Vitamin D deficiency exacerbates alcohol-related liver injury via gut barrier disruption and hepatic overload of endotoxin

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Highlights

- Vitamin D deficiency is observed in ALD.
- Vitamin D deficiency exacerbates gut barrier disruption and hepatic overload of LPS.
- Hence, vitamin D deficiency plays a role in the pathogenesis of ALD.

Abstract

Endogenous lipopolysaccharide (LPS) that translocates via the disrupted intestinal barrier plays an essential role in the progression of alcohol-related liver disease (ALD). Vitamin D deficiency is observed in ALD, and it participates in regulating gut barrier function. The current study aimed to examine the association between vitamin D deficiency and endotoxemia in patients with ALD-related cirrhosis. Moreover, the effect of vitamin D deficiency on ethanol (EtOH)- and carbon tetrachloride (CCl_4)-induced liver injury relevant to gut barrier disruption in mice was investigated.

Patients with ALD-related cirrhosis (Child–Pugh Class A/B/C; n = 56/15/7) had lower 25(OH)D levels and higher endotoxin activities than non-drinking healthy controls (n = 19). The serum 25(OH)D levels were found to be negatively correlated with endotoxin activity ($R = -0.481$, $p < 0.0001$). The EtOH/CCl_4-treated mice developed hepatic inflammation and fibrosis, which were significantly enhanced by vitamin D-deficient diet. Vitamin D deficiency enhanced gut hyperpermeability by inhibiting the intestinal expressions of tight junction proteins including ZO-1, occludin, and claudin-2/5/12/15 in the EtOH/CCl_4-treated mice. Consequently, it promoted the accumulation of lipid peroxidases, increased the expression of NADPH oxidases, and induced Kupffer cell infiltration and LPS/toll-like receptor 4 signaling-mediated proinflammatory response. Based on the in vitro assay, vitamin D-mediated vitamin D receptor activation inhibited EtOH-stimulated paracellular permeability and the downregulation of tight junction proteins via the upregulation of caudal-type homeobox 1 in Caco-2 cells.

Hence, vitamin D deficiency exacerbates the pathogenesis of ALD via gut barrier disruption and hepatic overload of LPS.

1. Introduction

Alcohol-related liver disease (ALD) is caused by long-term alcohol consumption and is characterized by a spectrum of disorders such as acute or chronic hepatitis, fatty liver disease, progressive fibrosis, and hepatocellular carcinoma [1]. ALD is the most common non-viral cause of high liver-related morbidity and mortality rates [2]. Several drugs including nalmefene are currently used to reduce the adverse effects of alcohol consumption among patients with ALD. However, only few people can successfully abstain from excessive alcohol consumption [3].

In terms of ALD pathogenesis, intra-organ cross-talks are responsible for the development of hepatic steatosis, inflammation, and fibrosis [4]. In particular, several studies have shown the functional involvement of the gut–liver axis in the progression of ALD [5-7]. Excessive alcohol consumption causes intestinal hyperpermeability caused by the disrupted tight junction, leading to the translocation of gut-derived endotoxin lipopolysaccharide (LPS) [8]. LPS activates macrophages recruited in the liver via the toll-like receptor 4 (TLR4) signaling pathway and help these cells to produce proinflammatory cytokines [9]. Hence, the inhibition of LPS translocation into the liver by maintaining intestinal barrier integrity may prevent alcohol-induced liver injury, and the regulators of intestinal barrier function should be determined to identify appropriate therapeutic strategies against ALD.

Among various substances, vitamin D is required in calcium homeostasis and bone metabolism. Vitamin D deficiency, defined as a serum 25-hydroxy vitamin D (25(OH)D) level of <50 nmol/L, is still considered a global public health issue [10]. This condition is closely associated with alcohol-related diseases including ALD [11]. Decrease in 25(OH)D levels were correlated with a high mortality rate

in patients with ALD [12]. A previous report has shown that vitamin D deficiency can exacerbate oxidative stress and parenchymal inflammation in patients with hepatitis C [13]. Vitamin D supplementation may improve hepatic reserve in patients with ALD-related cirrhosis [14]. Notably, recent studies have revealed that vitamin D plays a key role in regulating gut immune homeostasis [15,16]. 1,25-dihydroxycholecalciferol (1,25 $(OH)_2D_3$), the active form of vitamin D, can possibly regulate various genes correlated with gut barrier integrity and inflammatory response via the activation of vitamin D receptor (VDR), which is highly expressed in the small intestine and colon [17]. Lee et al. have found that 1,25 $(OH)_2D_3$ supplementation improves bacterial translocation and decreases intestinal permeability in a cirrhotic rat model [18]. However, data on the effects of vitamin D deficiency on the development of alcohol-related liver fibrosis correlated with intestinal barrier disruption are limited.

The current study aimed to investigate the impact of vitamin D deficiency on ALD-related liver injury relevant to intestinal permeability and LPS translocation into the liver. Initially, a clinical survey was performed to evaluate the association between vitamin D deficiency and endotoxemia in patients with ALD-related cirrhosis. Next, a basic study was conducted to assess the effect of vitamin D deficiency on murine alcohol-related liver injury induced by ethanol (EtOH) and carbon tetrachloride (CCl_4).

2. Materials and methods

2.1 Patients

This retrospective observational study enrolled a single-center cohort between January 2015 and December 2021 at Nara Medical University Hospital. In total, 86 consecutive patients with ALD-related liver cirrhosis were enrolled. The ALD criteria were alcohol consumption of >60 g/day for male or

>40 g/day for female for ≥ 5 years and negative results for hepatitis virus markers, antinuclear antibodies, and antimitochondrial antibodies. Liver cirrhosis was diagnosed depending on clinical data including laboratory tests, endoscopic findings and medical imaging features without liver biopsy based on the 2020 evidence-based clinical practice guidelines of the Japan Society of Gastroenterology and the Japan Society of Hepatology [19]. Moreover, 19 non-drinkers without chronic liver disease were recruited as controls. The exclusion criteria were severe cardiac, respiratory or renal dysfunction, invasive cancer within the last 5 years of sample collection, clinical or biochemical signs of infection 28 days prior to sample collection, concomitant inflammatory bowel diseases, irritable bowel syndrome or osteoporosis, and previous history of parathyroidectomy, gastrectomy, enterectomy, or liver or kidney transplantation. Patients taking nonabsorbable disaccharides, probiotics, antibiotics, or vitamin D supplements 28 days prior to sample collection were not included. 8 patients were excluded for meeting exclusion criteria, and 78 patients with ALD-related liver cirrhosis were include. The program comprised general laboratory tests and measurement of serum 25(OH)D levels and whole blood endotoxin activity.

All procedures conducted in this study involving human participants were based on the ethical standards of the Medical Ethics Committee of Nara Medical University, Kashihara, Japan (authorization numbers: 2868) and the 1964 Declaration of Helsinki and its later amendments. All patients (n = 97) provided a written informed consent prior to study enrollment.

2.2 Assessment of endotoxin activity

Whole blood endotoxin activity was assessed using the Endotoxin Activity Assay kit (Spectral Diagnostics, Toronto, Canada), as described in a previous study [20]. Briefly, 40 μL of whole blood sample was incubated in duplicate with

saturating concentrations of murine IgM monoclonal antibody raised against the lipid A of *Escherichia coli* J5 and was then stimulated with zymosan. The respiratory burst activity was determined as light release from the lumiphor luminol using of a chemiluminometer. The LPS/anti-LPS complex primes the neutrophils of patients to enhance response to stimulation with zymosan. Endotoxin activity was exhibited in relative units derived from the integral of the basal and maximally stimulated (4600 pg/mL LPS) chemiluminescent response (on a scale of 0–1). Values were presented as endotoxin activity units and the mean of triplicate measurements. Endotoxin activity values of 0.4 and 0.6 were approximately equivalent to endotoxin concentrations of 25–50 and 100–200 pg/mL of *E. coli* 055:B5 LPS, respectively.

2.3 Animals and experimental design

Ten-week-old female C57BL/6J mice (CLEA Japan, Osaka, Japan) were caged with free access to food and water under 23°C ± 3°C with 50% ± 20% humidity and a 12-h light/dark cycle.

To establish an animal model of ALD-related liver fibrosis, we employed EtOH and CCl_4- according to our previous reports [21,22]. The mice were randomly divided into four experimental groups. The vehicle + VtDS group (n = 10) was fed with vitamin D-sufficient diet (VtDS) without EtOH (Research Diets, New Brunswick, NJ, the USA). The vehicle + VtDD group (n = 10) was fed with vitamin D-deficient diet (VtDD) without EtOH (Supplementary Table 1). The vehicle groups received intraperitoneal injection with corn-oil twice weekly. The EtOH/CCl_4 + VtDS group (n = 10) was fed with VtDS with a 2.5% EtOH-containing Lieber–DeCarli liquid diet (Research Diets). The EtOH/CCl4 + VtDD group (n = 10) was fed with VtDD EtOH diet. The EtOH/CCl_4 groups received intraperitoneal injection of CCl_4 (FUJIFILM, Wako Pure Chemical Corporation,

Osaka, Japan) twice a week (1 mL/kg body weight). After 8 weeks of the experimental intervention, the mice were euthanized. For sample collection, all mice were anesthetized by intravenous injection with 150 mg/kg of pentobarbital sodium. Blood was collected from the cervical artery, and liver and ileum were harvested immediately after sacrifice. Serum levels of biochemical markers were assessed at SRL, Inc. (Tokyo, Japan). Animal care and experimental procedures were performed according to the guideline from ethics committee of Experimental Animal Care of Nara Medical University, Kashihara, Japan (No.12810).

2.4 Histological and immunohistochemical analyses

Liver and ileum specimens were fixed in 10% formalin, incubated overnight at room temperature, and embedded in paraffin. Then, 5-μm-thick liver sections were stained with hematoxylin and eosin (H&E) and Sirius Red (Narabyouri Research Co., Nara, Japan). Hepatic pathological scores were independently evaluated by two pathologists in 10 random fields from each slide at a magnification of 400-fold according to the criteria based on previous report [23]. Cell apoptosis of hepatocytes was determined by TUNEL assay using the In situ Apoptosis Detection Kit (Takara Bio Inc, Kusatsu, Japan) on the liver sections according to the manufacturer's instruction. Immunohistochemical staining of α-smooth muscle actin (α-SMA), F4/80, and COL-1 was performed, as previously described [22]. For immunofluorescence, zonula occludens-1 (ZO-1), occludin, and claudin-2 were applied as primary antibodies on the ileum sections, as previously described [22]. Supplementary Table 2 shows the list of primary antibodies. The primary antibody was detected using Alexa Fluor-labeled secondary antibody (Invitrogen); subsequently, nuclear staining was performed using 4',6-diamidino-2-phenylindole Fluoromount-G mounting medium (Vector

Laboratories, Inc.). Images were captured using BX53 (Olympus, Tokyo, Japan) for histology and immunohistochemistry and BZ-X700 (Keyence, Osaka, Japan) for immunofluorescence. Semi-quantitative analysis was performed for 10 fields per each section at a magnification of 400-fold using ImageJ 64-bit Java 1.8.0 (National Institutes of Health, Bethesda, MD, the USA).

2.5 Mouse serum 25(OH)D concentration

Mouse serum 25(OH)D concentrations were measured using the Mouse Rat 25-OH Vitamin D ELISA (Eagle Bioscience, Amherst, NH, the USA), according to the manufacturer's instruction.

2.6 Hepatic triglyceride concentration

Hepatic triglyceride concentration was measured in 100 mg of frozen liver tissue per mouse using the Triglyceride-Glo™ Assay (Promega, Madison, WI, USA), respectively, according to the manufacturer's instructions.

2.7 Hepatic hydroxyproline, antioxidant enzyme, and malondialdehyde concentration

Mouse hepatic concentrations of hydroxyproline, catalase (CAT), superoxide dismutase (SOD), and malondialdehyde (MDA) were assessed in frozen liver tissue using appropriate assay kits according to each manufacturer's instruction. These include Hydroxyproline Assay Kit (Cell Biolabs, Inc., San Diego, CA, the USA), Mouse Catalase ELISA Kit (CUSABIO, Houston, TX, the USA), Mouse Super Oxidase Dismutase ELISA Kit (CUSABIO), and OxiSelectTM TBARS Assay Kit (Cell Biolabs, Inc.).

2.8 Hepatic activity of aldehyde dehydrogenase 2 and cytochrome P450

2E1

The mouse hepatic activity of aldehyde dehydrogenase 2 (ALDH2) was evaluated using the ALDH2 activity assay kit (Abcam), according to the manufacturer's instruction. Mouse intrahepatic cytochrome P450 2E1 (CYP2E1) activity was assessed by assessing p-nitrophenol hydroxylation in liver homogenates, as described in a previous study [24].

2.9 FITC-dextran intestinal permeability assay

Intestinal permeability was assessed using the fluorescein isothiocyanate (FITC)-dextran assay with simple modifications, as described in a previous study [25]. Other experimental groups (n = 5 for each group) were used for this assay. The mice received gavage at a dose of 600 mg/kg body weight of FITC-dextran (4 kDa) (TdB Labs, Uppsala, Sweden) after fasting for 6 h. Then, 4 h after the administration, blood was collected from the portal vein. Subsequently, the plasma level of FITC-labeled dextran was determined via fluorescence measurement at an excitation wavelength of 490 nm and an emission wavelength of 520 nm.

2.10 Culture of human intestinal epithelial cells

The effect of vitamin D on enterocytes was analyzed using the human intestinal epithelial cell line Caco-2 (Riken BRC, Ibaraki, Japan). The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 1% penicillin-streptomycin, 1% nonessential amino acids, 25 mmol/L glucose, and 10% fetal bovine serum at 37°C under 5% CO_2, as described in a previous study [26]. After 10–20 passages, cells were incubated with different concentration of EtOH (0–60 mM), and they were treated for other assays with EtOH (60 mM) and/or different doses (10^{-9}–10^{-7} M) of calcitriol (FUJIFILM,

Wako Pure Chemical Corporation), an active form of vitamin D_3 and ZK159222 (0.1 μM, MedChemExpress, Monmouse Junction, NJ, the USA), a VDR antagonist.

2.11 Transepithelial electrical resistance

The paracellular permeability of cultured Caco-2 was determined by measuring transepithelial electrical resistance (TEER) using Millicell-ERS ohmmeter with electrodes (Millipore Corporation, Bedford, MA, the USA), as described in a previous study [27]. The electrical resistance of the monolayer was calculated by multiplying the resistance of the monolayer with the effective surface area (Ω cm^2).

2.12 RNA isolation, cDNA synthesis, and real-time PCR

Total cellular RNA was isolated from liver tissues and cultured Caco-2 cells with the RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instruction. cDNA was prepared with 2 μg of total RNA with the High-Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA, the USA), according to the manufacturer's instructions. Real-time PCR was performed with the StepOnePlus Real-time PCR system and SYBR Green (Applied Biosystems). Supplementary Table 3 presents the primer sequences. Gene expression levels were normalized to the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. mRNA levels were calculated by means of the $2^{-\Delta\Delta CT}$ method and were expressed as fold changes relative to the controls of each experiment.

2.13 Protein extraction and Western blotting

Frozen mouse liver tissues and harvested Caco-2 protein extracts were

prepared in T-PER Tissue Protein Extraction Reagent containing proteinase and phosphatase inhibitors (Thermo Scientific, Rockford, IL, the USA). The protein concentration was determined using a protein assay (Biorad, Hercules, CA, the USA). Subsequently, proteins (50 μg per lane) were resolved on a sodium dodecyl sulfate-polyacrylamide gel (NuPAGE; Thermo Scientific). For immunoblotting, proteins were transferred to a polyvinylidene difluoride membrane. Blots were incubated for 1 h at room temperature in blocking buffer containing 5% bovine serum albumin in Tris-buffered saline supplemented with Tween-20, incubated overnight at 4°C with primary antibodies (as shown in Supplementary Table 2), and incubated with Amersham ECL horseradish peroxidase-conjugated immunoglobulin G F(ab)2 fragment antibody (1:5000; GE Healthcare Life Sciences, Piscataway, NJ, the USA) for 1 h at room temperature. Signals were detected using Clarity Western-enhanced chemiluminescence substrate (BioRad) and were quantified using ImageJ 64-bit Java 1.8.0 (NIH).

2.14 Statistical analyses

For basic study, the two-tailed unpaired Student's *t*-test (post hoc test) was used to compare between-group differences. For the clinical study, quantitative parameters were compared using a one-way analysis of variance with a post-hoc Tukey's tests, Kruskal-Wallis test and categorical data were analyzed using the Fisher exact test, as appropriate. Spearman's rank correlation coefficient was applied to evaluate the association between serum 25(OH)D levels and endotoxin activity. Statistical analyses were performed using Prism version 9.1.2 (GraphPad Software, La Jolla, CA, the USA). A p value of < 0.05 was statistically significant. Data were presented as means ± standard deviation or medians (interquartile range). For the in vitro study, each experiment was

repeated three times with four replicates.

3. Results

3.1 Serum 25(OH)D level is negatively correlated with endotoxin activity in patients with ALD-related cirrhosis

Initially, we evaluated the serum 25(OH)D levels and endotoxin activity of patients with ALD-related cirrhosis (Figure 1A). Table 1 shows the demographic and clinical characteristics of the patients. As shown in Figure 1B, the serum 25(OH)D level of patients with ALD-related cirrhosis was significantly lower, as evidenced by decreased hepatic reserve, than that of non-drinking controls. However, the endotoxin activity of patients with ALD-related cirrhosis increased with decreased hepatic function (Figure 1C). Based on these results, serum 25(OH)D levels were negatively correlated with endotoxin activity in total patients ($R = -0.481$, $p < 0.0001$) (Figure 1D). Moreover, we confirmed that the negative correlation between them were observed in the patients with Child-Pugh class A ($R = -0.473$, $p < 0.001$) (Figure 1E), class B ($R = -0.847$, $p = 0.0162$) (Figure 1F), and class C ($R = -0.581$, $p = 0.0292$) (Figure 1G).

Thus, vitamin D deficiency was associated with endotoxemia in ALD-related cirrhosis regardless of hepatic function.

3.2 Vitamin D deficiency exacerbates hepatic injury and hepatocyte apoptosis in the ALD mice

Figure 2A shows the effects of vitamin D deficiency on ALD-related liver injury caused by EtOH and CCl_4 treatment in mice based on an *in vivo* examination. The EtOH/CCl_4 + VtDS group presented with a significant delay in body weight gain compared with the vehicle + VtDS group. This phenomenon was more evident in the EtOH/CCl_4 + VtDD group (Figure 2B). The energy intake of the

EtOH/CCl_4-treated mice was significantly lower than that of the vehicle-treated mice. Meanwhile, feeding of VtDD did not affect the energy intake of both the vehicle- and EtOH/CCl_4-treated mice (data not shown). As shown in Figure 2C, serum 25(OH)D levels were decreased in VtDD-fed groups as compared to VtDS-fed groups. Interestingly, the EtOH/CCl_4 + VtDS group had a lower serum 25(OH)D level than the Vehicle + VtDS group. Hence, EtOH and CCl_4 treatment could induce vitamin D deficiency. The VtDD-fed mice with low serum 25(OH)D levels presented with low calcium levels. However, their serum phosphorus levels did not change (Figure 2D).

Next, the effects of vitamin D deficiency on alcohol-related liver injury were examined. The serum aspartate aminotransferase and alanine aminotransferase levels of the EtOH/CCl_4 + VtDS group were significantly higher than those of the EtOH/CCl_4 + VtDD group (Figure 2E). The EtOH/CCl_4-treated mice had high serum triglyceride levels. However, VtDD feeding did not affect the serum triglyceride levels (Figure 2F). As shown in Figure 2G, the relative liver weights of the EtOH/CCl_4-treated mice increased, and the relative liver weight of the VtDD-fed mice did not change. In accordance with serum triglyceride level, the hepatic TG level was unchanged in the EtOH/CCl_4 + VtDD group as compared to the EtOH/CCl_4 + VtDS group (Figure 2H). H&E staining revealed that the EtOH/CCl_4 + VtDS group had extensive inflammation and hemorrhagic liver necrosis. However, these pathological changes were advanced in the EtOH/CCl_4 + VtDD group (Figures 2I and 2J). The EtOH/CCl_4 + VtDS group had TUNEL-positive hepatocyte apoptosis in the liver. Nevertheless, this condition was more evident in the EtOH/CCl_4 + VtDD group (Figures 2I and 2K).

3.3 Vitamin D deficiency exacerbates liver fibrosis development in the

ALD mice

The development of liver fibrosis in the experimental groups was assessed. Results showed that the EtOH/CCl_4 + VtDS group had an expansion of fibrous septa based on Sirius Red staining and a parallel increase in the number of α-SMA-positive activated hepatic stellate cells and COL-1-immunopositive extracellular matrix deposition (Figures 3A–3D). Notably, these histological features of hepatic fibrogenesis progressed in the EtOH/CCl_4 + VtDD group (Figures 3A–3D). Correspondingly, the hepatic expressions of COL-1 and α-SMA protein and the hepatic concentration of hydroxyproline increased in the EtOH/CCl_4 + VtDD group compared with the EtOH/CCl_4 + VtDS group (Figures 3E and 3F). The EtOH/CCl_4 + VtDS group had increased hepatic mRNA levels of fibrosis-related genes (i.e., *Acta2*, *Col1a1*, and *Tgfb1*). Further, the EtOH/CCl_4 + VtDD group had a more significant expression of these profibrotic markers (Figure 3G). By contrast, the vehicle + VtDD group did not present with fibrogenic changes (Figures 3A–3G).

3.4 Effects of vitamin D deficiency on alcohol metabolism and oxidative stress in the ALD mice

To identify the mechanism underlying the exacerbation of EtOH/CCl_4-mediated hepatic injury in mice with vitamin D deficiency, we evaluated the effect of vitamin D deficiency on alcoholic metabolism and oxidative stress in the liver of the experimental groups. The hepatic mRNA level of *Aldh2* in the EtOH/CCl_4-treated mice decreased, which was consistent with the ALDH2 activity in the liver (Figures 4A and 4B). The hepatic CYP2E1 activity of the EtOH/CCl_4-treated mice increased (Figure 4C). Meanwhile, there were no difference in the activities of these metabolic enzymes between the EtOH/CCl_4 + VtDS and EtOH/CCl_4 + VtDD groups (Figures 4B and 4C).

The EtOH/CCl_4-treated mice had lower hepatic concentrations of antioxidant enzymes CAT and SOD than the vehicle-treated mice. The decrease in these enzymes was more evident in the EtOH/CCl_4 + VtDD group (Figures 4D and 4E). By contrast, the EtOH/CCl_4-treated mice had a high level of hepatic MDA, a major indicator of lipid peroxidation, and the EtOH/CCl_4 + VtDD group had a significantly high hepatic MDA levels (Figure 4F).

Moreover, we found that the antioxidant genes (i.e., *Hmox1*, *Nqo1*, and *Gstm3*) were upregulated in the liver of EtOH/CCl_4 + VtDS group suggesting the response to EtOH and CCl_4 exposure (Figure 4G). Notably, the expression of these antioxidant genes did not increase in the EtOH/CCl_4 + VtDD group (Figure 4G). Compared with the vehicle-treated mice, the EtOH/CCl_4-treated mice had higher mRNA levels of the hepatic nicotinamide adenine dinucleotide phosphate oxidases (*Noxs*) (i.e., *Nox1*, *2,* and *4*). Among these genes, the expressions of *Nox1* and *Nox2* were further increased in the EtOH/CCl_4 + VtDD group (Figure 4H).

3.5 Vitamin D deficiency promotes Kupffer cell infiltration and LPS/TLR4 activation in the ALD mice

The proinflammatory status of the liver in the experimental mice was investigated based on the exacerbation of EtOH/CCl_4-induced hepatic inflammation and fibrosis caused by vitamin D deficiency. The F4/80-positive Kupffer cells infiltrated the liver of the EtOH/CCl_4 + VtDS group, and Kupffer cell infiltration was more extensive in the liver of the EtOH/CCl_4 + VtDD group (Figure 5A). Moreover, the EtOH/CCl_4 + VtDD group had higher mRNA levels of *Tnfa*, *Ccl2*, and *Il1b* than the EtOH/CCl_4 + VtDS group. Therefore, vitamin D deficiency was suggested to accelerate the proinflammatory response to EtOH and CCl_4 exposure (Figure 5B). Next, the effect of vitamin D deficiency on

hepatic LPS/TLR4 signaling was further assessed. As shown in Figure 5C, the EtOH/CCl_4 + VtDS group exhibited an upregulation of hepatic LPS-binding protein (*Lbp*), a relevant component of innate immunity synthesized in response to LPS. Along with the upregulation of *Lbp*, the hepatic expressions of *Tlr4* and its coreceptor *Cd14* detecting LPS, increased in the EtOH/CCl_4 + VtDS group (Figures 5D and 5E). Notably, the high levels of LPS/TLR4-related genes were more evident in the EtOH/CCl_4 + VtDD group. Therefore, vitamin D deficiency could induce hepatic overload of LPS (Figures 5C–5E). Consequently, in the EtOH/CCl_4-treated mice, the hepatic protein level of IκBα decreased, which, in turn, increased the phosphorylated NF-κB p65 levels. This indicated the induction of IκBα degradation and p65 phosphorylation caused by the LBP/CD14/TLR4 pathway (Figures 5F and 5G). In accordance with the increased hepatic overload of LPS, the EtOH/CCl_4 + VtDD group had enhanced IκBα degradation and p65 phosphorylation in the liver (Figures 5F and 5G).

3.6 Vitamin D deficiency promotes intestinal barrier disruption in the ALD mice

Vitamin D deficiency significantly promoted the hepatic accumulation of LPS, as indicated by the upregulation of *Lbp* (Figure 5C). To identify the source of hepatic LPS accumulation, we focused on the disrupted intestinal barrier function. As shown in Figures 6A–6D, the intestinal expressions of ZO-1, occludin and claudin-2, which are the components of tight junction protein (TJP), were significantly reduced in the EtOH/CCl_4 + VtDS group, and the EtOH/CCl_4 + VtDD group experienced more evident reduction in these expressions. Recent reports have shown that TJP markers and claudin-2/5/12/15 are highly relevant to intestinal barrier function regulated by the vitamin D/VDR axis [28-30]. According to these data, the intestinal mRNA

expressions of *Cldn2*, *Cldn5*, *Cldn12*, *Cldn15*, *Zo1*, and *Ocln* significantly decreased in the EtOH/CCl_4 + VtDD group (Figures 6E–6J). For the functional assessment of intestinal permeability, we examined the flux via the leak pathway, which is responsible for the paracellular mobilization of larger molecules, including LPS. The EtOH/CCl_4 + VtDS group experienced elevated plasma levels of FITC-dextran that leaked from the intestinal tract into the portal vein. Conversely, the group had decreased TJP expressions (Figure 6K). Moreover, the portal FITC-dextran levels of the EtOH/CCl_4 + VtDS group increased by more than 4-fold compared with that of the vehicle + VtDS group (Figure 6K).

3.7 Vitamin D suppresses the EtOH-induced barrier dysfunction in human enterocytes

We further analyzed the molecular mechanism of intestinal barrier function regulated by vitamin D using Caco-2 human enterocytes. The incubation with EtOH decreased the TEER values in Caco-2 cells in a dose-dependent manner (Figure 7A). Correspondingly, EtOH stimulation reduced both the mRNA and protein levels of TJPs including ZO-1, occludin, and claudin-2 (Figures 7B and 7C). Notably, the decrease in TEER values stimulated by EtOH was efficiently inhibited by treatment with calcitriol, an active form of vitamin D_3 in a dose-dependent manner (Figure 7D). Moreover, the VDR inhibitor ZK159222 efficiently negated the calcitriol-mediated recovery of electrical resistance and TJP expressions in EtOH-stimulated Caco-2 cells (Figures 7E and 7F). Based on these results, vitamin D supplementation could possibly have protective effects on intestinal barrier function via VDR activation in human enterocytes. A previous report has shown that the claudin-2 gene is a direct target of the transcription factor VDR, and VDR can enhance claudin-2 promoter activity in

the CDX1 binding site in Caco-2 cells [31]. In accordance with these findings, we found that treatment with calcitriol induced CDX1 expression, which was counteracted by treatment with ZK159222 in parallel with claudin-2 expression (Figure 7F).

4. Discussion

The current study showed that vitamin D deficiency significantly exacerbates EtOH- and CCl_4-induced liver injury in mice. In this model, the vitamin D-deficient groups presented with advanced hepatic inflammation and fibrosis. As an underlying mechanism, this study highlighted the disruption of intestinal barrier integrity caused by vitamin D insufficiency, thereby leading to increased hepatic exposure of LPS (Figure 8). Our initial data showed that a low serum 25(OH)D level is correlated with decreased hepatic reserve and increased endotoxin activity in patients with cirrhosis who presented with ALD. These results support our hypothesis that vitamin D insufficiency could be responsible for the pathological progression of ALD by disrupting intestinal barrier integrity.

Alcohol has a direct toxic impact on the intestinal mucosa of individuals acute and chronic alcohol consumption [32]. Alcohol binging impairs intestinal epithelium, and chronic EtOH exposure suppress the antimicrobial properties of the mucus, which, in turn, promotes bacterial translocation and endotoxemia with intestinal hyperpermeability [33]. Indeed, recent clinical surveys have shown that patients with ALD present with elevated serum LPS levels, which are correlated with liver injury [34,35]. Other reports have revealed that EtOH-fed mice had intestinal barrier impairment, thereby leading to elevated serum LPS levels, which exacerbate hepatic inflammation characterized by a massive macrophage infiltration and caused the upregulation of TLR4 in the liver [36,37]. Our current EtOH/CCl_4-treated mice model was found to promote LPS exposure

to the liver, as evidenced by hepatic *Lbp* expression, which is consistent with reduced intestinal TJP expression and enhanced plasma flux of FITC-dextran. These pathological mechanisms were substantiated by our cell-based assay, which showed that EtOH dose-dependently dampened epithelial resistance and decreased TJP expressions in a human intestinal epithelial cell line.

Notably, we found that the serum 25(OH)D level of the EtOH/CCl_4-treated mice decreased. This is similar to the results of our clinical study and other reports showing that vitamin D deficiency was observed in patients with ALD [38,39]. We hypothesized that vitamin D deficiency impaired the homeostasis of the gut–liver axis in this model. The vitamin D/VDR axis plays an important role in regulating intestinal integrity by shielding pathogenic bacterial invasion, thereby suppressing inflammation and maintaining barrier function [17]. In the intestinal epithelial cells, once 1,25$(OH)_2D_3$ binds with VDR, VDR heterodimerizes with the retinoid X receptor in the nuclei and then binds to the vitamin D-response element in the promoter of the target genes including claudin-2/5/12/15 [28,40]. In accordance with these findings, our results showed that vitamin D deficiency significantly accelerated the intestinal hyperpermeability leading to LPS/TLR4-mediated proinflammatory response and fibrosis development in the liver of EtOH/CCl_4-treated mice. Moreover, our in vitro study showed that calcitriol inhibited the EtOH-stimulated increase of paracellular permeability with restoration of TJPs including ZO-1, occludin, and claudin-2 in Caco-2 cells. These findings suggested that vitamin D directly regulates the intestinal barrier function in enterocytes. A recent study has shown that VDR is a transcriptional factor that regulates the expression of claudin-2 in human gut enterocytes [31]. Consistently, our study also showed that treatment with calcitriol upregulated the expression of CDX1 in parallel with claudin-2, which was offset by a VDR inhibitor in EtOH-treated Caco-2 cells. This finding

supports the notion that vitamin D restores claudin-2 expression by activating VDR.

The current study showed that vitamin D deficiency exacerbated the accumulation of hepatic oxidative stress in the EtOH/CCl_4-treated mice. Notably, vitamin D deficiency accelerated lipid peroxidation by dampening the antioxidant capacities in the EtOH/CCl_4-treated mice. This finding is supported the notion that vitamin D deficiency inhibited the hepatic expressions of some antioxidant genes (*Hmox1*, *Nqo1*, and *Gstm3*) in response to EtOH and CCl_4 exposure. Moreover, the current study showed that vitamin D deficiency promoted the upregulation of NOXs (*Nox1* and *Nox2*), which are key enzymatic sources of cellular oxidative stress in ALD pathogenesis [41]. Several research have shown that NOXs play an essential role in modulating the protective effect of vitamin D against oxidative stress [42-44]. Hu et al. have revealed that vitamin D deficiency augmented the alcohol-induced accumulation of NOXs and consequently attenuated the antioxidant capacity in the liver [45]. Furthermore, a recent study has revealed that NOXs can mediate the LPS/TLR4 pathway in human and murine hepatocytes and can contribute to the capacity of LPS to trigger the pathology of ALD [46]. Thus, further studies should be conducted to elucidate whether vitamin D deficiency-mediated upregulation of NOXs causes LPS/TLR4 signaling activation in the current model.

Our data also revealed that vitamin D deficiency accelerated EtOH/CCl_4-induced hepatocyte apoptosis. Hepatocyte apoptosis is known to be observed in alcohol-related liver injury [47,48]. Recent study has shown that long-term vitamin D deficiency caused apoptosis in hepatocytes [49]. Moreover, it is recognized that the chronic exposure of LPS augments the release of inflammatory cytokines from Kupffer cell leading to hepatocyte apoptosis as well as ROS accumulation and oxidative stress [50]. Accordingly, we suggested the

possible involvement of multiple mechanisms in the hepatocyte pro-apoptosis in the present model.

Furthermore, we found that ALD mice showed a higher serum triglyceride level and hepatic lipid accumulation in agreement with previous studies [51] . Of interest, the impact of vitamin D deficiency on ALD-induced lipid accumulation was limited in the current model. The role of vitamin D deficiency on hepatic lipid metabolism in chronic liver disease is controversially reported [52,53]. In addition, as with our results, recent study has suggested that vitamin D deficiency had a little effect on alcohol induced elevation of both hepatic triglyceride content and lipid accumulation [45]. Indeed, there remains with debate concerning the role of vitamin D on lipid metabolism and it is necessary to be analyzed in detail by using pleural models.

The current study had several limitations. Although this research showed that vitamin D deficiency was negatively correlated with endotoxemia in patients with ALD-related cirrhosis and could impair intestinal barrier integrity in the EtOH/CCl_4-treated mice, its impact on the gut microbiota was not fully elucidated. Various animal models have reported that an altered microbiota is associated with vitamin D deficiency [54,55]. Assa et al. have shown a higher quantity of *Bacteroidetes* in vitamin D-deficient mice [56]. Ooi et al. have shown that VDR or 1,25$(OH)_2D_3$ deficiency can cause dysbiosis and promote gut injury [57]. In addition, they revealed that compared with the feces of wild-type mice, the feces of cytochrome P knockout and VDR knockout mice had a greater abundance of *Bacteroidetes* and *Proteobacteria* and a lesser abundance of *Firmicutes*. By contrast, according to a rodent study using thioacetamide (TAA)-induced cirrhotic rats, treatment with vitamin D_3 enriched the abundance of *Muribaculaceae,* which belongs to the order of *Bacteroidales*, and generated short-chain fatty acid [18]. Meanwhile, limited study has evaluated the

relationship between the status of vitamin D and gut microbiota in humans, particularly the patients with ALD. Thus, further analyses must be performed to elucidate the possible impacts of vitamin D on gut microbiota.

The current study examined the effects of vitamin D deficiency on the development of ALD-related liver fibrosis in mice. Results showed that vitamin D deficiency enhanced the development of EtOH- and CCl_4-induced liver fibrosis, which might be involved in hepatic oxidative stress and LPS/TLR4-mediated proinflammatory response. Moreover, gut barrier disruption and exacerbation of LPS portal translocation could be correlated with the exacerbation of hepatic pathology caused by vitamin D deficiency in ALD.

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Figure legends

Figure 1. Serum 25(OH)D and endotoxin activity in cirrhotic patients with ALD.

(A): Flow chart of experimental design. (B): Serum 25(OH)D level in the patients with ALD. (C): Blood endotoxin activity in the patients with ALD. (D-G): Correlation between serum 25(OH)D level and blood endotoxin activity among the total patients (D), CP-class A (E), CP-class B (F), and CP-class C (G) with ALD. Data are mean ± SD (ND/CP-A/CP-B/CP-C; n=19/56/15/7). * $p < 0.05$; ** $p < 0.01$, indicating a significant difference between groups. ND; non-drinking patients without chronic liver disease, CP-class; Child-Pugh class.

Figure 2. Vitamin D deficiency on liver injury and hepatocyte apoptosis in ALD mice.

(A): Experimental protocols. (B): Body weight during experiment period. (C): Serum 25(OH)D level. (D-F): Serum levels of (D): calcium, phosphorus, (E): aspartate transaminase (AST), alanine aminotransferase (ALT) and (F): triglyceride (TG). (G): Representative macroscopic liver images and ratio of liver weight to body weight. (H): Hepatic concentrations of TG. (I): Representative microphotographs of liver sections stained with H&E and TUNEL. Scale bar: 50 μm. (J): Hepatic pathological scores at a 400-fold magnification. (K): TUNEL-positive hepatocytes in high-power field (HPF). Data are mean ± SD (n=10). [a] $p<0.01$ vs Veh+VtDS, [b] $p<0.01$ vs E/C+VtDS (B). * $p < 0.05$; ** $p < 0.01$, indicating a significant difference between groups (C-H, J and K). VtDS; vitamin D sufficient diet-fed groups, VtDD; vitamin D deficient diet-fed groups, Veh; vehicle-treated groups, E/C; EtOH and CCl_4-treated groups. N.S; not significant, N.D; not detected.

Figure 3. Vitamin D deficiency on liver fibrosis in ALD mice.

(A): Representative microphotographs of liver sections stained with Sirius-Red, α-SMA, and COL-1. Scale bar: 50 μm. (B-D): Semi-quantitation of (B): Sirius-Red-stained fibrotic area and (C): α-SMA and (D): COL-1 immuno-positive area in high-power field (HPF). (E): Western blots for COL-1 and α-SMA protein levels in liver tissues. Actin was used as the loading control. (F): Hepatic concentration of hydroxyproline. (G): Hepatic mRNA expression levels of *Acta2*, *Col1a1* and *Tgfb1*. *Gapdh* was used as internal control. Quantitative values are indicated as fold changes to the values of Veh+VtDS group. Data are mean ± SD (B-D, F and G; n=10, E; n=3). * $p < 0.05$; ** $p < 0.01$, indicating a significant difference between groups. VtDS; vitamin D sufficient diet-fed groups, VtDD; vitamin D deficient diet-fed groups, Veh; vehicle-treated groups, E/C; EtOH and CCl_4-treated groups. N.D; not detected.

Figure 4. Vitamin D deficiency on alcohol metabolism and oxidative stress in ALD mice.

(A): Hepatic mRNA expression level of *Aldh2*. (B and C): Hepatic activity of (B): aldehyde dehydrogenase 2 (ALDH2) and (C): cytochrome P450 2E1 (CYP2E1). (D-F): Hepatic concentration of (D): catalase (CAT), (E): superoxide dismutase (SOD) and (F): malondialdehyde (MDA). (G and H): Hepatic mRNA expression levels of (G): *Hmox1*, *Nqo1*, *Gstm3* and (H): *Nox1*, *Nox2*, *Nox4*. *Gapdh* was used as internal control. Quantitative values are indicated as fold changes to the values of Veh+VtDS group (A, B, G and H). Data are mean ± SD (n=10). * $p < 0.05$; ** $p < 0.01$, indicating a significant difference between groups. VtDS; vitamin D sufficient diet-fed groups, VtDD; vitamin D deficient diet-fed groups, Veh; vehicle-treated groups, E/C; EtOH and CCl_4-treated groups.

Figure 5. Vitamin D deficiency on Kupffer cell infiltration and LPS/TLR4 signaling pathway in ALD mice.

(A): Representative microphotographs of liver sections stained with F4/80 immuno-positive Kupffer cells and semi-quantitation in high-power field (HPF). Scale bar: 50 μm. (B-E): Hepatic mRNA expression levels of (B): *Tnfa*, *Ccl2, Il1b* and (C): *Lbp*, (D): *Cd14*, (E): *Tlr4*. *Gapdh* was used as internal control. (F): Western blots for IκBα, phosphor-p65 (p-p65) and p65 protein levels in liver tissues. Actin was used as the loading control. (G): Quantitative IκBα protein expression and phosphorylation rate of p65. Quantitative values are indicated as fold changes to the values of Veh+VtDS group (B-E and G). Data are mean ± SD (A-E; n=10, G; n=3), ** p <0.01, indicating a significant difference between groups. VtDS; vitamin D sufficient diet-fed groups, VtDD; vitamin D deficient diet-fed groups, Veh; vehicle-treated groups, E/C; EtOH and CCl_4-treated groups.

Figure 6. Vitamin D deficiency on intestinal barrier function in ALD mice.

(A): Representative microphotographs of ileum sections immunofluorescent stained with ZO-1, Occludin and Claudin-2. Scale bar: 50 μm. (B-D): Semi-quantitation of (B): ZO-1, (C): Occludin and (D): Claudin-2 immuno-positive area in high-power field (HPF). (E-J): Intestinal mRNA expression levels of (E): *Zo1*, (F): *Ocln*, (G): *Cldn2*, (H): *Cldn5*, (I): *Cldn12*, and (J): *Cldn15*. *Gapdh* was used as internal control. (K): Blood levels of FITC-dextran (4kDa) four hours after oral administration. Quantitative values are indicated as fold changes to the values of Veh+VtDS group (B-J). Data are mean ± SD (n=10), ** p <0.01, indicating a significant difference between groups. VtDS; vitamin D sufficient diet-fed groups, VtDD; vitamin D deficient diet-fed groups, Veh; vehicle-treated groups,

E/C; EtOH and CCl_4-treated groups.

Figure 7. Effects of Vitamin D_3 on in vitro EtOH-stimulated Caco-2 cells. (A): In vitro paracellular permeability in EtOH (0-60 mM)-stimulated Caco-2 cells determined as transepithelial electrical resistance (TEER). (B): Relative mRNA expression levels of TJP markers including *ZO1*, *OCLN*, and *CLDN2* in EtOH (0-60 mM)-stimulated Caco-2 cells. Cells were incubated with EtOH for 120 min. (C): Western blots for ZO-1, Occludin and Claudin-2 cellular protein levels in EtOH (0-60 mM)-stimulated Caco-2 cells. (D): TEER in EtOH (60 mM) and/or an active form of vitamin D_3, calcitriol (CAL) (10^{-9}-10^{-7} M)-treated Caco-2 cells. (E): TEER in EtOH (60 mM), CAL (10^{-7} M) and/or a VDR antagonist, ZK159222 (10^{-7} M)-treated Caco-2 cells. Cells were incubated with EtOH for 120 min. (F): Relative mRNA expression levels of TJP markers in EtOH, CAL and/or ZK159222-treated Caco-2 cells. (G): Western blots for Claudin-2 and CDX1 cellular protein levels in EtOH, CAL and/or ZK159222-treated Caco-2 cells. The mRNA expression levels were measured by RT-qPCR, and *GAPDH* was used as internal control. Quantitative values are indicated as fold changes to the values of non-treatment group (B and F). Actin was used as internal control for western blotting (C and G). Data are mean ± SD (n=8). [a] $p < 0.05$, [b] $p < 0.01$ vs non-treated groups, [c] $p < 0.01$ vs EtOH (+)/CAL(-)/ZK159222(-)-treated group, [d] $p < 0.01$ vs EtOH (+)/CAL(+)/ZK159222(-)-treated group.

Figure 8. Graphic summary of the impact of vitamin D deficiency on intestinal permeability and ALD-related liver injury.