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The effect of vitamin D deficiency and 1,25(OH)$_2$D$_3$ treatment on oxidative stress and Nrf2-antioxidant signaling in ethanol-induced hepatotoxicity

D vitamini eksikliği ve 1,25(OH)$_2$D$_3$ uygulamasının etanole bağlı karaciğer hasarında oksidatif stres ve Nrf2-antioksidan sinyal sistemi üzerine etkisi

İlknur Bingül[1], A. Fatih Aydın[1], Canan Küçükgergin[1], Işın Doğan-Ekici[2], Semra Doğru-Abbasoğlu[1], Müjdat Uysal[1]

Abstract

Aim: Vitamin D deficiency (VDD) is suggested to enhance hepatotoxicity in chronic liver diseases. However, there is limited knowledge about the association between VDD and alcoholic liver damage. Therefore, the effect of VDD on ethanol (EtOH)-induced hepatotoxicity was investigated in this study. Moreover, the role of the Nrf2-antioxidant signaling pathway in the hepatoprotective potential of 1,25(OH)$_2$D$_3$ was also searched in EtOH-treated rats.

Methods: Male Wistar rats were fed on VDD-diet for 12 weeks. EtOH (5-20%) was applied in drinking water in increasing concentrations for the last 8 weeks. In addition, one group of rats were injected with 1,25(OH)2D3 (5µg/kg; twice a week; i.p.) during this period. Hepatic triglyceride and hydroxyproline levels, inflammation markers, lipid peroxides, protein carbonyls, mRNA expressions of Nrf2, superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px), SOD and GSH-Px activities, glutathione levels and histopathology were examined.

Results: EtOH application caused steatosis and fibrosis, elevated hepatic TG, lipid peroxide, protein carbonyls and hydroxyproline levels and inflammation markers. VDD did not aggravate EtOH-induced liver damage, steatosis and inflammation, but reactive oxygen species and lipid peroxide levels were slightly increased in VDD+EtOH group. Gene expressions of Nrf2-SOD-GSH-Px, enzyme activities and glutathione levels were also higher in VDD+EtOH group than EtOH group. Additionally, 1,25(OH)$_2$D$_3$ elevated mRNA expressions and activities of SOD and GSH-Px in EtOH-treated rats.

Conclusion: Our results indicate that VDD diet did not cause an additive effect on EtOH-induced hepatotoxicity. Moreover, it was detected that the activation of Nrf2-antioxidant signaling pathway may play a role in the protective effect of 1,25(OH)$_2$D$_3$ against EtOH-induced hepatotoxicity.

Keywords: Vitamin D deficiency, 1,25(OH)$_2$D$_3$, ethanol, liver damage, oxidative stress, Nrf2.

Öz

Amaç: D vitamini eksikliğinin (VDE) kronik karaciğer hastalıklarında karaciğer hasarını arttırdığı ileri sürülmektedir. Bununla birlikte, VDE ile alkole bağlı karaciğer hasarı arasındaki ilişkiye ait bilgiler sınırlıdır. Bu nedenle, bu çalışmada VDE'nin etanol (EtOH) ile indüklenen karaciğer hasarı üzerindeki etkisi araştırıldı. Ayrıca, EtOH uygulanan sıçanlarda 1,25(OH)$_2$D$_3$'ün karaciğeri koruyucu potansiyelinde Nrf2-antioksidan sinyal yolunun rolü de araştırıldı.

Yöntemler: Erkek Wistar sıçanlar 12 hafta süreyle VDE diyet ile beslendi. Son 8 hafta içme suyunda artan konsantrasyonlarda EtOH (% 5-20) verildi. Ayrıca, bir grup sıçana bu süreçte 11,25(OH)$_2$D$_3$ (5µg/kg; haftada iki kez; i.p.) uygulandı. Karaciğerde trigliserit (TG) ve hidroksiprolin düzeyleri, inflamasyon göstergeleri, lipid peroksitler, protein karboniller, Nrf2, süperoksit dismutaz (SOD) ve glutatyon peroksidaz (GSH-Px)'ın mRNA ekspresyonları, SOD ve GSH-Px aktiviteleri, glutatyon düzeyleri ve histopatolojik incelemeler yapıldı.

Bulgular: EtOH uygulaması steatoz ve fibroza, karaciğerde TG, lipit peroksit, protein karbonil ve hidroksiprolin düzeyleri ile inflamasyon göstergelerinde artışa neden oldu. VDE, EtOH'a bağlı karaciğer hasarını, steatoz ve fibrozu yoğunlaştırmadı. Ancak, reaktif oksijen türleri ve lipit peroksit düzeylerinde VDE+EtOH grubunda ılımlı artış bulundu. Nrf2-SOD-GSH-Px'in gen ekspresyonları, enzim aktiviteleri ve glutatyon düzeyleri de VDE+EtOH grubunda EtOH grubundan daha yüksekti. Ayrıca, 1,25(OH)$_2$D$_3$, EtOH uygulanan sıçanlarda karaciğerde SOD ve GSH-Px'ın mRNA ekspresyonlarını ve aktivitelerini arttırdı.

Sonuç: Bulgularımız VDE diyetin EtOH ile indüklenen karaciğer hasarı üzerinde ek bir etki oluşturmadığını gösterdi. Ayrıca EtOH'a bağlı karaciğer hasarında 11,25(OH)$_2$D$_3$'ün koruyucu etkisinde Nrf2-antioksidan sinyal yolunun aktivasyonunun rol oynayabileceği saptandı.

Anahtar Kelimeler: D vitamini eksikliği, 1,25(OH)$_2$D$_3$, etanol, karaciğer hasarı, oksidatif stres, Nrf2.

[1] **Department of Medical Biochemistry, Istanbul Faculty of Medicine, Istanbul University, Istanbul, Turkey.**

[2] **Department of Pathology, Acibadem University Medical Faculty, Acibadem Mehmet Ali Aydınlar University, Istanbul, Turkey.**

İB: 0000-0002-6432-3541
AFA: 0000-0002-3336-4332
CK: 0000-0002-1797-5889
IDE: 0000-0003-4062-9519
SDA: 0000-0003-3467-9763
MU: 0000-0002-8802-8766

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Sorumlu yazar / Corresponding author:
İlknur Bingül
Adres/Address: Department of Medical Biochemistry, Istanbul Faculty of Medicine, Istanbul University, Çapa-Istanbul, Turkey.
e-mail: ilknur.bingul@istanbul.edu.tr
Tel/Phone:+90533 358 33 04

Introduction

Vitamin D (Vit D), a hormone precursor, is present in two forms, vit D2 (ergocalciferol) and vit D3 (cholecalciferol). Dietary and synthesized Vit D undergo hydroxylations to 25-hydroxyvitamin D [25(OH)D] in the liver and then to 1,25-dihydroxyvitamin D [1,25(OH)2D], the active form of Vit D, in the kidney. Serum 25(OH)D levels are used as an indicator of Vit D stores [1].

Vitamin D deficiency (VDD) is an important public health problem in the world. VDD may induce insulin resistance (IR), oxidative stress, inflammation, and mitochondrial dysfunction [2] and it is accepted as a risk factor for chronic diseases such as bone diseases, immune disorders, cardiovascular diseases, diabetes mellitus [1,2], although there are some different opinions on this issue [1]. VDD is also common in chronic liver diseases such as viral hepatitis, cirrhosis, and fatty liver diseases and may induce fat accumulation in hepatocytes, as well as inflammation and fibrosis in the liver [3].

Fatty liver diseases are classified as alcoholic liver disease (ALD) and non-alcoholic fatty liver disease (NAFLD) [4]. Vit D_3 or $1,25(OH)_2D_3$ treatments were detected to be effective in the amelioration of hepatic lesions in dietary models of NAFLD in rodents [5-7]. Moreover, some studies have shown that VDD may be an aggravating factor in the formation and progression of hepatic lesions in NAFLD [8,9], but others did not confirm this effect of VDD [6,10].

On the other hand, the relationship between ALD and vit D has generally been investigated in patients with ALD and the results are inconsistent. [11,12]. In vitro [13,14] and experimental [15] studies investigating the effect of Vit D_3 or $1,25(OH)_2D_3$ on ethanol (EtOH)-induced hepatotoxicity are also quite limited. Moreover, there is only one experimental study on the relationship between VDD and ALD [16]. Since we have recently reported that $1,25(OH)_2D_3$ treatment alleviated hepatic lesions by decreasing ROS formation, lipid and protein oxidation products in the liver of EtOH-treated rats [7], the current study was primarily aimed to investigate whether the VDD diet could affect EtOH-induced hepatotoxicity and oxidative stress in the liver of rats.

Vit D_3 and $11,25(OH)_2D_3$ are not only direct-acting antioxidants, but they are also effective by increasing the gene expression of proteins/enzymes in the antioxidant system [17,18]. It has been suggested that the nuclear factor-erythroid-2-related factor 2 (Nrf2) transcription factor and downstream target genes such as superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and glutathione (GSH) biosynthetic enzymes may play a role in the antioxidant potential of Vit D_3 and $1,25(OH)_2D_3$ in some oxidative stress-induced pathologies [17, 19, 20]. Since there is no experimental study in this subject, we also investigated the effect of both $1,25(OH)_2D_3$ treatment and VDD on Nrf2-SOD-GSH-Px system in EtOH-treated rats. Our results indicate that the VDD diet did not cause an additive effect on EtOH-induced hepatotoxicity and the induction of Nrf2-antioxidant signaling pathway were effective in the protective role of $1,25(OH)_2D_3$ in EtOH-induced hepatotoxicity.

Material and methods

Chemicals

Chemicals were obtained from Sigma- Aldrich (Saint-Louise, MI, USA). $1,25(OH)_2D_3$ (Ostriol, 2µg/mL) was donated by VEM ILAC San. A.S. (Istanbul, Turkey).

Animals

Male Wistar rats (140-160 g) used in this study were obtained from Aziz Sancar Experimental Medical Research Institute of Istanbul University. The animals were provided with food and water *ad libitum.* They were housed in polypropylene cages (three to four per cage) at 22 ^{0}C, with 12- h light and 12-h darkness without shielding from ultraviolet B radiation (290-320 mn). All procedures were carried out in accordance with the protocols of the Animal Care and Use Committee of the University of Istanbul (Approval number: March 29, 2018-2018/28)

Diets and experimental design

Rats were divided into five groups as follows: Control (n=6), VDD (n=8), EtOH (n=7), VDD+EtOH (n=8) and EtOH+$1,25(OH)_2D_3$ (n=7) groups. Control, EtOH and EtOH +$1,25(OH)_2D_3$ groups were fed on PicoLab Rodent diet 5053 (2300 IU Vit D3/kg added), however, VDD and VDD+EtOH groups were fed on Modified LabDiet 5053 (Vit D3 not added) for 12 weeks as previously reported [21]. These diets were supplied from LabDiet (St. Louis-Missouri, USA).

EtOH (5-20%; v/v) was applied in drinking water in increasing concentrations for the last 8 weeks to EtOH, VDD+EtOH and EtOH+$1,25(OH)_2D_3$ groups. These groups were treated with 5% (v/v) and 10 % (v/v) EtOH for the first and second weeks, respectively. For the last 6 weeks, 20% (v/v) EtOH was administered. However, in EtOH+1,25 $(OH)_2D_3$ group, rats were injected with 1,25 $(OH)_2D_3$ (5µg/kg; twice a week) intraperitoneally [5, 7] for the last 8 weeks along with EtOH.

Blood and tissue samples

The animals were fasted overnight and anesthetized with ketamine (35 mg/kg, i.p., Pfizer, USA) and xylazine HCl (15 mg/kg, i.p., Bioveta, Czech Republic). Blood samples were taken into dry tubes by cardiac puncture and centrifuged at 1500xg for 10 min to separate the sera. Liver tissues were removed, washed with chilled 0.9% NaCl and kept in ice. The liver index was calculated as liver weight /body weightx100.

Liver tissues were homogenized in ice-cold phosphate-buffered saline (PBS; 0.01M, pH:7.4). Homogenates were centrifuged at 600x g for 10 min at 4°C to remove unbroken tissue samples and nuclear fraction. Post-nuclear supernatant (PNS) was used for biochemical analyses in the liver. A part of PNS was centrifuged at 10000xg for 20 min at 4^{o}C to obtain post-mitochondrial fraction (PMF). This fraction was used for the determination of activities of antioxidant enzymes. All materials were stored at -80^{o}C until analyzed.

Determinations in serum

Serum fasting glucose, triglyceride (TG), calcium and inorganic phosphorus levels, and alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were measured using a Cobas Integra 800 autoanalyzer (Roche Diagnostics, Mannheim, Germany). Serum $25(OH)D_3$ and insulin levels were determined by ELISA kits (Abbkine, Wuhan-China). The homeostasis model the assessment (HOMA) index was used to evaluate insulin resistance (IR) and calculated using the formula: fasting insulin concentration (pmol/L) x fasting glucose concentration (mmol/L)/135. High HOMA scores indicate IR (low insulin sensitivity).

Determinations in the liver

Triglyceride (TG) levels

Hepatic lipids were extracted with a mixture of chloroform: methanol (2:1) [22]. After the evaporation process, the extracts were dissolved in alcohol: ether (3:1). TG levels were measured by the kits provided from Biolabo Biochemistry and Coagulation (Maizy, France).

Tumor necrosis factor-alpha (TNF-α) and hydroxyproline (Hyp) levels

The liver tissue was homogenized in PBS (0.01M, pH: 7.4). The homogenates (10%; w/v) were sonicated for 30 seconds and then centrifuged at 5000xg for 5 minutes at +4°C. Supernatants were used to measure levels of TNF-α (Abbkine, Wuhan, China) and Hyp (Bioassay Technology Laboratory, Shanghai, China) by sandwich ELISA method.

Myeloperoxidase (MPO) activity

Liver tissue was homogenized in chilled phosphate buffer (50 mM; pH: 6.0) containing hexadecyltrimethylammonium bromide (HTAB). After freezed-thawed three times, and centrifuged at 15000xg. The supernatant was used for the measurement of MPO enzyme activity [23].

Reactive oxygen species (ROS), lipid and protein oxidation products

The formation of 2′,7′-dichlorofluorescein was determined in the liver homogenate incubated with 1 mM 2′,7′-dichlorodihydrofluorescein diacetate at 37 °C for 30 min in a fluorometric microplate reader (Fluoroskan Ascent FL., Thermo Scientific Inc, USA) with an excitation of 485 nm and emission of 538 nm [24].

Hepatic lipid peroxidation was evaluated by determining thiobarbituric acid reactive substances (TBARS) and diene conjugate (DC) levels. TBARS levels were determined according to Buege and Aust [25]. The mixture of liver homogenates and Buege-Aust reagent incubated in a boiling water bath for 15 min were cooled and then centrifuged at 1000xg for 10 min. The absorbances of supernatants were measured at 532 nm. To determine DC levels, liver lipids were extracted in chloroform/methanol (2:1) and then redissolved in cyclohexane, and absorbances at 233 nm were recorded [25].

Protein carbonyl (PC) levels were evaluated according to the method of Reznick and Packer [26] which is based on the measurement of protein hydrazones. Results were calculated from the maximum absorbance (360 nm) using a molar extinction coefficient of 22,000 M^{-1} cm^{-1}.

Antioxidant system parameters

Hepatic ferric reducing anti- oxidant power (FRAP) levels were determined spectrophotometrically to evaluate antioxidant power in the liver. The electron donating antioxidants found in liver tissue reduce a ferric-tripyridyltriazine complex to the ferrous form. The reaction is observed by measuring the change in absorption at 593 nm [27]. Hepatic glutathione (GSH) levels were determined by using 5,5-dithiobis-(2-nitrobenzoate) at 412 nm [28]. Superoxide dismutase (SOD) activity was determined according to Mylorie et. al. [29] and calculated using a standard curve prepared by bovine SOD. Glutathione peroxidase (GSH-Px) activity was measured using cumene hydroperoxide as a substrate [30]. The reaction was followed spectrophotometrically (340 nm) at 37°C, and activity was calculated using the extinction coefficient of NADPH ($6.22x10^3$ $M^{-1}cm^{-1}$).

mRNA expressions of Nrf2, SOD and GSH-Px

The homogenate of liver tissue was performed using a handheld homogenizer (SCILOGEX D160, USA). Total RNA was isolated (NucleoSpin RNA Isolation Kit, #740955, Macherey-Nagel, Germany) and then cDNA was synthesized (SCRIPT cDNA Synthesis Kit, Jena Bioscience, GmbH, Jena, Germany) using 5 ng RNA. Nrf2 (F: 5'-GTGGATCTGTCAGCTACTCCC-3'; R: 5'-CTGGGAATATCCAGGGCAAGC-3'), SOD (F: 5'-GGTCCAGCGGATGAAGAG-3'; R: 5'-GGACACATTGGCCACACC-3'), GSH-Px (F: 5'-CGACATCGAACCCGATATAGA-3'; R: 5'-ATGCCTTAGGGGTTGCTAGG-3') and the housekeeping gene GAPDH (F: 5'-CAGGGCTGCCTTCTCTTGTG-3'; R: 5'-AACTTGCCGTGGGTAGAGTC-3') primers were obtained from LGC Biosearch Technologies (Denmark). Quantitative real-time polymerase chain reaction (qPCR) was carried out using qPCR Green Master with UNG (Jena Bioscience, GmbH, Jena, Germany) in a real-time PCR system (Bio-Rad CFX Connect, California, USA). The $2^{-\Delta\Delta Ct}$ method were used for quantifying the expression levels of mRNA.

Protein levels

Protein levels of the liver tissue were measured spectrophotometrically using bicinchoninic acid [31].

Histopathologic examination

Livers were fixed in 10% buffered formalin solution for histopathological examinations. Tissues were embedded in paraffin and sectioned and stained with hematoxylin and eosin (H&E) and Masson's trichrome (MTC). Fibrosis scores were evaluated according to the protocol described in our previous study [32].

Statistical analysis

Statistical analysis was evaluated by using the Statistical Package for The Social Sciences program (21.0; SPSS Inc., Chicago, IL, USA) program. All variables were expressed as mean ± standard deviation (SD). Data distributions and test of normality were investigated by the Kolmogorov-Smirnov test. One-way ANOVA test (post-hoc Tukey's test) was used to assess the parameters with normal distribution. The homogeneity of variances was evaluated with the Levene test. Kruskal-Wallis test (post-hoc Mann Whitney-U test) was used to compare the parameters without normal distribution. In all cases, a difference was considered significant when $p < 0.05$.

Results

Final body and liver weights and liver index

Final body weight, liver weight and liver index remained unchanged in EtOH and VDD+EtOH groups as compared to the control group (data not shown).

Effect of VDD feeding on serum and hepatic parameters in rats

In the VDD group, there were significant decreases in serum $25(OH)D_3$ and significant increases in serum glucose, HOMA, TG and TC levels were detected as compared to controls. In addition, hepatic TG and TNF-α levels and MPO activity also increased. However, there were no changes in hepatic Hyp levels, prooxidant and antioxidant parameters and histopathological findings (Tables 1-3 and Figures 1-3).

Effect of VDD feeding on serum parameters in EtOH-treated rats

Serum 25(OH)D levels remained unchanged in the EtOH group, but it diminished significantly (47.5 %; $p<0.01$ and 45.2%; $p<0.001$) in the VDD+EtOH group in comparison with control and EtOH groups, respectively. Calcium and phosphorus levels did not alter in both groups.

Serum glucose, insulin, HOMA, and TC levels did not alter, but only TG levels increased in EtOH and VDD+EtOH groups in comparison with controls. While insulin, HOMA and TG levels did not change, glucose and TC levels were higher in the VDD +EtOH group than the EtOH group.

ALT and AST activities were found to be increased in EtOH and VDD+EtOH in comparison with control. However, there were no differences in ALT and AST activities between the two groups (Table 1).

Effect of VDD feeding on hepatic histology in EtOH-treated rats

In hematoxylin and eosin (H&E) and Masson's trichrome (MTC) staining of liver sections, the EtOH and VDD+EtOH groups exhibited microvesicular steatosis not exceeding 5%. In the EtOH group, 4/7 of the cases showed mild portal and periportal

Table 1. Effect of VDD on serum parameters in EtOH-treated rats (Mean±SD)

Variable	Control (n=6)	VDD (n=8)	EtOH (n=7)	VDD+EtOH (n=8)
25(OH)D (ng/mL)	28.4±3.09	15.7±1.07^{a2}	27.2±6.58	14.9±2.38^{a2,b1}
Calcium (mmol/L)	2.42±0.16	2.50±0.18	2.60±0.19	2.65±0.34
Phosphorus (mmol/L)	2.55±0.18	2.58±0.26	2.47±0.24	2.61±0.75
Glucose (mmol/L)	7.53±1.44	9.76±1.04^{a3}	6.50±1.91	9.51±1.32^{b2}
Insulin (pmol/L)	29.2±4.37	29.3±2.98	24.5±11.5	27.2±3.62
HOMA	1.61±0.33	2.10±0.21^{a2}	1.22±0.75	1.89±0.21
TC (mmol/L)	1.67±0.18	2.04±0.19^{a3}	1.62±0.28	1.98±0.25^{b3}
TG (mmol/L)	0.48±0.09	0.71±0.05^{a2}	0.89±0.26^{a3}	0.80±0.18^{a2}
ALT (U/L)	43.3±13.1	53.6±8.53	114.0±33.1^{a2}	99.1±27.2^{a2}
AST (U/L)	108.0±10.2	114.6±19.0	228.5±75.6^{a2}	194.5±49.6^{a2}

VDD: Vitamin D deficiency; EtOH: ethanol; 25(OH)D: 25-hydroxyvitamin D; HOMA: homeostasis model the assessment index; TC: total cholesterol; TG: triglyceride; ALT: alanine aminotransferase; AST: aspartate aminotransferase
a2 p<0.01; a3 p<0.05 as compared to controls
b1 p<0.001; b2 p<0.01; b3 p<0.05 as compared to EtOH group

fibrous expansion and 3/7 of the cases showed fibrous expansion of most portal areas with occasional portal to portal (P-P) bridging. However, In the VDD+EtOH group, only 2/7 of the cases showed mild portal fibrous expansion (Figure 1). Fibrosis scores were calculated as 1.85±1.06 and 0.25±0.46 in EtOH and VDD+EtOH groups, respectively. According to this, the fibrosis score decreased significantly (p<0.01) in the VDD+EtOH group in comparison with the EtOH group.

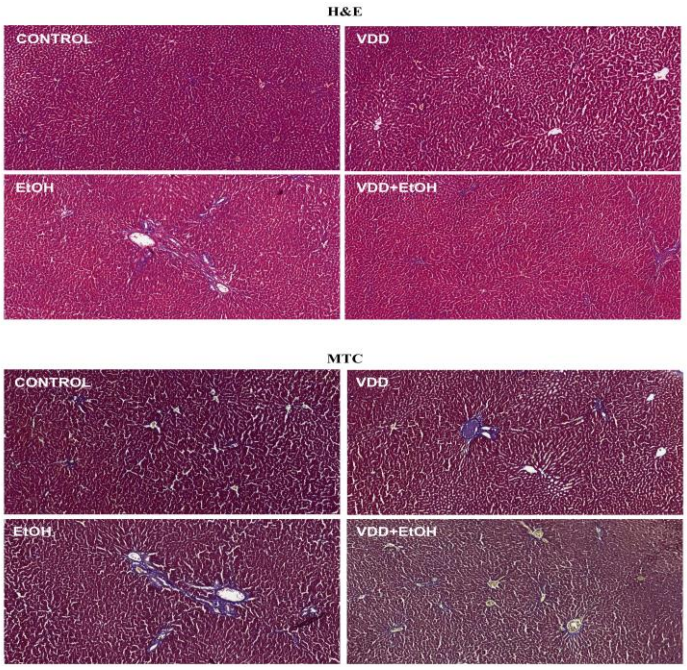

Figure 1. Effect of VDD on hepatic histology in EtOH-treated rats (Mean±SD). VDD: Vitamin D deficiency; EtOH: ethanol; H&E: hematoxylin and eosin; MTC: Masson's trichrome

Effect of VDD feeding on hepatic parameters in EtOH-treated rats

Hepatic TG, Hyp and TNF-α levels and MPO activity elevated significantly in EtOH and VDD+EtOH groups. However, there were no changes in these parameters between the two groups (Table 2).

Table 2. Effect of VDD on hepatic TG, Hyp and TNF-α levels and MPO activity in EtOH-treated rats (Mean±SD)

Variable	Control (n=6)	VDD (n=8)	EtOH (n=7)	VDD+EtOH (n=8)
TG (µmol/g tissue)	15.8±2.83	22.5±3.42^{a1}	22.8±1.77^{a2}	24.3±3.55^{a1}
Hyp (ng/mL protein)	2.02±0.27	2.27±0.28	2.78±0.25^{a1}	2.45±0.18^{a3}
TNF-α (ng/mg protein)	2.75±0.57	3.71±0.38^{a3}	6.33±0.89^{a1}	7.09±0.45^{a1}
MPO (µmol/min/mg protein)	5.17±0.96	7.21±0.72^{a1}	7.74±0.73^{a1}	8.56±0.81^{a1}

VDD: Vitamin D deficiency; EtOH: ethanol; Hyp: hydroxyproline; TNF-α: tumor necrosis factor-alpha; MPO: myeloperoxidase
a1 p<0.001; a2 p<0.01; a3 p<0.05 as compared to controls

Hepatic TBARS, DC, and PC levels elevated in both groups. However, ROS and TBARS levels, but not DC and PC levels were higher in the VDD+EtOH group than the EtOH group (Figure 2).

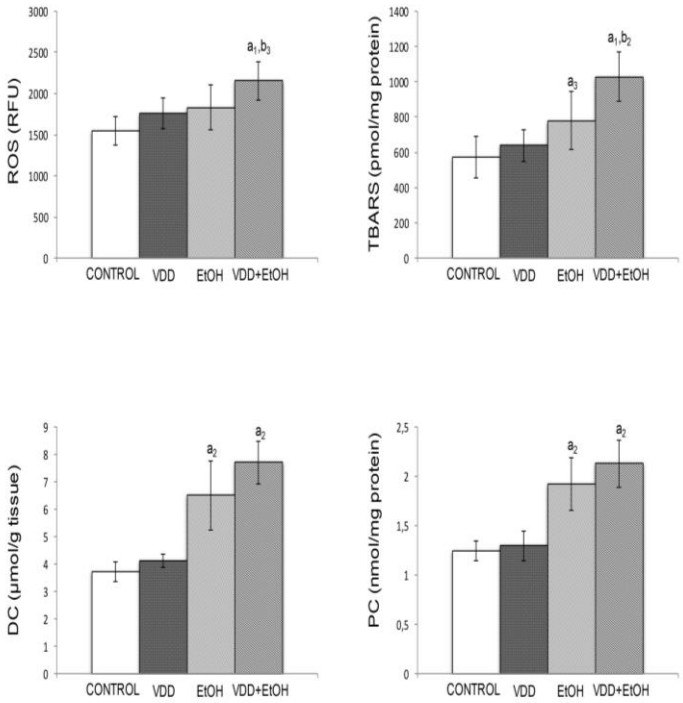

Figure 2. Effect of VDD on hepatic ROS, TBARS, DC and PC levels in EtOH-treated rats (Mean±SD).
VDD: Vitamin D deficiency; EtOH: ethanol; ROS: reactive oxygen species; TBARS: thiobarbituric acid reactive substances; DC: diene conjugate; PC: protein carbonyls
a1 p<0.001; a2 p<0.01; a3 p<0.05 as compared to controls
b2 p<0.01; b3 p<0.05 as compared to EtOH group

Hepatic Nrf2, SOD, and GSH-Px mRNA expressions and SOD and GSH-Px activities diminished significantly, but FRAP and GSH levels remained unchanged in EtOH group as compared to controls. These values were significantly higher in the VDD+ETOH group than the EtOH group (Figure 3 and Table 3).

Effect of $1,25(OH)_2D_3$ on hepatic Nrf2, SOD and GSH-Px mRNA expressions in EtOH-treated rats

$1,25(OH)_2D_3$ treatment did not alter EtOH-induced Nrf2 mRNA downregulation. However, this treatment restored EtOH-induced SOD and GSH-Px mRNA downregulations. In addition, SOD and GSH-Px activities were significantly higher in the EtOH+$1,25(OH)_2D_3$ group than EtOH group. However, there were no changes in GSH and FRAP levels in $1,25(OH)_2D_3$-treated ETOH rats (Figure 4 A/B).

Table 3. Effect of VDD on hepatic SOD and GSH-Px activities as well as FRAP and GSH levels in EtOH-treated rats. (Mean±SD)

Variable	Control (n=6)	VDD (n=8)	EtOH (n=7)	VDD+EtOH (n=8)
SOD (U/mg protein)	20.1±2.89	18.7±1.43	16.3±2.36^{a3}	22.4±2.56^{b1}
GSH-Px (nmol/min/ mg protein)	520.9±64.1	433.0±71.9	389.1±78.5^{a2}	497.9±35.9^{b3}
FRAP (nmol/mg protein)	80.3±11.9	76.9±15.8	68±13.3	87.4±6.75^{b3}
GSH (nmol/mg protein)	23.9±3.02	24.9±6.30	21.4±5.17	31.2±5.30^{b2}

VDD: Vitamin D deficiency; EtOH: ethanol; SOD: superoxide dismutase; GSH-Px: glutathione peroxidase; FRAP: ferric reducing antioxidant power; GSH: glutathione
a2 p<0.01; a3 p<0.05 as compared to controls
b1 p<0.001; b2 p<0.01; b3 p<0.05 as compared to EtOH group

Discussion

Excessive alcohol drinking is a leading cause of chronic liver disease. The liver is the main organ where EtOH metabolism takes place. EtOH is metabolized to acetaldehyde (AA) by alcohol dehydrogenase and cytochrome P450 (CYP2E1), which is further metabolized by aldehyde dehydrogenase to acetate. Impaired lipid homeostasis, ROS-induced oxidative stress, increases in AA-protein adducts formation and inflammatory cytokine levels, endotoxemia, and mitochondrial damage were suggested to play an important role in the pathomechanism of EtOH-induced hepatotoxicity [33]. In our study, EtOH (5-20%, v/v) was applied in drinking water in increasing concentrations for 8 weeks as previously reported. Our results have shown that chronic EtOH application led to lipid accumulation, increases in inflammation (TNFα, MPO) and fibrosis (Hyp) markers in the liver of rats. Additionally, increases in lipid (TBARS, DC) and protein oxidation (PC) products and significant decreases Nrf2, SOD and GSH-Px mRNA expressions as well as SOD and GSH-Px activities were detected in the liver of EtOH-treated rats. Our findings are accordance with previous studies [7,34-37].

There is only one study about the effect of VDD on EtOH-induced hepatotoxicity. This study has reported that VDD aggravated the hepatic injury by increasing ROS-induced oxidative stress, pro-inflammatory cytokines and chemokines and decreasing GSH levels in the liver of mice fed with 4% (w/v) ethanol containing liquid diet for 6 weeks [16]. They have also detected that VDD attenuated EtOH-induced upregulations of hepatic SOD and GSH-Px. Therefore, we wanted to provide additional information to the topic by investigating the effect of VDD on EtOH-induced liver damage. Since 25(OH)D levels were reported to fall below 20 ng/ml in mice fed on VDD diet as early as 1 month [21], in this study, EtOH application started after 1 month of VDD diet administration and continued 8 weeks. When the findings obtained in EtOH and VDD+EtOH groups were compared, it was found that VDD did not have an augmenting effect on EtOH-induced increases in serum transaminase activities, hepatic TG, TNF-α and Hyp levels and MPO activity. Some factors such as use of the Lieber-De Carli ethanol liquid diet, the simultaneous application of the VDD diet and EtOH, and the use of mice as experimental animals may be responsible for the different findings between the two studies. In line with our findings in ALD, some authors have also detected that the VDD diet did not aggravate IR, hepatic lipid accumulation, and inflammation, even attenuated them, in rodents fed high fat- [10] and high fructose [6]-diets, which are dietary models of NAFLD.

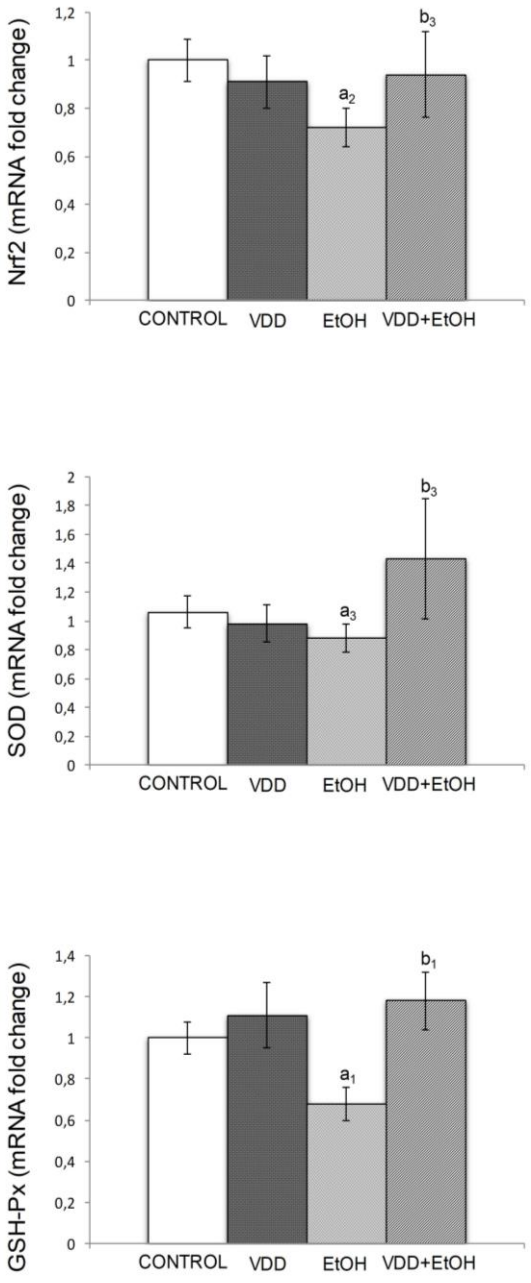

Figure 3. The effect of VDD on hepatic Nrf2, SOD and GSH-Px mRNA expressions in EtOH-treated rats (Mean±SD)
VDD: Vitamin D deficiency; EtOH: ethanol; Nrf2: nuclear factor-erythroid-2-related factor 2; SOD: superoxide dismutase; GSH-Px: glutathione peroxidase
a1 p<0.001; a2 p<0.01; a3 p<0.05 as compared to controls
b1 p<0.001; b3 p<0.05 as compared to EtOH group

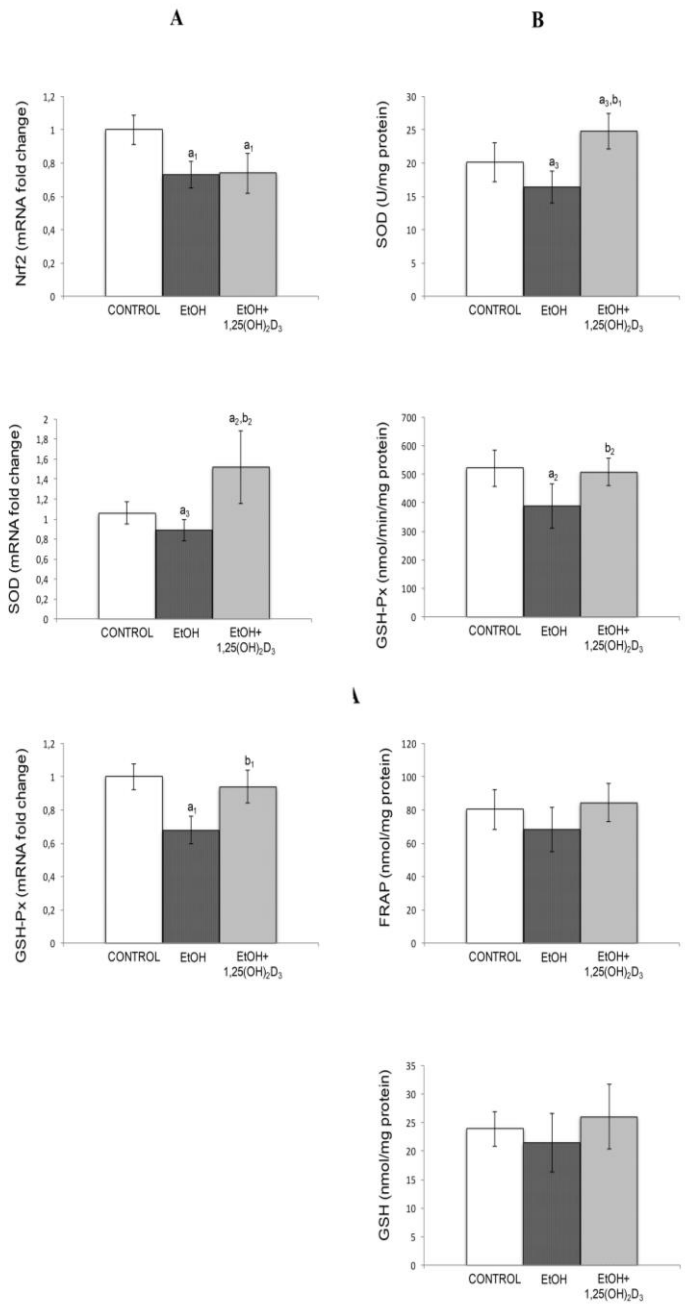

Figure 4. Effect of $1,25(OH)_2D_3$ on hepatic Nrf2, SOD and GSH-Px mRNA expressions (A) as well as SOD and GSH-PX activities (B) in EtOH-treated rats (Mean±SD).
$1,25(OH)_2D_3$: 1,25-dihydroxyvitamin D3; Nrf2: nuclear factor-erythroid-2-related
factor 2; SOD: superoxide dismutase; GSH-Px: glutathione peroxidase
a1 $p<0.001$; a2 $p<0.01$; a3 $p<0.05$ as compared to controls
b1 $p<0.001$; b2 $p<0.01$ as compared to EtOH group

Nrf2 is a transcription factor located in the cytoplasm under normal conditions. Kelch- like ECH-associated protein 1 (Keap 1) is a sensor of oxidative stress and couples Nrf2. Mild oxidative stress could induce the uncoupling of Nrf2 and Keap 1 and then Nrf2 transfers into nucleus to play its role in the antioxidant protection. Thus, it provides the transcription of several antioxidant and cytoprotective genes through the antioxidant response element (ARE) by entering into the nucleus [38]. In this study, hepatic ROS and TBARS, but not DC and PC levels were significantly higher in VDD+EtOH group than EtOH group. However, a tendency for increased DC and PC levels was also detected. In these conditions, hepatic Nrf2, SOD and GSH-Px mRNA expressions, SOD and GSH-Px activities, and GSH and FRAP levels were also found to be higher in the VDD+EtOH group than EtOH group. Our results indicate that activation of Nrf2-antioxidant signaling may protect against further increases in EtOH-induced hepatotoxicity and inflammation and oxidative stress in rats fed on VDD.

It has been accepted that VDD may have a promoting role in chronic diseases such as metabolic syndrome, diabetes mellitus, cardiovascular and liver diseases [1,2]. However, there are contradictory findings in rodents fed on the VDD diet alone. Some studies have shown that VDD-diet did not affect IR, serum transaminase activities, hepatic lipid accumulation, histology, inflammation, and oxidative stress parameters in rodents [10,16]. However, others have detected that VDD-diet caused IR, fatty liver and mild fibrotic changes, inflammation and oxidative stress in the liver [6,9,17]. In our study, although significant increases in HOMA values, hepatic TG and inflammation parameters were detected, there were no changes in serum transaminase levels, hepatic histopathological findings, and pro-oxidant and antioxidant parameters in rats fed on VDD alone.

Vit D3 and 1,25(OH)2D3 are known to have antioxidant potential. This potential is related to their structural similarity to cholesterol and ergosterol [18]. However, Vit D and 1,25(OH)2D3 are also effective in the regulation of gene expression of proteins/enzymes in the antioxidant system by increasing Nrf2 transcription factor [17,18]. In vitro studies showed that 1,25(OH)2D3 increased the translocation of Nrf2 to the nucleus and induced the expression of several antioxidant enzymes in human endothelial [39] and epithelial [40] cells incubated by oxidative stressors. Similarly, 1,25(OH)2D3 treatment was also observed to increase Nrf2-antioxidant signaling in the liver of rats fed on a high fat diet [5], in the renal tissue of diabetic rats [19], and in the brain tissue of rats with experimental Alzheimer's disease [20]. However, there is a limited knowledge on the changes in Nrf2-antioxidant signaling pathway in EtOH-induced toxicity. Some in vitro studies have shown that 1,25 (OH)2D3 resulted in a protective effect on EtOH-induced cytotoxicity and oxidative stress by upregulating Nrf2 [13] or facilitating the translocation of Nrf2 into the nucleus [14], and thus improving endogenous antioxidant defense system. However, there is no experimental study on this issue. In this study, for the first time, 1,25(OH)2D3 treatment was found not to alter mRNA expressions of Nrf2, but it elevated SOD and GSH-Px mRNA expressions, as well as SOD and GSH-Px activities in the liver of EtOH-treated rats. Our results indicate that, besides direct antioxidant potential, 1,25(OH)2D3 may play a protective role against EtOH-induced oxidative stress probably by augmenting Nrf2 translocation into the nucleus. According to this, the activation of the Nrf2-antioxidant signaling pathway may also play an active role in the protective effect of 1,25(OH)2D3 against EtOH-induced hepatotoxicity and oxidative stress, which was detected in our previous study [7].

In conclusion, our results indicate that the VDD diet did not augment EtOH-induced hepatotoxicity. Moreover, for the first time 1,25(OH)2D3-induced Nrf2-antioxidant signaling pathway was detected to be effective in the protection against EtOH-induced hepatotoxicity in rats.

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