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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).

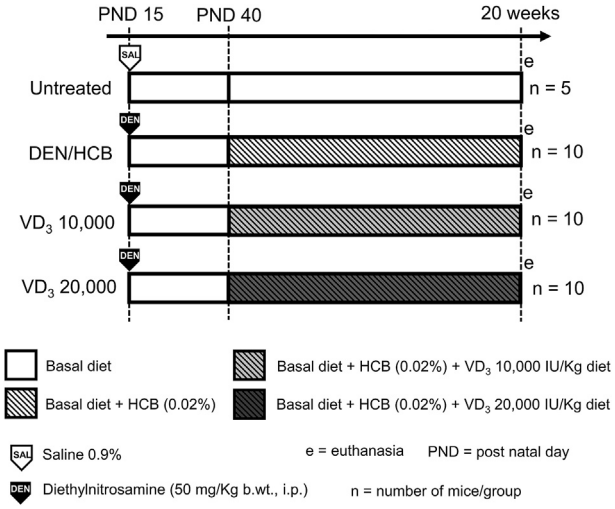

Fig. 1. Experimental design (for details, see "Materials and Methods").

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).