that the decrease of vitamin A uptake from food is not the only factor in the VAD that seems to be induced by THPS. In coherence with this, serum retinol (statistically

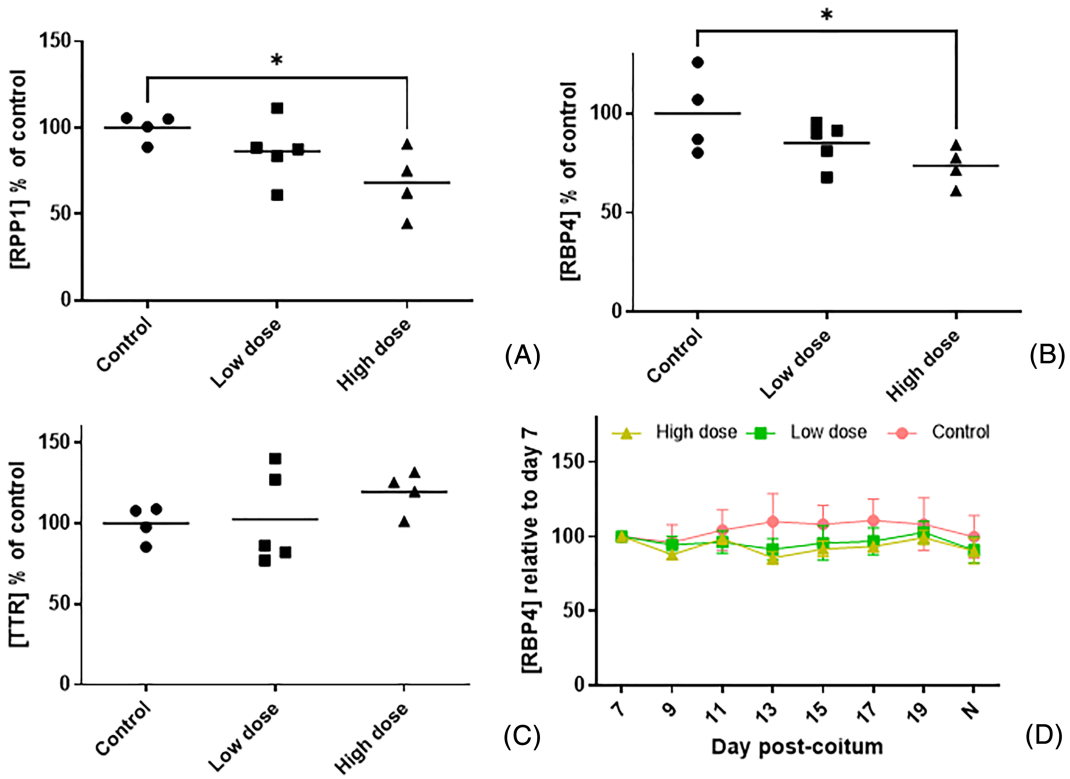

FIGURE 8 The effect of THPS on in vivo expression of proteins related to vitamin A homeostasis (A) RBP1 content of rabbit livers, (B) RBP4 content of rabbit livers, (C) TTR content of rabbit livers. (D) Serum content of RBP4, relative to day 7 (±SEM). Nonpregnant rabbits were excluded. Individual data points and the average of $n = 4–5$ livers were depicted per dose group. Data were calculated relative to the controls. Statistical significance was assessed by one-way ANOVA (liver data) or by using a mixed effect model (serum) with Dunnett's post hoc test.
* indicates $p < 0.05$, compared with control.

significant) and RBP4 (trend), which are important for embryo development (Quadro et al., 2005), were decreased in vivo.

Remarkably, we observed more vitamin A related changes in the rat liver slices compared to rabbit liver slices, whereas in vivo teratogenicity was only found in rabbits. Apart from some experimental differences between the rat and the rabbit ex vivo studies (shorter incubation time and less toxic THPS concentrations were used in rabbit slices), this observation could be explained by the fact that rats in vivo do not show a decrease in food consumption during gestation. Based on these findings THPS seems to cause VAD in a two-hit fashion. The first hit is by decreasing the food intake of rabbit dams, which lowers the circulation of retinyl esters available for the embryos. Second, there are indications that THPS interferes with the mobilization of retinol from the liver in dams, which is required to ensure retinoid delivery to the embryos during fasting. Possibly, because the first hit does not occur in rats, embryos were largely unaffected in the rat teratogenicity study. The fact that teratogenicity was only observed in studies with THPX compounds at dose levels that caused a marked maternal toxicity and food intake reduction is consistent with a double-hit mode of action.

The mechanism by which THPS interferes with retinol mobilization is still unsolved. Liver damage has been associated with disturbance of vitamin A homeostasis and signalling: Acute liver failure accompanied by acute phase response (APR) has been reported to decrease the gene expression of RBP4 (Higgins et al., 2003; Qian et al., 1995; Vatakuti et al., 2016) and TTR (Vatakuti et al., 2016). Furthermore, the cytokines induced by lipopolysaccharide (LPS), a model compound to induce liver inflammation and APR, are known to downregulate several enzymes of the cytochrome P-450 enzyme system, particularly of the CYP2 family (Higgins et al., 2003). Additionally, there are several studies that show that APR decreases retinoid concentrations in blood (Stephensen & Gildengorin, 2000). There is evidence that APR and subsequent changes in gene expression of acute phase proteins is induced via repression of RXR. Repression of RXR function was found to be due to the inhibition of RXR gene expression (possibly by increased break-down of the mRNA; Beigneux et al., 2000) shortly (2–4 h) after exposure to LPS. LPS has been shown to induce birth defects (amongst others microphthalmia, anophthalmia, and reduced limb size [micromelia]), similar to THPX species (Carey et al., 2003). Despite these similarities, and although we observed liver toxicity in the form of degenerative liver changes and increased plasma GGT, we found no indications of acute liver failure or liver inflammation (IL6 levels in serum and liver were unchanged during this study; data not shown); most of the liver enzymes remained unchanged and no necrosis was observed upon

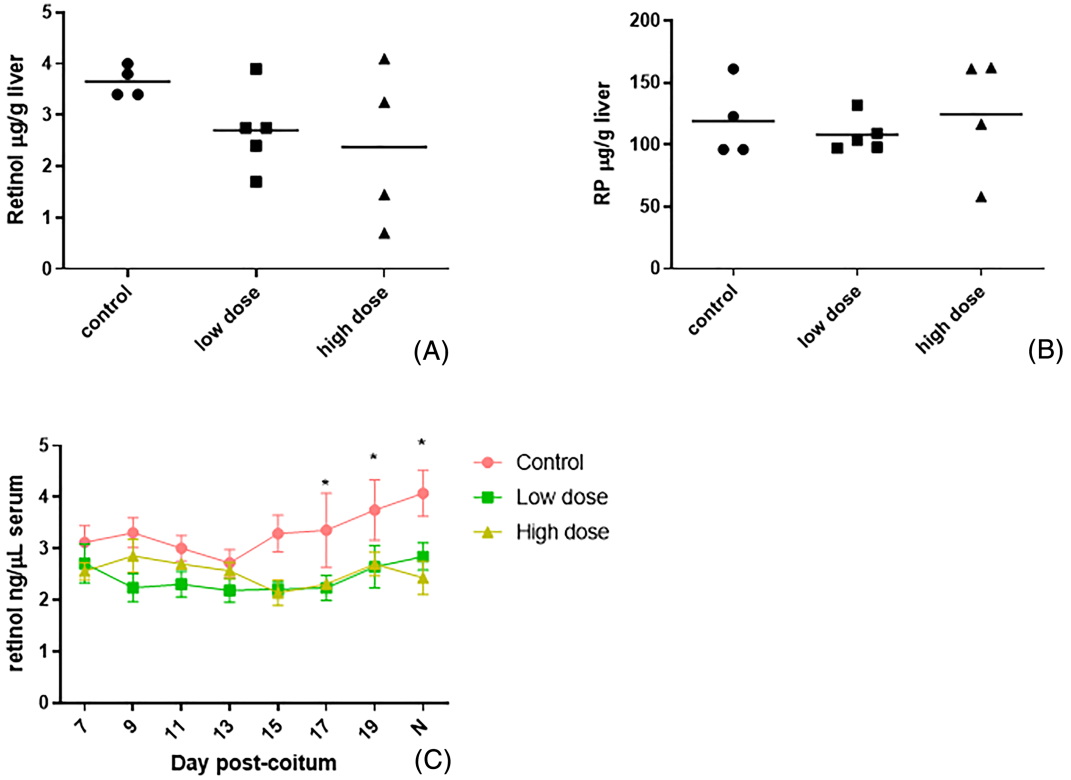

FIGURE 9 The effect of THPS on the content of (A) retinol (B) retinyl palmitate in liver and (C) retinol in serum. Nonpregnant rabbits were excluded. Individual data points and the average of $n = 4–5$ livers were depicted per dose group for the livers. For serum, the average content ± SEM is shown. Statistical significance was assessed by one-way ANOVA (liver data) or by using a mixed effect model (serum) with Dunnett's post hoc test. * indicates $p < 0.05$, compared to control (on day 17, serum retinol was significantly lower in the low dose group compared to the control, and on day 19 and the day of necropsy, serum retinol levels in the high dose group were significantly lower than in the control group).

histomorphological examination. For this, it seems that APR is not the cause of vitamin A dysregulation in the present study.

THPS or its metabolites could also interfere with the action of the enzymes involved in the formation of retinol and/or retinoic acid, the ligand for RXR. One of the major metabolites of THPS is formaldehyde. Other small aldehydes, such as acetaldehyde, a metabolite of ethanol, can interfere with retinylaldehyde dehydrogenases (Clugston & Blaner, 2012; Kot-Leibovich & Fainsod, 2009; Shabtai et al., 2018), which are indispensable in the formation of retinoic acids (Duester, 2009; Duester et al., 2003). Strikingly, also ethanol decreases plasma and liver retinol levels, plasma RBP and liver RA content, and it also induces triglyceride accumulation in the liver, similar to what was seen in some rabbits after THPS exposure in the present study (Clugston & Blaner, 2012). Moreover, maternal exposure to ethanol (Stromland, 1985) or methanol (Sweeting et al., 2011) (of which formaldehyde is a major metabolite), is known to lead to microphthalmia in offspring. Finally, fatty changes in the liver that are not related to alcohol exposure (non-alcoholic fatty liver disease) are also associated with changes in vitamin A metabolism (Saeed et al., 2021; Saeed, Dullaart, et al., 2017). Further studies are required to determine if THPS poses its actions through (one) of these mechanisms.

In conclusion, exposure of rabbits to a high dose of THPS (46.0 mg/kg) results in substantial reduction of food intake and adverse liver effects in dams. In addition, we report deregulation of vitamin A homeostasis and signalling, indicated by changes in liver and plasma retinol (in vivo study) and in (gene) expression of proteins involved in retinoid metabolism and transport (both in vivo and ex vivo). The interference with vitamin A homeostasis could act in synergy with food deprivation leading to observed birth defects in rabbits following THPS exposure.

CONFLICTS OF INTEREST

This study was financed by Solvay S.A., whereas THPS is a Solvay product. The authors Steiblen, de Groot, Groome, and van Miert are/were employed at Solvay.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Inge Anne Maria de Graaf https://orcid.org/0000-0002-0760-8356

REFERENCES

Barker, L. (1991). *THPS: Oral (gavage) teratotology study in the rabbit*. Harrogate North-Yorkshire UK.

Beigneux, A. P., Moser, A. H., Shigenaga, J. K., Grunfeld, C., & Feingold, K. R. (2000). The acute phase response is associated with retinoid X receptor repression in rodent liver. *Journal of Biological Chemistry*, *275*(21), 16390–16399. https://doi.org/10.1074/jbc.M000953200

Bellovino, D., Lanyau, Y., Garaguso, I., Amicone, L., Cavallari, C., Tripodi, M., & Gaetani, S. (1999). MMH cells: An in vitro model for the study of retinol-binding protein secretion regulated by retinol. *Journal of Cellular Physiology*, *181*(1), 24–32. https://doi.org/10.1002/(SICI)1097-4652(199910)181:1<24::AID-JCP3>3.0.CO;2-0

Bligh, E. G., & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology*, *37*, 911–917. https://doi.org/10.1139/y59-099

Cappon, G. D., Fleeman, T. L., Chapin, R. E., & Hurtt, M. E. (2005). Effects of feed restriction during organogenesis on embryo-fetal development in rabbit. *Birth Defects Research. Part B, Developmental and Reproductive Toxicology*, *74*(5), 424–430. https://doi.org/10.1002/bdrb.20058

Carey, L. C., Berbee, P. L., Coyle, P., Philcox, J. C., & Rofe, A. M. (2003). Zinc treatment prevents lipopolysaccharide-induced teratogenicity in mice. *Birth Defects Research. Part a, Clinical and Molecular Teratology*, *67*(4), 240–245. https://doi.org/10.1002/bdra.10035

Clagett-Dame, M., & Knutson, D. (2011). Vitamin A in reproduction and development. *Nutrients*, *3*(4), 385–428. https://doi.org/10.3390/nu3040385

Clugston, R. D., & Blaner, W. S. (2012). The adverse effects of alcohol on vitamin A metabolism. *Nutrients*, *4*(5), 356–371. https://doi.org/10.3390/nu4050356

de Graaf, I. A., Olinga, P., de Jager, M. H., Merema, M. T., de Kanter, R., van de Kerkhof, E. G., & Groothuis, G. M. (2010). Preparation and incubation of precision-cut liver and intestinal slices for application in drug metabolism and toxicity studies. *Nature Protocols*, *5*(9), 1540–1551. https://doi.org/10.1038/nprot.2010.111

Dixon, J. L., & Goodman, D. S. (1987). Studies on the metabolism of retinol-binding protein by primary hepatocytes from retinol-deficient rats. *Journal of Cellular Physiology*, *130*(1), 14–20. https://doi.org/10.1002/jcp.1041300104

Duester, G. (2009). Keeping an eye on retinoic acid signaling during eye development. *Chemical and Biological Interactions*, *178*(1–3), 178–181. https://doi.org/10.1016/j.cbi.2008.09.004

Duester, G., Mic, F. A., & Molotkov, A. (2003). Cytosolic retinoid dehydrogenases govern ubiquitous metabolism of retinol to retinaldehyde followed by tissue-specific metabolism to retinoic acid. *Chemical and Biological Interactions*, *143-144*, 201–210. https://doi.org/10.1016/s0009-2797(02)00204-1

Higgins, M. A., Berridge, B. R., Mills, B. J., Schultze, A. E., Gao, H., Searfoss, G. H., Baker, T. K., & Ryan, T. P. (2003). Gene expression analysis of the acute phase response using a canine microarray. *Toxicological Sciences*, *74*(2), 470–484. https://doi.org/10.1093/toxsci/kfg142

Kato, M., Blaner, W. S., Mertz, J. R., Das, K., Kato, K., & Goodman, D. S. (1985). Influence of retinoid nutritional status on cellular retinol- and cellular retinoic acid-binding protein concentrations in various rat tissues. *Journal of Biological Chemistry*, *260*(8), 4832–4838. https://doi.org/10.1016/S0021-9258(18)89146-3

Kim, Y. K., & Quadro, L. (2010). Reverse-phase high-performance liquid chromatography (HPLC) analysis of retinol and retinyl esters in mouse serum and tissues. *Methods in Molecular Biology*, *652*, 263–275. https://doi.org/10.1007/978-1-60327-325-1_15

Kinkel, A. D., Fernyhough, M. E., Helterline, D. L., Vierck, J. L., Oberg, K. S., Vance, T. J., & Dodson, M. V. (2004). Oil red-O stains non-adipogenic cells: A precautionary note. *Cytotechnology*, *46*(1), 49–56. https://doi.org/10.1007/s10616-004-3903-4

Kot-Leibovich, H., & Fainsod, A. (2009). Ethanol induces embryonic malformations by competing for retinaldehyde dehydrogenase activity during vertebrate gastrulation. *Disease Models & Mechanisms*, *2*(5–6), 295–305. https://doi.org/10.1242/dmm.001420

Kubo, Y., Arimura, A., Watanabe, Y., Nakayasu, K., & Kanai, A. (2000). Effect of vitamin A palmitate on vitamin A-deficient rabbits. *Japanese Journal of Ophthalmology*, *44*(2), 189. https://doi.org/10.1016/s0021-5155(99)00206-3

Maden, M. (2001). Vitamin A and the developing embryo. *Postgrade Medical Journal*, *77*(910), 489–491. https://doi.org/10.1136/pmj.77.910.489

O'Byrne, S. M., & Blaner, W. S. (2013). Retinol and retinyl esters: Biochemistry and physiology. *Journal of Lipid Research*, *54*(7), 1731–1743. https://doi.org/10.1194/jlr.R037648

Qian, X., Samadani, U., Porcella, A., & Costa, R. H. (1995). Decreased expression of hepatocyte nuclear factor 3 alpha during the acute-phase response influences transthyretin gene transcription. *Molecular and Cellular Biology*, *15*(3), 1364–1376. https://doi.org/10.1128/MCB.15.3.1364

Quadro, L., Hamberger, L., Gottesman, M. E., Wang, F., Colantuoni, V., Blaner, W. S., & Mendelsohn, C. L. (2005). Pathways of vitamin A delivery to the embryo: Insights from a new tunable model of embryonic vitamin A deficiency. *Endocrinology*, *146*(10), 4479–4490. https://doi.org/10.1210/en.2005-0158

Ronne, H., Ocklind, C., Wiman, K., Rask, L., Obrink, B., & Peterson, P. A. (1983). Ligand-dependent regulation of intracellular protein transport: Effect of vitamin A on the secretion of the retinol-binding protein. *Journal of Cell Biology*, *96*(3), 907–910. https://doi.org/10.1083/jcb.96.3.907

Ross, A. C., & Zolfaghari, R. (2004). Regulation of hepatic retinol metabolism: Perspectives from studies on vitamin A status. *Journal of Nutrition*, *134*(1), 269S–275S. https://doi.org/10.1093/jn/134.1.269S

Saeed, A., Bartuzi, P., Heegsma, J., Dekker, D., Kloosterhuis, N., de Bruin, A., Jonker, J. W., van de Sluis, B., & Faber, K. N. (2021). Impaired hepatic vitamin A metabolism in NAFLD mice leading to vitamin A accumulation in hepatocytes. *Cellular and Molecular Gastroenterology and Hepatology*, *11*(1), 309–325. https://doi.org/10.1016/j.jcmgh.2020.07.006

Saeed, A., Dullaart, R. P. F., Schreuder, T., Blokzijl, H., & Faber, K. N. (2017). Disturbed vitamin A metabolism in non-alcoholic fatty liver disease (NAFLD). *Nutrients*, *10*(1), 29. https://doi.org/10.3390/nu10010029

Saeed, A., Hoekstra, M., Hoeke, M. O., Heegsma, J., & Faber, K. N. (2017). The interrelationship between bile acid and vitamin A homeostasis. *Biochimica et Biophysica Acta, Molecular and Cell Biology of Lipids*, *1862*(5), 496–512. https://doi.org/10.1016/j.bbalip.2017.01.007

Shabtai, Y., Bendelac, L., Jubran, H., Hirschberg, J., & Fainsod, A. (2018). Acetaldehyde inhibits retinoic acid biosynthesis to mediate alcohol teratogenicity. *Scientific Reports*, *8*, 347. https://doi.org/10.1038/s41598-017-18719-7

Smith, W. C., Nakshatri, H., Leroy, P., Rees, J., & Chambon, P. (1991). A retinoic acid response element is present in the mouse cellular retinol binding protein I (mCRBPI) promoter. *European Molecular Biology Organization Journal*, *10*(8), 2223–2230. https://doi.org/10.1002/j.1460-2075.1991.tb07758.x

Stephensen, C. B., & Gildengorin, G. (2000). Serum retinol, the acute phase response, and the apparent misclassification of vitamin A status in the third National Health and Nutrition Examination Survey. *American Journal of Clinical Nutrition*, *72*(5), 1170–1178. https://doi.org/10.1093/ajcn/72.5.1170

Stromland, K. (1985). Ocular abnormalities in the fetal alcohol syndrome. *Acta Ophthalmologica. Supplement*, *171*, 1–50.

Sweeting, J. N., Siu, M., Wiley, M. J., & Wells, P. G. (2011). Species- and strain-dependent teratogenicity of methanol in rabbits and mice. *Reproductive Toxicology*, *31*(1), 50–58. https://doi.org/10.1016/j.reprotox.2010.09.014

Thatcher, J. E., & Isoherranen, N. (2009). The role of CYP26 enzymes in retinoic acid clearance. *Expert Opinion of Drug Metabolism and Toxicology*, *5*(8), 875–886. https://doi.org/10.1517/17425250903032681

Topletz, A. R., Tripathy, S., Foti, R. S., Shimshoni, J. A., Nelson, W. L., & Isoherranen, N. (2015). Induction of CYP26A1 by metabolites of retinoic acid: Evidence that CYP26A1 is an important enzyme in the elimination of active retinoids. *Molecular Pharmacology*, *87*(3), 430–441. https://doi.org/10.1124/mol.114.096784

van Esch, G. J. (2000). Flame retardants: tris(2-butoxyethyl) phosphate, tris (2-ethylhexyl) phosphate and tetrakis(hydroxymethyl) phosphonium salts. Environmental Health Criteria 218.

Vatakuti, S., Pennings, J. L., Gore, E., Olinga, P., & Groothuis, G. M. (2016). Classification of Cholestatic and necrotic hepatotoxicants using transcriptomics on human precision-cut liver slices. *Chemical Research in Toxicology*, *29*(3), 342–351. https://doi.org/10.1021/acs.chemrestox.5b00491

How to cite this article: Estrada-Ortiz, N., Starokozhko, V., van Steenwijk, H., van der Heide, C., Permentier, H., van Heemskerk, L., Prins, G. H., Heegsma, J., Faber, K. N., Bressers, S., Steiblen, G., de Groot, A., Groome, S., van Miert, E., Groothuis, G., & de Graaf, I. A. M. (2022). Disruption of vitamin A homeostasis by the biocide tetrakis(hydroxymethyl) phosphonium sulphate in pregnant rabbits. *Journal of Applied Toxicology*, *42*(12), 1921–1936. https://doi.org/10.1002/jat.4364