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Vitamin D_3 supplementation attenuates the early stage of mouse hepatocarcinogenesis promoted by hexachlorobenzene fungicide

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ABSTRACT

Hexachlorobezene (HCB), a fungicide widely distributed in the environment, promotes the development of hepatocellular preneoplastic lesions (PNL) and tumors in rodents. In contrast, vitamin D_3 (VD_3) supplementation presents a potential role for the prevention/treatment of chronic liver diseases. Thus, we investigated whether VD_3 supplementation attenuates the early stage of HCB-promoted hepatocarcinogenesis. Female Balb/C mice were injected a single dose of diethylnitrosamine (DEN, 50 mg/kg) at postnatal day 15. From day 40 onwards, mice were fed with a standard diet containing 0.02% HCB alone or supplemented with VD_3 (10,000 or 20,000 IU/Kg diet) for 20 weeks. Untreated mice were fed just standard diet. After this period, mice were euthanized and liver and serum samples were collected. Compared to the untreated group, DEN/HCB treatment decreased total hepatic glutathione levels and glutathione peroxidase (GSH-Px) activity while increased lipid peroxidation, p65 protein expression, cell proliferation/apoptosis and the PNL development. In contrast, dietary VD_3 supplementation enhanced vitamin D receptor (VDR) protein expression, total glutathione levels and GSH-Px activity while diminished lipid hydroperoxide levels. Also, VD_3 supplementation decreased p65 protein expression, hepatocyte proliferation, the size and the liver area occupied by PNL. Therefore, our findings indicate that VD_3 supplementation attenuates the early stage of HCB-promoted hepatocarcinogenesis.

1. Introduction

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related deaths worldwide (GLOBOCAN, 2012). The highest incidence and mortality rates for HCC are found in Asian and African countries, also presenting increasing rates in the United States (USA) in the past decades (Siegel et al., 2016). HCC development involves several major etiological agents, mainly chronic hepatitis B and C virus infections (Sanyal et al., 2010). Other non-viral risk factors include alcohol and aflatoxin intake, non-alcoholic fatty liver disease (NAFLD) and tobacco and pesticides exposure (Hamed and Ali, 2013).

Globally, hexachlorobenzene (HCB), an aromatic chlorinated hydrocarbon, was extensively used in agriculture as a pesticide and fungicide and its production exceeded 100.000 tons (Barber et al., 2005). HCB primary emissions to atmosphere probably peaked in the 1970s, when it was discontinued in most countries (Barber et al., 2005). Nowadays, this persistent organochlorine pesticide continues to be released to the environment across from volatilization of "old" HCB on the soil from past contamination, old dump sites and inappropriate manufacture and disposal of wastes of chlorinated solvents, aromatics and pesticides (Barber et al., 2005). HCB exposure is associated with a wide variety of toxic effects in humans and animals (Reed et al., 2007; Mrema et al., 2013). Experimentally, HCB showed to promote the development of hepatocellular preneoplastic (PNL) and neoplastic lesions in both carcinogen-initiated and non-initiated rodents (Stewart et al., 1989; Carthew and Smith, 1994; Gustafson et al., 2000). This carcinogenic effect seems to be associated to an increase in cell proliferation and subsequent loss of the homeostatic balance between cell

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proliferation and death in rodent liver (Giribaldi et al., 2011; Portaz et al., 2015). In addition, HCB showed to increase oxidative stress in rodent liver by reducing antioxidant enzyme activity (Almeida et al., 1997). For these reasons, HCB is usually used as a classical promoting agent in chemically-induced rodent models of hepatocarcinogenesis (Gustafson et al., 2000; Portaz et al., 2015). In these models, the altered hepatocyte foci (AHF), considered as hepatocellular PNL, are easily identified and quantified in short and medium-term bioassays, allowing the investigation of potential modifying factors on early stage of hepatocarcinogenesis, including dietary micronutrients (Klaunig and Kamendulis, 1999; Ogawa, 2009; Romualdo et al., 2016).

Vitamin D (VD) plays a vital role in calcium and phosphorus bone homeostasis as well as it is implicated in the modulation of immunological function, hormone secretion, and cellular proliferation and differentiation (Christakos et al., 2016). VD is acquired from both dietary sources, as vitamin D_2 or D_3 (VD_3), and skin synthesis under the exposition to sunlight, as VD_3 (Christakos et al., 2016). In the liver, VD_3 is metabolized to 25-hydroxyvitamin D_3 [25(OH)D_3]. The 25(OH)D_3 is the major circulating form of VD, typically used as a biomarker to assess serum VD status (Borel et al., 2015; Christakos et al., 2016). Then, 25(OH)D_3 is transported to the kidney and metabolized to calcitriol [1,25(OH)D_3], the active form of VD that mediates its biological effects through the vitamin D receptor (VDR) (Borel et al., 2015; Christakos et al., 2016). Epidemiological studies revealed widespread prevalence of VD insufficiency and deficiency worldwide, especially in several Asian and African countries (Hilger et al., 2014). In the past two decades, VD deficiency increased from 5% to 10% in the USA population (Ganji et al., 2012). Recently, VD insufficiency and/or deficiency have been associated with increased risk or poor prognosis for chronic liver disease development, including hepatitis B, NAFLD, cirrhosis and HCC development (Farnik et al., 2013; Dasarathy et al., 2014; Finkelmeier et al., 2014, 2015). On the other hand, VD_3 supplementation showed to improve the antiviral treatment to hepatitis C (Bitetto et al., 2011). In addition, increased serum 25(OH)D levels were associated with a 49% reduction in the risk for human HCC development (Fedirko et al., 2014).

Considering the concomitance of the increasing incidence and mortality for HCC and the VD insufficiency/deficiency in several human populations, and also emphasizing the potential role of VD_3 supplementation in the prevention/treatment of human chronic liver diseases, we evaluated whether dietary VD_3 supplementation attenuates the early stage of mouse hepatocarcinogenesis promoted by HCB, a persistent organochlorine pollutant.

2. Methods

2.1. Experimental design

Female Balb/C mice were obtained from Multidisciplinary Center for Biological Investigation on Laboratory Animal Science (CEMIB, UNICAMP, Campinas – SP, Brazil). Mice were submitted to a classical neonatal model of hepatocarcinogenesis by receiving a single intraperitoneal (i.p.) injection of DEN [50 mg/kg body weight (b.wt.) in 0.9% saline, Sigma-Aldrich, USA] or saline vehicle at postnatal day (PND) 15 (Fig. 1) (Romualdo et al., 2016). From PND 40 onwards, the female mice were allocated into four groups receiving different experimental diets: standard diet (untreated group) (n = 5 mice); standard diet containing 0.02% HCB (w/w, DEN/HCB group) (n = 10 mice) (British Drug House Chemicals, UK); standard diet containing 0.02% HCB and supplementation with 10,000 or 20,000 IU of VD_3 (BASF, Germany) *per* Kg of chow, respectively (VD_3 10,000 and VD_3 20,000 groups) (n = 10 mice, both) during 20 weeks. For this study, females were chosen since female mice exposed to 0.02% HCB presented a statistically significant increase in the incidence of "liver cell tumors (hepatomas)" compared to male mice (Cabral and Shubik, 1986).

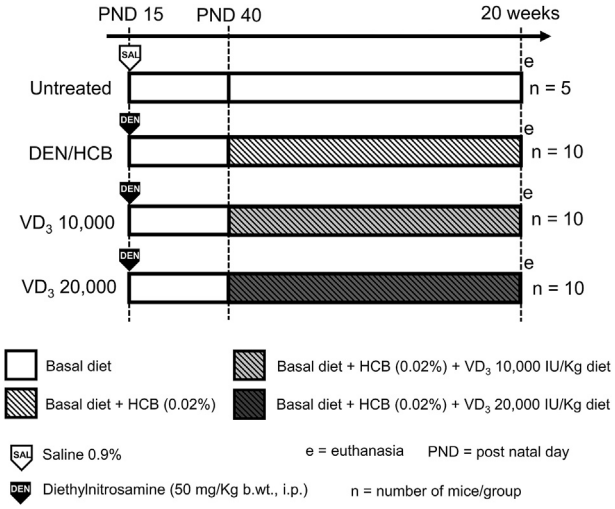

Fig. 1. Experimental design (for details, see "Materials and Methods").

The animals were euthanized by exsanguination under ketamine/xylazine anesthesia (100/16 mg/kg b.wt. i.p.) 20 weeks after the introduction of the experimental diets (Fig. 1). At necropsy, liver, spleen and kidneys were removed and weighted. Liver was washed in saline solution (0.9% NaCl) and samples from all lobes were collected and processed for histopathological and immunohistochemical analysis. Additional liver samples were collected, snap frozen in liquid nitrogen and stored at −80 °C to further performance of western blot and biochemical analysis. Moreover, blood samples were collected by cardiac puncture and serum samples were stored at −20 °C to further determination of serum total 25(OH)D levels. For all described analysis, the number of liver or serum samples analyzed was five (n = 5) for the untreated group and ten (n = 10) for the other groups (DEN/HCB; VD_3 10,000 and VD_3 20,000 groups). Food and water were provided *ad libitum*. Body weight and food consumption were recorded twice a week during all the experimental period. The mice were kept in a room with ventilation (16–18 air changes/hour), relative humidity (45–65%), controlled temperature (20–24 °C) and light/dark cycle 12:12 h. The animal experiment was carried out under protocols approved by Botucatu Medical School/UNESP Ethics Committee on Use of Animals (CEUA) (Protocol number 1073/14) and all animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" (National Research Council, 2011).

2.2. Diets

Standard diet formulation used contained 1000 IU/Kg diet of VD_3 (Presence, In vivo, Brazil), meeting the recommended concentrations for rodents according to the criteria described in the "Nutrient Requirements of Laboratory Animals" (National Research Council, 1995) and corresponding to the average requirement for humans (400 IU/day) (Ross et al., 2011). VD_3 supplemented diets were designed to contain 10 (VD_3 10,000) and 20 (VD_3 20,000)-fold higher VD_3 than the standard diet, similarly to high dose VD_3 supplementation regimens usually applied to humans (Vashi et al., 2010; Alzaman et al., 2016). Besides, the VD_3 supplementation regimens chosen are below the toxicity levels associated with excessive VD_3 intake in humans (above 20,000 IU/day) (Heaney, 2008).

2.3. Histopathological analysis

Liver samples were fixed in 10% buffered formalin for 24 h at room temperature, stored in 70% ethanol and embedded in paraffin. Five-micron thick liver sections from paraffin embedded blocks were stained with hematoxylin and eosin (HE). Liver lesions were identified using well-established criteria (Thoolen et al., 2010). The incidence of these lesions was calculated for each group. For hepatocellular PNL analysis, we calculated ***(a)*** *AHF number/liver area*, counting all AHF and dividing by the liver section area analyzed (cm^2), ***(b)*** *AHF size*, by individually measuring all AHF (mm^2), and ***(c)*** *AHF area*, dividing the sum of all AHF sizes by the liver area analyzed (mm^2/cm^2). Each liver section and all AHF were measured using KS300 image analysis system (Carl Zeiss, Germany).

2.4. Immunohistochemistry

Immunoreactivity for Ki-67, transforming growth factor alpha (TGF-α) and β-catenin were detected using a universal labeled Streptavidin-Biotin system (LSAB System-HRP, Dako Cytomation, Denmark). Briefly, deparaffinated 5-μm liver sections on silane-treated microscope slides were subject to antigen retrieval in 0.01 M citrate buffer (pH 6.0) at 120 °C for 5 min in a Pascal Pressure Chamber (Dako Cytomation, Denmark). After blockade of endogenous peroxidase with 3% H_2O_2 in phosphate-buffered saline (PBS) for 10 min, the slides were treated with low-fat milk for 60 min and incubated in a humidified chamber overnight at 4 °C with anti-Ki-67 (ab16667, 1:100 dilution, Abcam, UK), cleaved caspase-3 (5A1E, dilution 1:50, Cell Signaling Technology, USA), β-catenin (ab32572, 1:400 dilution, Abcam, UK) or TGF-α (ab9585, 1:1000 dilution, Abcam, UK) primary antibodies. The slides were then incubated with a biotinylated universal link and streptavidin HPR for 20 min each. The reaction was visualized with 3′3-diaminobenzidine (DAB) chromogen (Sigma–Aldrich, USA) and counterstained with Harris hematoxylin.

For Ki-67 and cleaved caspase-3 analysis, 40 and 20 random fields were assessed *per* section, respectively comprising all lobes (40 × objective). Ki-67 and cleaved caspase-3-positive hepatocytes were counted and divided by the analyzed liver area (mm^2). TGF-α staining intensity was graded based on Miller et al. (1995), according to the following scale: (−) negative; (+) weak; (++) moderate and (+++) strong staining intensity. Ultimately, for β-catenin, the intensity and pattern of staining (membranous, cytoplasmic and/or nuclear) were evaluated according to Mercer et al. (2014). All semi-quantitative analyses were performed in Olympus cellSens software (Olympus Corporation, Japan).

2.5. Biochemical analysis

Liver samples were homogenized in 50 mM phosphate buffer (pH 7.4) using a motor–driven teflon glass Potter Elvehjem (100 × g/min). The homogenized tissue was centrifuged (12000×*g*, -4 °C, 15 min). The supernatant was used to determine total protein, glutathione peroxidase (GSH-Px) activity and total glutathione (total GSH) and lipid hydroperoxide levels. The GSH-Px activity was assayed by following the oxidation of 0.16 mM NADPH in the presence of glutathione reductase, which catalyzed the reduction of oxidized glutathione (GSSG) formed by the GSH-Px (Nakamura et al., 1974). The total glutathione was assayed with 0.6 mM 5,5′-dithiobis-(2-nitrobenzoic) acid (DTNB, Sigma-Aldrich, USA) and 1 U of glutathione reductase in buffer 0.1 M Tris–HCl, pH 8.0 containing 0.5 mM EDTA (Tietze, 1969). Ultimately, lipid hydroperoxide levels were measured in the liver through hydroperoxide-mediated oxidation of Fe^{2+}, with 100 μL of sample and 900 μL of a reaction mixture containing 250 μM $FeSO_4$, 25 mM H_2SO_4, 100 μM xylenol orange and 4 mM butylated hydroxytoluene in 90% (v/v) methanol (Jiang et al., 1991). All determinations were performed using a microplate reader (25 °C) (μQuant-Gen5 2.0 software, Bio-Tec Instruments, USA).

2.6. Total serum 25(OH)D determination

Total serum 25(OH)D levels were determined by a high performance liquid chromatography (HPLC) method (Turpeinen et al., 2003), using a commercial Kit (Chromsystems Instruments & Chemicals GmbH, Germany). First, a volume of 150 μL of each serum sample was vortexed for 20s, cool down at 4 °C for 10 min, centrifuged at 15,000 × g for 5 min and 75 μL of the supernatant was injected into the Waters HPLC 2695 system (Waters Corporation, USA). This analyzer uses a simple isocratic HPLC system, with a HPLC pump, injector and a UV detector. In summary, serum protein was precipitated, and through selective solid phase extraction, interfering components were removed and the analytes were concentrated.

2.7. Western blot

Liver samples were homogenized in lysis buffer (1% Triton X-100 and 2 μL/100 mL protease inhibitor cocktail, Sigma-Aldrich, USA) (4 °C, 2 h). The extracted material was centrifuged (4000 rpm, 4 °C, 20 min) and the supernatant was collected for protein quantification by Bradford's method. Aliquots of liver homogenates containing 70 μg of total protein were heated (95 °C, 5 min) in sample-loading buffer and then electrophoretically separated in a 12% SDS–PAGE gel under reducing conditions and transferred to nitrocellulose membranes (Sigma-Aldrich, USA). Membranes were blocked with low-fat milk in TBS-T (0.05 M Tris, 0.15 M NaCl, pH 7.2, 1% Tween-20) (1 h). The nitrocellulose membranes were subsequently incubated with anti-NF-κB p65 (sc-372, 65 kDa, 1:1000 dilution, Santa Cruz Biotechnology, USA), Nrf2 (ab31163, 68 kDa, 1:1000 dilution, Abcam, UK), VDR (sc-13133, 60-48 KDa, 1:500 dilution, Santa Cruz Biotechnology, USA) or actin (sc1615, 43 kDa, 1:1000 dilution, Santa Cruz Biotechnology, USA) primary antibodies in 5% BSA solution overnight. After 5 wash steps with PBS-T, membranes were incubated with specific horseradish conjugated secondary antibodies, according to the primary antibodies used (2 h). Finally, after 5 wash steps, the membranes were submitted to immunoreactive protein signals (GE Healthcare Life Sciences, UK). Signals were captured by a G:BOX Chemi system (Syngene, UK) controlled by an automatic software (GeneSys, Syngene, UK). Band intensities were quantified using densitometry analysis software (Image J software, Austria). Finally, NF-κB (p65), VDR and Nrf2 protein expressions were reported as fold change according to actin expression, used as a normalizer. All western blot experiments were performed twice.

2.8. Statistical analysis

Data were analyzed by ANOVA and *post hoc* Tukey's test or Kruskal-Wallis test and *post hoc* Dunn's method. In addition, the incidence of histopathological findings was analyzed by Fisher's test. Significant differences were assumed when $p < 0.05$. All statistical analyses were performed using GraphPad Prism software (GraphPad, USA).

3. Results

3.1. VD_3 supplementation does not alter body weight and selected organ weights

Since HCB has showed to induce adverse effects on different

organs, including potential endocrine, reproductive and immunological toxicities (Reed et al., 2007; Mrema et al., 2013), the noxious effects of HCB feeding on body weight and selected organ weights were evaluated. All groups receiving HCB feeding presented lower final body weight ($p = 0.005$) and higher absolute and relative spleen ($p = 0.01$ and 0.003, respectively) and liver ($p < 0.001$, for both) weights compared to the untreated group (Table 1). This hepatomegaly observed in all HCB-fed groups has been associated with frank centrilobular hypertrophy (Gustafson et al., 2000). In agreement, HCB feeding has also induced splenomegaly in rats due to hyperplasia in splenic B cell compartments (Schielen et al., 1995; Ezendam et al., 2004). The VD_3 dietary interventions did not alter body weight and spleen and liver weights compared to the DEN/HCB-treated group. Finally, mean values of food consumption, absolute and relative thymus and kidney weights were similar among the groups.

3.2. VD_3 10,000 supplementation enhances serum 25(OH)D total levels

Circulating 25(OH)D levels are frequently applied to assess VD status in humans, reflecting VD bioavailability (Borel et al., 2015; Christakos et al., 2016). As expected, both dietary VD_3 supplementations led to higher serum 25(OH)D levels than untreated and DEN/HCB group levels (Table 1). In special, VD_3 10,000 IU intervention significantly increased 25(OH)D levels in comparison to DEN/HCB-treated and untreated groups ($p = 0.016$).

The conversion of serum 25(OH)D_3 to active calcitriol is determined mainly by two renal hydroxylases enzymes, 1α-hydroxylase and 25(OH)D_3 -24-hydroxylase (24-OHase) (Christakos et al., 2016). Especially, 24-OHase expression occurs in all VD responsive tissues and under high VD levels. This enzyme may act as a negative regulatory factor, inactivating calcitriol by chain hydroxylation reactions and stimulating VD renal excretion (Omdahl et al., 2002; Barletta et al., 2004; Borel et al., 2015). Thus, the VD_3 20,000 IU supplementation used herein could have activated this regulatory mechanism, resulting in a non-concentration–response relationship of 25(OH)D serum levels in comparison to dietary VD_3 10,000 IU.

3.3. VD_3 supplementation decreases the size and the liver area occupied by PNL

All DEN/HCB-treated groups showed increased incidence of preneoplastic AHF (showing basophilic, eosinophilic and clear cell phenotype), microgoticular steatosis and centrilobular hepatocellular hypertrophy (Fig. 2A) when compared to untreated group ($p < 0.05$), that developed none of these lesions (Table 2). The HCB-induced centrilobular hypertrophy occurs due to sustained induction of liver MFO enzymatic system (Gustafson et al., 2000) while the microgoticular steatosis may emerge due to HCB-induced oxidative stress, lipid peroxidation and alterations in lipid metabolism (De Catabbi et al., 1997; Morita et al., 2012).

In contrast, both dietary VD_3 interventions did not alter the incidence of different types of hepatocellular lesions (Table 2), as well as the number of AHF *per* liver area (Fig. 2B). Nonetheless, both VD_3 treatments reduced the size (mm^2) and the liver area (mm^2/cm^2) occupied by PNL when compared to DEN/HCB-treated group ($p = 0.002$ and $p = 0.001$, respectively) (Fig. 2B).

3.4. VD_3 supplementation reduces hepatocyte proliferation

VD is implicated to play key roles in the control of cell proliferation (Samuel and Sitrin, 2008). Besides, the HCB carcinogenic effect appears to be related to the increase of cell proliferation and subsequent loss of the balance between proliferation and apoptosis in the liver (Giribaldi et al., 2011; Portaz et al., 2015). In the present study, the hepatocyte proliferation (Ki-67) was significantly higher in all DEN/HCB-treated groups than the untreated group ($p < 0.001$) (Fig. 3). Contrarily, all VD_3 interventions reduced cell proliferation in comparison to DEN/HCB-treated group ($p < 0.001$) (Fig. 3). Moreover, all DEN/HCB-treated groups showed increased apoptosis (cleaved caspase-3) compared to the untreated group ($p < 0.001$) (Supplementary Data 1). However, both VD interventions did not alter this DEN/HCB-mediated effect (Supplementary Data 1).

Both transforming growth factor alfa (TGF-α) and β-catenin expressions are potent stimuli to hepatocyte proliferation, participating in liver growth and regeneration events (Fausto, 1991; Webber et al., 1994; Micsenyi et al., 2004; Tan et al., 2006). Notably, the aberrant activation of both pathways is often related to different stages of human and rodent chemically-induced hepatocarcinogenesis (Moser et al., 1997; Stahl et al., 2005; Kitano et al., 2006; Yeh et al., 2007).

All liver sections from DEN/HCB-treated groups (DEN/HCB, VD_3 10,000 and VD_3 20,000) showed similar strong cytoplasmic TGF-α staining (+++), mainly in hypertrophied centrilobular hepatocytes, while the untreated group did not show liver TGF-α expression (Fig. 4). Ultimately, all liver sections of all groups showed typical *membranous* β-catenin staining pattern (Fig. 4).

Table 1
Effects of Vitamin D_3 supplementation on experimental period parameters and total serum 25(OH)D levels in DEN/HCB-induced hepatocarcinogenesis.

Parameters	Groups/Treatments[a]			
	Untreated	DEN/HCB	VD_3 10,000	VD_3 20,000
n (number of mice/group)	5	10	10	10
Final body wt. (g)	28.40 ± 1.14 a	24.50 ± 1.90 b	25.58 ± 2.99 b	23.75 ± 1.28 b
Food intake (g/mice/day)	4.26 ± 0.79	4.33 ± 0.93	4.40 ± 0.81	4.38 ± 0.81
Thymus wt. (g)	0.69 ± 0.06	0.67 ± 0.13	0.70 ± 0.10	0.69 ± 0.08
Relative thymus wt. (%)	0.36 ± 0.03	0.34 ± 0.07	0.37 ± 0.05	0.38 ± 0.06
Spleen wt. (g)	0.12 ± 0.01 b	0.15 ± 0.02 a	0.14 ± 0.03 a	0.14 ± 0.02 a
Relative spleen wt. (g)	0.41 ± 0.03 b	0.57 ± 0.07 a	0.55 ± 0.09 a	0.57 ± 0.06 a
Liver wt. (g)	1.07 ± 0.05 b	2.34 ± 0.36 a	2.29 ± 0.25 a	2.31 ± 0.27 a
Relative liver wt. (%)	3.76 ± 0.09 b	9.52 ± 0.46 a	9.01 ± 0.94 a	9.71 ± 0.91 a
Kidney wt. (g)	0.16 ± 0.02	0.17 ± 0.12	0.17 ± 0.01	0.17 ± 0.10
Relative kidney wt. (%)	0.68 ± 0.09	0.69 ± 0.07	0.66 ± 0.07	0.64 ± 0.05
Serum 25(OH)D (ng/mL)	19.40 ± 2.22 b	17.88 ± 2.59 b	29.30 ± 4.25 a	23.45 ± 6.55 ab

Vales are Mean ± S.D.
[a] DEN = diethylnitrosamine (50 mg/kg b.wt., i.p); HCB= Hexachlorobenzene (0.02%); VD_3 10,000 or 20,000 = 10,000 or 20,000 IU of Vitamin D_3 supplementation on standard diet (Kg), respectively. Different letters correspond to statistical difference among the groups by ANOVA *post hoc* Tukey's test ($p < 0.05$).

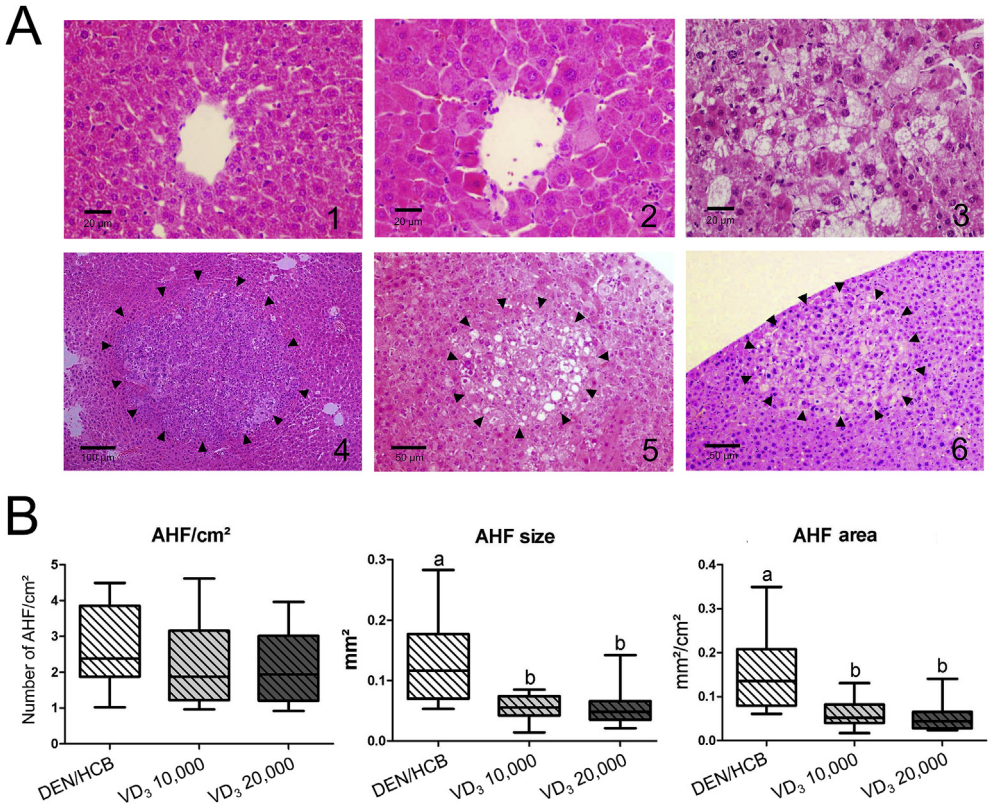

Fig. 2. **(A)** Representative photomicrographs of (1) centrilobular region of normal liver (40 × objective); (2) centrilobular hyperplasia (40 × objective); (3) microgoticular steatosis (40 × objective); (4) basophilic AHF (10 × objective); (5) clear cell AHF (20 × objective); (6) eosinophilic AHF (20 × objective). **(B)** Effects of Vitamin D_3 supplementation on the number of AHF *per* cm^2, AHF size (mm^2) and area (cm^2/mm^2) in DEN/HCB-induced hepatocarcinogenesis. Values are presented as box and whiskers. AHF = Altered Hepatocyte Foci; DEN = diethylnitrosamine (50 mg/kg b.wt., i.p.); HCB= Hexachlorobenzene (0.02%); VD_3 10,000 or 20,000 = 10,000 or 20,000 IU of Vitamin D_3 supplementation on standard diet (Kg), respectively. n = 5 (untreated group) or 10 mice (other groups)/group. Different letters correspond to statistical difference among the groups by Kruskal Wallis *post hoc* Dunn's Method ($p < 0.05$).

Table 2
Effects of Vitamin D_3 supplementation on the incidence of liver lesions in DEN/HCB-induced hepatocarcinogenesis.

Groups/Alterations[a]	Untreated	DEN/HCB	VD_3 10,000	VD_3 20,000
Microgoticular steatosis	0/5 (0) b	6/10 (60%) a	9/10 (90%) a	10/10 (100%) a
Centrilobular hypertrophy	0/5 (0) b	10/10 (100%) a	9/10 (90%) a	10/10 (100%) a
Altered hepatocyte foci	0/5 (0) b	10/10 (100%) a	7/10 (70%) a	10/10 (100%) a

Values represent the proportion of affected mice (percentage).
[a] DEN = diethylnitrosamine (50 mg/kg b.wt., i.p); HCB= Hexachlorobenzene (0.02%); VD_3 10,000 or 20,000 = 10,000 or 20,000 IU of Vitamin D_3 supplementation on standard diet (Kg), respectively. n = 5 (untreated group) or 10 mice (other groups)/group. Different letters correspond to statistical difference among groups by Fisher's Exact test ($p < 0.05$).

3.5. VD_3 supplementation increases total GSH levels, GSH-Px activity and decreases lipid peroxidation

In the liver, there is an increase in oxidative stress levels during HCB biotransformation (Almeida et al., 1997; To-Figueras et al., 1997). One of the major metabolic pathways of HCB is related to its conjugation with glutathione (GSH), a potent endogen electron-donator that acts as a GSH-Px enzyme substrate (To-Figueras et al., 1997). In general, GSH and GSH-Px are important non-enzymatic and enzymatic antioxidant agents, respectively, that protect cells against oxidative damage on protein, lipids and DNA (Lu, 2013; Brigelius-Flohé and Maiorino, 2013).

In our experimental conditions, DEN/HCB exposure resulted in a significant decrease in hepatic total GSH levels and GSH-Px activity when compared to the untreated group ($p < 0.001$, for both) (Fig. 5A and B). Moreover, prolonged dietary exposure to HCB increased liver lipid hydroperoxide levels when compared to the untreated group ($p < 0.001$) (Fig. 5C). Nonetheless, both dietary VD_3 interventions attenuated this HCB-mediated effect by increasing total GSH levels and GSH-Px activity in comparison to DEN/HCB-treated group ($p < 0.001$) (Fig. 5A and B). In addition, the VD_3 20,000 IU supplementation decreased liver lipid peroxidation compared to DEN/HCB group ($p < 0.001$) (Fig. 5C). Interestingly, lipid hydroperoxide levels in this group were similar to the untreated group.

3.6. VD_3 supplementation increases VDR and decreases p65 protein expression

After metabolism, active VD_3 directly binds the VDR, a VD_3-responsive transcription factor, triggering the expression of networks of target genes related to specific biological responses (Pike et al., 2012). As expected, both VD_3 interventions increased VDR protein expression, however, only 20,000 IU significantly increased VDR levels compared to untreated group ($p = 0.039$) (Fig. 6).

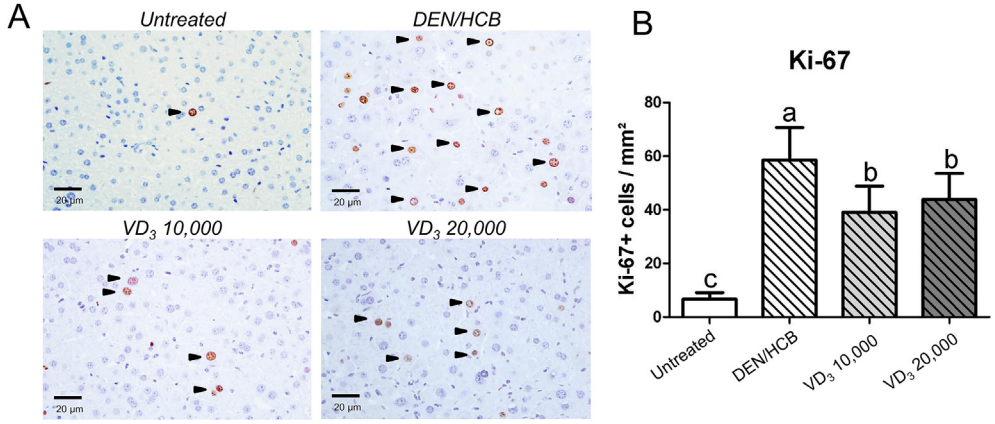

Fig. 3. Effects of VD_3 supplementation on cell proliferation (Ki-67) in DEN/HCB-induced hepatocarcinogenesis. **(A)** Representative photomicrographs of nuclear Ki-67 staining in hepatocytes (arrowheads) (40 × objective). **(B)** Semi-quantitative analysis. Values are presented as Mean + S.D. DEN = diethylnitrosamine (50 mg/kg b. wt., i.p.); HCB= Hexachlorobenzene (0.02%); VD_3 10,000 or 20,000 = 10,000 or 20,000 IU of Vitamin D_3 supplementation on standard diet (Kg), respectively. n = 5 (untreated group) or 10 mice (other groups)/group. Different letters correspond to statistical difference among the groups by ANOVA *post hoc* Tukey's test ($p < 0.05$).

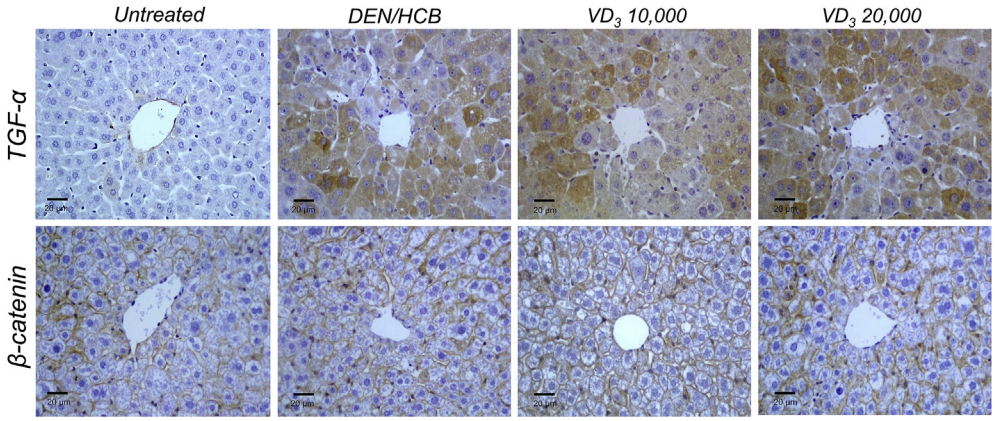

Fig. 4. Representative photomicrographs of TGF-α and β-catenin staining in centrilobular hepatocytes of all experimental groups (40 × objective). DEN = diethylnitrosamine (50 mg/kg b. wt., i.p.); HCB = Hexachlorobenzene (0.02%); VD_3 10,000 or 20,000 = 10,000 or 20,000 IU of Vitamin D_3 supplementation on standard diet (Kg), respectively.

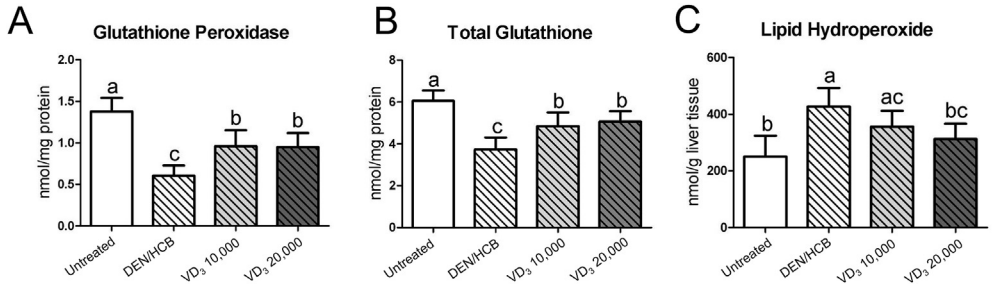

Fig. 5. Effects of Vitamin D_3 supplementation on **(A)** GSH-Px activity, **(B)** total glutathione and **(C)** lipid peroxidation levels in DEN/HCB-induced hepatocarcinogenesis. Values are presented as Mean + S.D. DEN = diethylnitrosamine (50 mg/kg b. wt., i.p.); HCB= Hexachlorobenzene (0.02%); VD_3 10,000 or 20,000 = 10,000 or 20,000 IU of Vitamin D_3 supplementation on standard diet (Kg), respectively. n = 5 (untreated group) or 10 mice (other groups)/group. Different letters correspond to statistical difference among the groups by ANOVA *post hoc* Tukey's test ($p < 0.05$).

The Nrf2 pathway stimulates the antioxidant response to stresses caused by reactive oxygen species (ROS) and electrophiles (Nguyen et al., 2009). Alterations in this pathway are commonly observed during early stages of rodent hepatocarcinogenesis (Zavattari et al., 2015). Under basal liver conditions, like the untreated group, Nrf2 is degraded (Nguyen et al., 2009), resulting in undetectable protein levels (Fig. 6). Oxidative stress and different compounds have shown to activate Nrf2 pathway (Nguyen et al., 2009). Indeed, as a result of the increase in oxidative stress due to DEN/HCB regimen (Fig. 5C), we detected Nrf2 protein expression in this group (Fig. 6). However, both VD_3 supplementations slightly increased Nrf2 protein expression compared to the DEN/HCB-treated group, but without a statistical difference (Fig. 6).

The activation of NF-κB (p65) pathway is an important hallmark of human hepatocarcinogenesis and it is also observed during chemically-induced mice hepatocarcinogenesis (Majumder et al.,

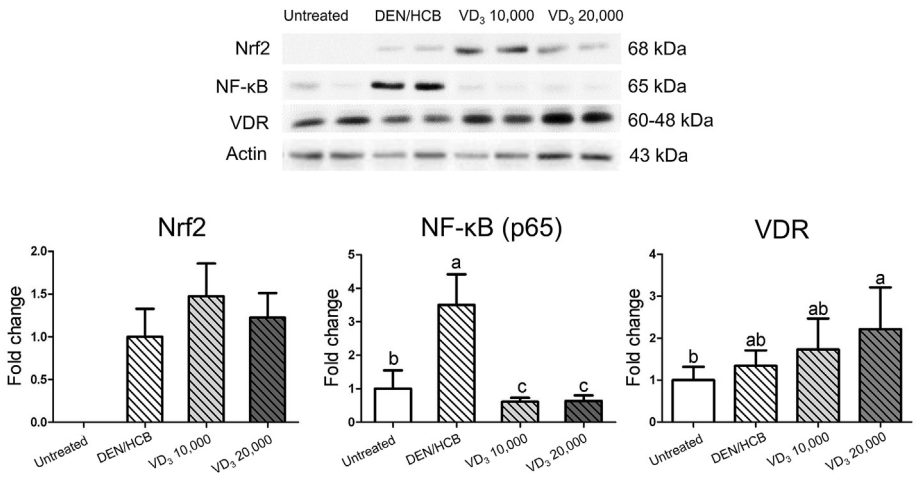

Fig. 6. Effects of Vitamin D_3 supplementation on VDR, Nrf2 and NF-κB (p65) protein expression by Western blot (band intensities and densitometry analysis) in DEN/HCB-induced hepatocarcinogenesis. Values are presented as Mean + S.D. DEN = diethylnitrosamine (50 mg/kg b. wt., i.p.); HCB= Hexachlorobenzene (0.02%); VD_3 10,000 or 20,000 = 10,000 or 20,000 IU of Vitamin D_3 supplementation on standard diet (Kg), respectively. n = 5 (untreated group) or 10 mice (other groups)/group. Different letters correspond to statistical difference among the groups by ANOVA *post hoc* Tukey's test ($p < 0.05$).

2010; Luedde and Schwabe, 2011). Compared to untreated group, DEN/HCB exposure led to a ~3.5 fold increase in NF-κB (p65) protein expression ($p < 0.001$) (Fig. 6). Contrarily, both dietary VD_3 interventions reversed this HCB-mediated effect by remarkably decreasing NF-κB (p65) protein expression in comparison to the DEN/HCB and untreated groups ($p < 0.001$) (Fig. 6).

4. Discussion

In the present study, we evaluated whether dietary supplementation of 10,000 or 20,000 IU of VD_3 attenuates the early stage of mouse hepatocarcinogenesis promoted by HCB, a persistent organochlorine pollutant. Compared to the untreated group, the DEN/HCB regimen resulted in a reduction in both total GSH levels and GSH-Px activity while increased lipid peroxidation in the liver. Moreover, the DEN/HCB regimen increased p65 protein expression, hepatocyte proliferation, apoptosis and the development of hepatocellular PNL. On the other hand, dietary VD_3 supplementation enhanced VDR protein expression (20,000 IU), total GSH levels and GSH-Px activity (10,000 and 20,000 IU) while diminished lipid hydroperoxide levels (20,000 IU). In addition, both VD_3 supplementations (10,000 and 20,000 IU) decreased p65 protein expression, hepatocyte proliferation, PNL size and the liver area occupied by PNL.

After oral exposure, HCB fungicide is metabolized in the liver, resulting on its association with GSH, a molecule that acts as a substrate for GSH-Px enzyme (To-Figueras et al., 1997). In fact, the prolonged dietary exposure to HCB contributed to the cellular depletion of total GSH and to the decrease of hepatic GSH-Px activity in consequence. In fact, the GSH-Px enzyme neutralizes lipid hydroperoxides, which are the end products of oxidative degradation of cell and organelle membrane lipids (Brigelius-Flohé and Maiorino, 2013). Indeed, the HCB-mediated adverse effects on total GSH/GSH-Px resulted in an increase in lipid hydroperoxide levels, an indicator of oxidative stress (Barrera, 2012). Contrastingly, dietary VD_3 supplementations attenuated HCB adverse effects by increasing total-GSH levels and GSH-Px activity. The up-regulation of total GSH/GSH-Px could directly reestablish liver antioxidant capacity and, then, reduce lipid hydroperoxide levels (Fig. 7). Previous findings have already established a positive correlation between VD_3 supplementation and the increase in the levels/activity of many antioxidant agents as superoxide dismutase (SOD), catalase and GSH-Px itself in rodents submitted to hepatic ischemia and reperfusion injury (Seif and Abdelwahed, 2014; Yang et al., 2015). The main regulator of antioxidant responses, including GSH-Px enzyme and GSH synthesis, is the Keap1/Nrf2/ARE pathway (Nguyen et al., 2009). Although the mechanism is not fully understood, it is suggested that VD_3 plays a critical role in the positive modulation of Keap1/Nrf2/ARE pathway through VDR-mediated mechanisms (Nakai et al., 2014). Here, despite of observing significant increases in VDR protein expression and total GSH/GSH-Px, VD_3 dietary interventions did not significantly increase Nrf2 protein expression compared to DEN/HCB-treated group. Therefore, further studies to elucidate the effects of VD_3 supplementation on the Keap1/Nrf2/ARE pathway are necessary.

The nuclear factor κB (NF-κB), formed by p50, p65, and IkB subunits, is a redox-sensitive transcription factor (Morgan and Liu, 2011). Increased oxidative stress activates NF-κB, which is

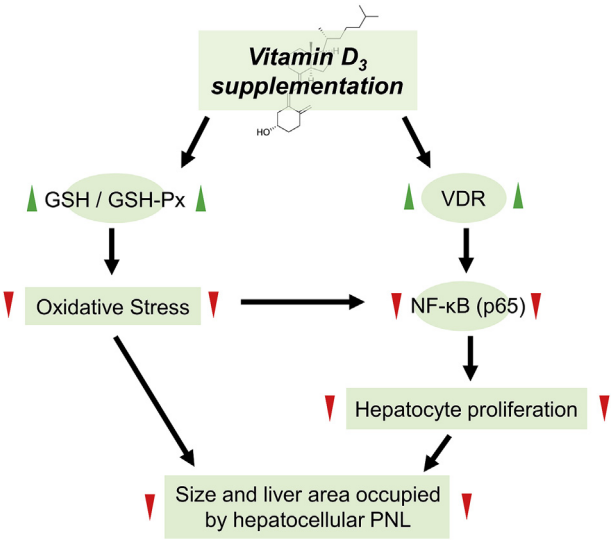

Fig. 7. The effects of VD_3 supplementation (blue) on the early stage of DEN/HCB-induced hepatocarcinogenesis. increase; decrease. PNL = preneoplastic lesions; VDR = Vitamin D Receptor. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

translocated to nucleus (including p65 subunit), increasing the expression of genes involved in inflammation, cell proliferation and survival (Pahl, 1999; Morgan and Liu, 2011). The findings showed a markedly increasing of NF-κB p65 subunit protein expression in the liver of DEN/HCB-treated mice, possibly as a result of the enhanced hepatic oxidative stress. Furthermore, the TGF-α protein promotes the activation of epidermal growth factor receptor (EGFR) (Seshacharyulu et al., 2013). When active, EGFR stimulates pro-proliferative signaling (Seshacharyulu et al., 2013). In HCB feeding studies, this herbicide has already demonstrated to increase microsomal EGFR content and activation in rat liver (Randi et al., 2003, 2008). Here, all HCB-promoted groups showed strong cytoplasmic TGF-α immunostaining. These results indicate that both NF-κB and TGF-α pathways may have provided the stimuli to increase hepatocyte proliferation during DEN/HCB-induced hepatocarcinogenesis.

On the other hand, both dietary VD_3 supplementations reversed this DEN/HCB-mediated adverse effect by notably decreasing p65 protein expression without altering TGF-α and β-catenin immunostaining in the liver. A recent *in vitro* study revealed that VD_3 inhibited NF-κB nuclear translocation through the VDR protein, which directly binds to IKKβ protein (Chen et al., 2013). The interaction between the C-terminal portions of both VDR and IKKβ proteins abolishes IKKβ activity, arresting p65/p50 nuclear translocation and, ultimately, decreasing NF-κB pathway activation (Chen et al., 2013). Thus, a VD_3-mediated increase in VDR, as well as the decrease in oxidative stress, may attenuate NF-κB pathway activation, leading to a decrease in p65 protein expression and hepatocyte proliferation (Fig. 7).

In the early stages of DEN/HCB-induced hepatocarcinogenesis model, the altered hepatic microenvironment associated with increased oxidative stress and cell proliferation favors the development of preneoplastic AHF (Almeida et al., 1997; Giribaldi et al., 2011; Portaz et al., 2015) (Fig. 7). Putative preneoplastic AHF have been considered suitable early endpoint biomarkers for the identification of potential modifying factors of hepatocarcinogenesis (Klaunig and Kamendulis, 1999; Ogawa, 2009). Under early promoting stimuli, these lesions accumulate progressive molecular alterations, which may contribute to AHF growth and ultimately, late progression into neoplastic lesions, as adenomas and HCC (Ogawa, 2009). Previous findings indicated that VD_3 oral treatment attenuated the development of liver PNL or hyperplastic nodules in rats initiated with DEN and promoted by sodium phenobarbital or streptozocin (Saha et al., 2002; Banakar et al., 2004). Here, we showed similar results as dietary VD_3 supplementation reduced the development of hepatocellular PNL by decreasing the size and the liver area occupied by these lesions. The positive modulation of total GSH/GSH-Px and subsequent decrease of hepatic oxidative stress, as well as the decrease of p65/hepatocyte proliferation, are proposed as potential modes of action to the VD_3-mediated beneficial effect (Fig. 7).

In conclusion, the present study demonstrates that dietary VD_3 supplementation attenuates the early stage of HCB-promoted hepatocarcinogenesis. To our knowledge, this is the first report of the beneficial effects of VD_3 supplementation in a mouse model of hepatocarcinogenesis promoted by HCB, a persistent organochloride pollutant. Our findings could reinforce the potential importance of VD_3 supplementation on the adjuvant treatment for human liver diseases.

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Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.fct.2017.06.030.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fct.2017.06.030.

References

Almeida, M.G., Fanini, F., Davino, S.C., Aznar, a E., Koch, O.R., Barros, S.B.D., 1997. Pro- and anti-oxidant parameters in rat liver after short term exposure to hexachlorobenzene. Hum. Exp. Toxicol. 16, 257–261. http://dx.doi.org/10.1177/096032719701600504.

Alzaman, N.S., Dawson-hughes, B., Nelson, J., Alessio, D.D., Pittas, A.G., 2016. Vitamin D status of black and white Americans and changes in vitamin D metabolites after varied doses of vitamin D supplementation. Am. J. Clin. Nutr. http://dx.doi.org/10.3945/ajcn.115.129478.

Banakar, M.C., Paramasivan, S.K., Chattopadhyay, M.B., Datta, S., Chakraborty, P., Chatterjee, M., Kannan, K., Thygarajan, E., 2004. 1α, 25-dihydroxyvitamin D_3 prevents DNA damage and restores antioxidant enzymes in rat hepatocarcinogenesis induced by diethylnitrosamine and promoted by phenobarbital. World J. Gastroenterol. 10, 1268–1275.

Barber, J.L., Sweetman, A.J., Wijk, D., Van Jones, K.C., 2005. Hexachlorobenzene in the global environment: emissions, levels, distribution, trends and processes. Sci. Total Environ. 349, 1–44. http://dx.doi.org/10.1016/j.scitotenv.2005.03.014.

Barletta, F., Dhawan, P., Christakos, S., Dhawan, P., Inte, S.C., 2004. Integration of hormone signaling in the regulation of human 25 (OH)D3 24-hydroxylase transcription. Am. J. Physiol. Endocrinol. Metab. 286, E598–E608.

Barrera, G., 2012. Oxidative stress and lipid peroxidation products in cancer progression and therapy. ISRN Oncol. 137289. http://dx.doi.org/10.5402/2012/137289.

Bitetto, D., Fabris, C., Fornasiere, E., Pipan, C., Fumolo, E., Cussigh, A., Bignulin, S., Cmet, S., Fontanini, E., Falleti, E., Martinella, R., Pirisi, M., Toniutto, P., 2011. Vitamin D supplementation improves response to antiviral treatment for recurrent hepatitis. C. Transpl. Int. 24, 43–50. http://dx.doi.org/10.1111/j.1432-2277.2010.01141.x.

Borel, P., Caillaud, D., Cano, N.J., 2015. Vitamin D bioavailability: state of the art. Crit. Rev. Food Sci. Nutr. 55 (9), 1193–1205. http://dx.doi.org/10.1080/10408398.2012.688897.

Brigelius-Flohé, R., Maiorino, M., 2013. Glutathione peroxidases. Biochim. Biophys. Acta 1830, 3289–3303. http://dx.doi.org/10.1016/j.bbagen.2012.11.020.

Cabral, J.R., Shubik, P., 1986. Carcinogenic activity of hexachlorobenzene in mice and hamsters. IARC Sci. Publ. 77, 411–446.

Carthew, P., Smith, A.G., 1994. Pathological mechanisms of hepatic tumour formation in rats exposed chronically to dietary hexachlorobenzene. J. App. Toxicol. 14, 447–452. http://dx.doi.org/10.1002/jat.2550140610.

Chen, Y., Zhang, J., Ge, X., Du, J., Deb, D.K., Li, Y.C., 2013. Vitamin D receptor inhibits NF-κB activation by interacting with IKKβ protein. J. Biol. Chem. 288, 19450–19458. http://dx.doi.org/10.1074/jbc.M113.467670.

Christakos, S., Dhawan, P., Verstuyf, A., Verlinden, L., Carmeliet, G., 2016. Vitamin D: metabolism, molecular mechanism of action, and pleiotropic effects. Physiol. Rev. 96, 365–408. http://dx.doi.org/10.1152/physrev.00014.2015.

Dasarathy, J., Periyalwar, P., Allampati, S., Bhinder, V., Hawkins, C., Brandt, P., Khiyami, A., McCullough, A.J., Dasarathy, S., 2014. Hypovitaminosis D is associated with increased whole body fat mass and greater severity of non-alcoholic fatty liver disease. Liver Int. 34 http://dx.doi.org/10.1111/liv.12312 e118–27.

De Catabbi, S.B., Sterin-Speziale, N., Fernandez, M.C., Minutolo, C., Aldonatti, C., San Martin De Viale, L., 1997. Time course of hexachlorobenzene-induced alterations of lipid metabolism and their relation to porphyria. Int. J. Biochem. Cell Biol. 29, 335–344. http://dx.doi.org/10.1016/S1357-2725(96)00096-9.

Ezendam, J., Hassing, I., Bleumink, R., Vos, J.G., Pieters, R., 2004. Hexachlorobenzene-induced immunopathology in Brown Norway rats is partly mediated by T cells. Toxicol. Sci. 78, 88–95. http://dx.doi.org/10.1093/toxsci/kfh034.

Farnik, H., Bojunga, J., Berger, A., Allwinn, R., Waidmann, O., Kronenberger, B., Keppler, O.T., Zeuzem, S., Sarrazin, C., Lange, C.M., 2013. Low vitamin D serum concentration is associated with high levels of hepatitis B virus replication in chronically infected patients. Hepatology 58, 1270–1276. http://dx.doi.org/10.1002/hep.26488.

Fausto, N., 1991. Growth factors in liver development, regeneration and carcinogenesis. Prog. Growth Factor Res. 3, 219–234. http://dx.doi.org/10.1016/0955-2235(91)90008-R.

Fedirko, V., Duarte-Salles, T., Bamia, C., Trichopoulou, A., Aleksandrova, K.,

Trichopoulos, D., Trepo, E., Tjønneland, A., Olsen, A., Overvad, K., Boutron-Ruault, M.C., Clavel-Chapelon, F., Kvaskoff, M., Kühn, T., Lukanova, A., Boeing, H., Buijsse, B., Klinaki, E., Tsimakidi, C., Naccarati, A., Tagliabue, G., Panico, S., Tumino, R., Palli, D., Bueno-de-Mesquita, H.B., Siersema, P.D., Peters, P.H., Lund, E., Brustad, M., Olsen, K.S., Weiderpass, E., Zamora-Ros, R., Sánchez, M.J., Ardanaz, E., Amiano, P., Navarro, C., Quirós, J.R., Werner, M., Sund, M., Lindkvist, B., Malm, J., Travis, R.C., Khaw, K.T., Stepien, M., Scalbert, A., Romieu, I., Lagiou, P., Riboli, E., Jenab, M., 2014. Prediagnostic circulating vitamin D levels and risk of hepatocellular carcinoma in European populations: a nested case-control study. Hepatology 60, 1222–1230. http://dx.doi.org/10.1002/hep.27079.

Finkelmeier, F., Kronenberger, B., Köberle, V., Bojunga, J., Zeuzem, S., Trojan, J., Piiper, A., Waidmann, O., 2014. Severe 25-hydroxyvitamin D deficiency identifies a poor prognosis in patients with hepatocellular carcinoma - a prospective cohort study. Aliment. Pharmacol. Ther. 39, 1204–1212. http://dx.doi.org/10.1111/apt.12731.

Finkelmeier, F., Kronenberger, B., Zeuzem, S., Piiper, A., Waidmann, O., 2015. Low 25-hydroxyvitamin D levels are associated with infections and mortality in patients with cirrhosis. Plos One 10, e0132119. http://dx.doi.org/10.1371/journal.pone.0132119.

Ganji, V., Zhang, X., Tangpricha, V., 2012. Serum 25-hydroxyvitamin D concentrations and prevalence estimates of hypovitaminosis D in the U.S. population based on assay-adjusted data. Am. J. Clin. Nutr. 142, 498–507. http://dx.doi.org/10.3945/jn.111.151977.1.

Giribaldi, L., Chiappini, F., Pontillo, C., Randi, A.S., Pisarev, D.L.K., De Alvarez, L., 2011. Hexachlorobenzene induces deregulation of cellular growth in rat liver. Toxicology 289, 19–27. http://dx.doi.org/10.1016/j.tox.2011.07.004.

GLOBOCAN, 2012. GLOBOCAN 2012: Estimated Cancer Incidence, Mortality and Prevalence Worldwide in 2012. Accessed 17.May.2016. http://globocan.iarc.fr/Default.aspx.

Gustafson, D.L., Long, ME., Thomas, R.S., Benjaming, A., Yang, R.S., 2000. Comparative hepatocarcinogenicity of hexachlorobenzene, pentachlorobenzene, 1,2,4,5-Tetrachlorobenzene, and 1,4-Dichlorobenzene: application of a medium-term liver focus bioassay and molecular and cellular indices. Toxicol. Sci. 53, 245–252. http://dx.doi.org/10.1093/toxsci/53.2.245.

Hamed, M.A., Ali, S.A., 2013. Non-viral factors contributing to hepatocellular carcinoma. World J. Hepatol. 5, 311–322. http://dx.doi.org/10.4254/wjh.v5.i6.311.

Heaney, R.P., 2008. Vitamin D: criteria for safety and efficacy. Nutr. Rev. 66 (10 Suppl. 2), S178–S181. http://dx.doi.org/10.1111/j.1753-4887.2008.00102.x.

Hilger, J., Friedel, A., Herr, R., Rausch, T., Roos, F., Wahl, D.A., Pierroz, D.D., Weber, P., Hoffmann, K., 2014. A systematic review of vitamin D status in populations worldwide. Br. J.Nutr 111, 23–45. http://dx.doi.org/10.1017/S0007114513001840.

Jiang, Z.Y., Woollard, A., Wolff, S., 1991. Lipid hydroperoxide measurement by oxidation of Fe3+ in the presence of xylenol orange. Lipids 26, 853–856.

Kitano, M., Wada, J., Ariki, Y., Kato, M., Wanibuchi, H., Morimura, K., Hidaka, T., Hosoe, K., Fukushima, S., 2006. Possible tumor development from double positive foci for TGF-alpha and GST-P observed in early stages on rat hepatocarcinogenesis. Cancer Sci. 97, 478–483. http://dx.doi.org/10.1111/j.1349-7006.2006.00210.x.

Klaunig, J.E., Kamendulis, L.M., 1999. Mechanisms of cancer chemoprevention in hepatic carcinogenesis: modulation of focal lesion growth in mice. Toxicol. Sci. 52, 101–106.

Lu, S.C., 2013. Glutathione synthesis. Biochim. Biophys. Acta 1830, 3143–3153. http://dx.doi.org/10.1016/j.bbagen.2012.09.008.

Luedde, T., Schwabe, R.F., 2011. NF-κB in the liver—linking injury, fibrosis and hepatocellular carcinoma. Nat. Rev. Gastroenterol. Hepatol. 8, 108–118. http://dx.doi.org/10.1038/nrgastro.2010.213.

Majumder, S., Roy, S., Kaffenberger, T., Wang, B., Costinean, S., Frankel, W., Bratasz, A., Kuppusamy, P., Hai, T., Ghoshal, K., Jacob, S.T., 2010. Loss of metallothionein predisposes mice to diethylnitrosamine-induced hepatocarcinogenesis by activating NF-κB target genes. Cancer Res. 70, 10265–10276. http://dx.doi.org/10.1158/0008-5472.CAN-10-2839.

Mercer, K.E., Hennings, L., Sharma, N., Lai, K., Cleves, M.a, Wynne, R. a, Badger, T.M., Ronis, M.J.J., 2014. Alcohol consumption promotes diethylnitrosamine-induced hepatocarcinogenesis in male mice through activation of the Wnt/β-catenin signaling pathway. Cancer Prev. Res. 7, 675–685. http://dx.doi.org/10.1158/1940-6207.CAPR-13-0444-T.

Micsenyi, A., Tan, X., Sneddon, T., Luo, J.-H., Michalopoulos, G.K., Monga, S.P.S., 2004. β-Catenin is temporally regulated during normal liver development. Gastroenterology 126, 1134–1146. http://dx.doi.org/10.1053/j.gastro.2003.12.047.

Miller, R.T., Cattley, R.C., Marsman, D.S., Lyght, O., Popp, J.a, 1995. TFGα is differentially expressed in liver foci induced by diethylnitrosamine initiation and peroxisome proliferator promotion. Carcinogenesis 16, 77–82.

Morgan, M.J., Liu, Z., 2011. Crosstalk of reactive oxygen species and NF-κB signaling. Cell Res. 21, 103–115. http://dx.doi.org/10.1038/cr.2010.178.

Morita, M., Ishida, N., Uchiyama, K., Yamaguchi, K., Itoh, Y., Shichiri, M., Yoshida, Y., Hagihara, Y., Naito, Y., Yoshikawa, T., Niki, E., 2012. Fatty liver induced by free radicals and lipid peroxidation. Free Rad. Res. 46, 758–765. http://dx.doi.org/10.3109/10715762.2012.677840.

Moser, G.J., Wolf, D.C., Goldsworthy, T.L., 1997. Quantitative relationship between transforming growth factor-alpha and hepatic focal phenotype and progression in female mouse liver. Toxicol. Pathol. 25, 275–283. http://dx.doi.org/10.1177/019262339702500305.

Mrema, E.J., Rubino, F.M., Brambilla, G., Moretto, A., Tsatsakis, A.M., Colosio, C., 2013. Persistent organochlorinated pesticides and mechanisms of their toxicity. Toxicology 307, 74–88. http://dx.doi.org/10.1016/j.tox.2012.11.015.

Nakai, K., Fujii, H., Kono, K., Goto, S., Kitazawa, R., Kitazawa, S., Hirata, M., Shinohara, M., Fukagawa, M., Nishi, S., 2014. Vitamin D activates the Nrf2-keap1 antioxidant pathway and ameliorates nephropathy in diabetic rats. Am. J. Hypertens. 27, 586–595. http://dx.doi.org/10.1093/ajh/hpt160.

Nakamura, W., Hojoda, S., Hayashi, K., 1974. Purification and properties of rat liver glutathione peroxidase. Biochim. Biophys. Acta 358, 251–261.

National Research Council (US) Subcommittee on Laboratory Animal Nutrition, 1995. Nutrient Requirements of Laboratory Animals, fourth revised ed. National Academies Press, Washington (DC).

National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011. Guide for the Care and Use of Laboratory Animals, eight ed. National Academies Press, Washington (DC).

Nguyen, T., Nioi, P., Pickett, C.B., 2009. The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress. J. Biol. Chem. 284, 13291–13295. http://dx.doi.org/10.1074/jbc.R900010200.

Ogawa, K., 2009. Molecular pathology of early stage chemically induced hepatocarcinogenesis. Pathol. Int. 59, 605–622. http://dx.doi.org/10.1111/j.1440-1827.2009.02416.x.

Omdahl, J.L., Morris, H.a, May, B.K., 2002. Hydroxylase enzymes of the vitamin D pathway: expression, function, and regulation. Annu. Rev. Nutr. 22, 139–166. http://dx.doi.org/10.1146/annurev.nutr.22.120501.150216.

Pahl, H.L., 1999. Activators and target genes of Rel/NF-kappaB transcription factors. Oncogene 18, 6853–6866. http://dx.doi.org/10.1038/sj.onc.1203239.

Pike, J.W., Meyer, M.B., Bishop, K.A., 2012. Regulation of target gene expression by the vitamin D receptor - an update on mechanisms. Rev. Endocr. Metab. Disord. 13, 45–55. http://dx.doi.org/10.1007/s11154-011-9198-9.

Portaz, A.C.T., Caimi, G.R., Sánchez, M., Chiappini, F., Randi, A.S., Kleiman de Pisarev, D.L., Alvarez, L., 2015. Hexachlorobenzene induces cell proliferation, and aryl hydrocarbon receptor expression (AhR) in rat liver preneoplastic foci, and in the human hepatoma cell line HepG2. AhR is a mediator of ERK1/2 signaling, and cell cycle regulation in HCB-treated HepG2 cells. Toxicology 336, 36–47. http://dx.doi.org/10.1016/j.tox.2015.07.013.

Randi, A.S., Sancovich, H.a, Ferramola De Sancovich, A.M., Loaiza, A., Kölliker Frers, R.a., Spinelli, F., Kleiman De Pisarev, D.L., 2003. Effect of in vivo administered hexachlorobenzene on epidermal growth factor receptor levels, protein tyrosine kinase activity, and phosphotyrosine content in rat liver. Biochem. Pharmacol. 65, 1495–1506. http://dx.doi.org/10.1016/S0006-2952(03)00107-2.

Randi, A.S., Sanchez, M.S., Alvarez, L., Cardozo, J., Pontillo, C., de Pisarev, D.L.K., 2008. Hexachlorobenzene triggers AhR translocation to the nucleus, c-Src activation and EGFR transactivation in rat liver. Toxicol. Lett. 177, 116–122. http://dx.doi.org/10.1016/j.toxlet.2008.01.003.

Reed, L., Buchner, V., Tchounwou, P.B., 2007. Environmental toxicology and health effects associated with hexachlorobenzene exposure. Rev. Environ. Health 18, 203–229.

Romualdo, G.R., Goto, R.L., Henrique Fernandes, A.A., Cogliati, B., Barbisan, L.F., 2016. Dietary zinc deficiency predisposes mice to the development of preneoplastic lesions in chemically-induced hepatocarcinogenesis. Food Chem. Toxicol. 96, 280–289.

Ross, A.C., Manson, J.E., Abrams, S.A., Aloia, J.F., Brannon, P.M., Clinton, S.K., Durazo-Arvizu, R.A., Gallagher, J.C., Gallo, R.L., Jones, G., Kovacs, C.S., Mayne, S.T., Rosen, C.J., Shapses, S.A., 2011. The 2011 report on dietary reference intakes for calcium and vitamin D from the institute of medicine: what clinicians need to know. J. Clin. Endocrinol. Metab. 96, 53–58. http://dx.doi.org/10.1210/jc.2010-2704.

Saha, B.K., Bhattacharya, R., Chatterjee, M., 2002. 1alpha, 25-Dihydroxyvitamin D_3 inhibits rat liver ultrastructural changes and the development of -glutamyl-transpeptidase-positive foci in diabetes-promoted hepatocarcinogenesis. Cell biochem. Funct. 20, 195–204.

Samuel, S., Sitrin, M.D., 2008. Vitamin D's role in cell proliferation and differentiation. Nutr. Rev. 66, S116–S124. http://dx.doi.org/10.1111/j.1753-4887.2008.00094.x.

Sanyal, A.J., Yoon, S.K., Lencioni, R., 2010. The etiology of hepatocellular carcinoma and consequences for treatment. The Oncologist 4 (15 Suppl. l), 14–22. http://dx.doi.org/10.1634/theoncologist.2010-S4-14.

Schielen, P., Van Rodijnen, W., Pieters, R.H., Seinen, W., 1995. Hexachlorobenzene treatment increases the number of splenic B-1-like cells and serum autoantibody levels in the rat. Immunology 86, 568–574.

Seif, A.A., Abdelwahed, D.M., 2014. Vitamin D ameliorates hepatic ischemic/reperfusion injury in rats. J. Physiol. Biochem. 1–8. http://dx.doi.org/10.1007/s13105-014-0335-2.

Seshacharyulu, P., Ponnusamy, M., Haridas, D., Jain, M., Ganti, A., Batra, S., 2013. Targeting the EGFR signaling pathway in cancer therapy. Expert Opin. Ther. Targets 16, 15–31. http://dx.doi.org/10.1517/14728222.2011.648617.Targeting.

Siegel, R.L., Miller, K.D., Jemal, A., 2016. Cancer statistics, 2016. CA Cancer J. Clin. 66, 7–30. http://dx.doi.org/10.3322/caac.21332.

Stahl, S., Ittrich, C., Marx-Stoelting, P., Köhle, C., Altug-Teber, O., Riess, O., Bonin, M., Jobst, J., Kaiser, S., Buchmann, A., Schwarz, M., 2005. Genotype-phenotype relationships in hepatocellular tumors from mice and man. Hepatology 42, 353–361. http://dx.doi.org/10.1002/hep.20768.

Stewart, F.P., Manson, M.M., Cabral, J.R.P., 1989. Hexachlorobenzene as a promoter of diethylnitrosamine-initiated hepatocarcinogenesis in rats and comparison with induction of porphyria. Rev. Environ. Health 10, 1225–1230.

Tan, X., Behari, J., Cieply, B., Michalopoulos, G.K., Monga, S.P.S., 2006. Conditional deletion of β-catenin reveals its role in liver growth and regeneration.

Gastroenterology 131, 1561–1572. http://dx.doi.org/10.1053/j.gastro.2006.08.042.
Thoolen, B., Maronpot, R.R., Harada, T., Nyska, A., Rousseaux, C., Nolte, T., Malarkey, D.E., Kaufmann, W., Küttler, K., Deschl, U., Nakae, D., Gregson, R., Vinlove, M.P., Brix, A.E., Singh, B., Belpoggi, F., Ward, J.M., 2010. Proliferative and nonproliferative lesions of the rat and mouse hepatobiliary system. Toxicol. Pathol. 38, 5S–81S. http://dx.doi.org/10.1177/0192623310386499.
Tietze, F., 1969. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. Anal. Biochem. 27, 502–522.
To-Figueras, J., Sala, M., Otero, R., Barrot, C., Santiago-Silva, M., Rodamilans, M., Herrero, C., Grimalt, J., Sunyer, J., 1997. Metabolism of hexachlorobenzene in humans: association between serum levels and urinary metabolites in a highly exposed population. Environ. Health Persp 105, 78–83.
Turpeinen, U., Hohenthal, U., Stenman, U.H., 2003. Determination of 25-hydroxyvitamin D in serum by HPLC and immunoassay. Clin. Chem. 49, 1521–1524. http://dx.doi.org/10.1373/49.9.1521.
Vashi, P.G., Trukova, K., Lammersfeld, C.a, Braun, D.P., Gupta, D., 2010. Impact of oral vitamin D supplementation on serum 25-hydroxyvitamin D levels in oncology. Nutr. J. 9, 60. http://dx.doi.org/10.1186/1475-2891-9-60.
Webber, E.M., Wu, J.C., Wang, L., Merlino, G., Fausto, N., 1994. Overexpression of transforming growth factor-alpha causes liver enlargement and increased hepatocyte proliferation in transgenic mice. Am. J. Pathol. 145, 398–408.
Yang, J., Chen, Q., Tian, S., Song, S., Liu, F., Wang, Q., Fu, Z., 2015. The role of 1,25-dyhydroxyvitamin D3 in mouse liver ischemia reperfusion injury: regulation of autophagy through activation of MEK/ERK signaling and PTEN/PI3K/Akt/mTORC1 signaling. Am. J. Transl. Res. 7, 2630–2645.
Yeh, M.M., Larson, A.M., Campbell, J.S., Fausto, N., Rulyak, S.J., Swanson, P.E., 2007. The expression of transforming growth factor-alpha in cirrhosis, dysplastic nodules, and hepatocellular carcinoma: an immunohistochemical study of 70 cases. Am. J. Surg. Pathol. 31 (5), 681–689.
Zavattari, P., Perra, A., Menegon, S., Kowalik, M.A., Petrelli, A., Angioni, M.M., Follenzi, A., Quagliata, L., Ledda-Columbano, G.M., Terracciano, L., Giordano, S., Columbano, A., 2015. Nrf2, but not β-catenin, mutation represents an early event in rat hepatocarcinogenesis. Hepatology 62, 851–862. http://dx.doi.org/10.1002/hep.27790.