

Vitamin D protects against necrotising enterocolitis in newborn mice by activating the ERK signalling pathway

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Abstract. Necrotising enterocolitis (NEC) is a serious intestinal disease that occurs in the neonatal period. The present study aimed to investigate the protective effect of vitamin D on NEC and the underlying mechanisms. Artificial feeding and hypoxia-cold stimulation were used to establish a mouse NEC model. IEC-6 cells were treated with lipopolysaccharide (LPS) to establish the *in vitro* NEC model. Changes in the levels of interleukin (IL)-6, IL-1 β and tumour necrosis factor (TNF)- α , and activities of malondialdehyde (MDA) and glutathione peroxidase (GPx) were investigated via ELISA kits. In addition, mRNA expression of IL-6, IL-1 β and TNF- α and protein expression of phosphorylated (p)-ERK1/2, Ki67, cleaved caspase-3 and Bcl-2 in intestinal tissues were determined via reverse transcription-quantitative PCR and western blotting. Cell proliferation and apoptosis were also analysed via MTT assay and flow cytometry. In NEC mice, vitamin D reduced intestinal tissue damage, decreased the mRNA expression of IL-6, IL-1 β and TNF- α , and decreased the protein expression of cleaved caspase-3 and MDA. Whereas, vitamin D increased the protein expression of Bcl-2 and Ki67 and GPx, as well as the p-ERK1/2/ERK1/2 ratio, in NEC mice. Furthermore, vitamin D improved cell viability, increased the ratio of p-ERK1/2/ERK1/2, inhibited apoptosis, and decreased the mRNA expression of IL-6, IL-1 β and TNF- α in LPS-treated IEC-6 cells. The dual-specificity mitogen-activated protein kinase kinase inhibitor PD98059 reversed the effects of vitamin D on the proliferation, apoptosis and inflammation of LPS-treated IEC-6 cells. Overall, vitamin D relieved NEC in mice. Vitamin D promoted proliferation, and inhibited apoptosis and inflammation of LPS-treated IEC-6 cells by activating the ERK signalling pathway.

Introduction

Necrotising enterocolitis (NEC) is one of the main causes of neonatal gastrointestinal disease-associated death, which primarily occurs in premature and low birth weight infants (1). The main clinical manifestations of NEC are abdominal distention, diarrhoea and blood in stool, and patients with severe forms of the disease may have intestinal perforation or stenosis, peritonitis, sepsis and intestinal fistula (2). With the development of perinatal medicine and the establishment of neonatal intensive care units, the survival rate of premature infants and infants with a very low birth weight has increased significantly, and the incidence rate of NEC has increased rapidly (3). A large number of *in vitro* studies and clinical studies have investigated NEC (4,5). However, the pathogenesis of the disease remains unclear. Therefore, it is necessary to explore the mechanisms underlying pathogenesis to inform effective strategies for the treatment of NEC.

Vitamin D participates in cell differentiation, proliferation and inflammation, and it acts as a key component in bone metabolism and calcium homeostasis by interacting with the vitamin D receptor (VDR) (6,7). Clinical research has shown that deficiency of serum vitamin D is a high-risk factor for inflammatory bowel disease (IBD) and is associated with IBD duration and prognosis (8). Vitamin D deficiency is common in preterm labour, especially in premature infants born before 32 weeks of gestation (9). Du *et al* (10) confirmed that 1,25-dihydroxyvitamin D protects the intestinal epithelial barrier by regulating the myosin light-chain kinase signalling pathway in a mouse model of 2,4,6-trinitrobenzene sulfonic acid-induced colitis. However, the possible protective mechanism of vitamin D against NEC remains unclear.

ERK1/2 is a member of the mitogen-activated protein kinase family of proteins, the members of which transmit regulatory signals involved in cell proliferation, apoptosis or differentiation (11-14). Bai *et al* (15) reported that sesamin enhances NADH-dependent flavin reductase subunit 2-mediated defence against oxidative stress and inflammation in ulcerative colitis by activating AKT/ERK. Dai *et al* (16) revealed that VSL#3 probiotics protect the intestinal epithelial barrier in acute colitis rats by activating ERK signalling pathways. Bai *et al* (17) demonstrated that serine/threonine-protein kinase Sgk1 protects intestinal epithelial cells against tumour necrosis factor

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(TNF)- α -induced apoptosis in colitis partly by activating the dual-specificity mitogen-activated protein kinase kinase (MEK)/ERK signalling pathway. These studies demonstrate that the ERK signalling pathway plays an important regulatory role in colitis; however, the specific regulatory role of this pathway in NEC remains unclear. In addition, Yuan *et al* (18) revealed that the VDR protects against neurological deficits and neuronal death in rats with global cerebral ischemia by activating the ERK signalling pathway. Therefore, it was hypothesised that vitamin D may protect against NEC by regulating the ERK signalling pathway.

The present study aimed to establish *in vitro* and *in vivo* models of NEC. The effects of vitamin D on NEC were then investigated, including pathological changes in intestinal tissue, oxidative stress, inflammation, proliferation and apoptosis of intestinal cells, and the ERK signalling pathway. The findings of the present research may provide a useful theoretical basis for the treatment of NEC.

Materials and methods

Establishment of an animal model of NEC. In total, 32 C57BL/6J mice (16 female and 16 male mice; age, 5–6 weeks; weight, 18–25 g) were used for the establishment of the NEC animal model *in vivo* based on previous studies (19–21). The mice were fostered in a cage and maintained on a 12:12 light/dark cycle at room temperature under dry atmosphere. C57BL/6J mice were purchased from Changzhou Cavens Laboratory Animal Co., Ltd (license no. SCXK(Su)2016-0010; Jiangsu, China). The mice were divided into four groups of 8 animals each: i) Control; ii) NEC; iii) vitamin D + control and; iv) vitamin D + NEC. Control mice were fed artificial formula (Abbott Pharmaceutical Co., Ltd.) and given normal saline by gavage. NEC mice were fed artificial formula and subjected to hypoxia-cold stimulation. Vitamin D + control mice were fed artificial formula and administered vitamin D (0.5 g/kg/day; Sigma-Aldrich; Merck KGaA) by gavage. Vitamin D + NEC mice were fed artificial formula, subjected to hypoxia-cold stimulation, and given 0.5 g/kg/day vitamin D by gavage. Artificial feeding and hypoxia-cold stimulation were used to establish the NEC model, as previously described (16). Briefly, mice were exposed to hypoxia with 5% O₂ and 95% N₂ for 1 min; then, they were immediately placed in a 4°C cold chamber for 10 min. The hypoxia-cold stimulation was controlled within 1 h after the meal and performed twice a day for 4 days. The body weight of the mice was measured every day. Upon appearance of clinical signs (abdominal distension, diarrhea and vomiting) of NEC distress (~96 h after modelling), the mice were anaesthetised by an intraperitoneal injection of 50 mg/kg pentobarbital sodium. Anaesthesia was confirmed when mice presented with symptoms including excitement, convulsion and fainting. They were then sacrificed by cervical dislocation. Intestinal tissues of the mice were collected for follow-up tests. All animal experiments were approved by the Institutional Animal Care and Use Ethics Committee (approval no. L2019-4; Foshan, China).

Histological injury score. The terminal ileum was fixed in 4% neutral buffered formalin solution for 24 h at room

temperature, embedded in paraffin and cut into sections with a thickness of 3 μ m. Pathological changes were detected using haematoxylin and eosin (H&E) staining. Briefly, after dewaxing and hydration, sections were stained with hematoxylin (Sigma-Aldrich; Merck KGaA) for 20 min at room temperature, and then stained with 0.5% eosin (Sigma-Aldrich; Merck KGaA) for 90 sec at room temperature. Sections were washed with 70% ethyl alcohol to remove the eosin, and were dehydrated with anhydrous alcohol for 1 min. Finally, these sections were treated with xylene I or II (Sigma-Aldrich; Merck KGaA) for 10 min at room temperature. After blocking the sections with neutral balsam at room temperature for 24 h, images were captured via an Olympus light microscope (magnification, x200; Nikon Corporation).

Histopathological changes were scored by two blinded evaluators based on the following criteria (22): 0, normal, no damage; 1, slight submucosal and/or lamina propria separation; 2, moderate separation of submucosa and/or lamina propria and/or oedema in submucosal and muscular layers; 3, severe separation of the submucosa and/or lamina propria and/or severe oedema in the submucosa and muscular layers, region villous sloughing; and 4, loss of villi, with necrosis. NEC was defined as a histological score ≥ 2 .

ELISA. Intestinal tissues were placed in cold physiological saline (4°C) to create a tissue homogenate. Next, the tissue homogenate was centrifuged at 10,000 \times g for 15 min at 4°C and the supernatant was collected to determine the levels of inflammatory cytokines, including interleukin (IL)-6, IL-1 β and TNF- α , using IL-6, IL-1 β and TNF- α ELISA kits (cat. nos. ab100713, ab100705 and ab208348, respectively; all Abcam) according to the manufacturer's instructions.

Malondialdehyde (MDA) and glutathione peroxidase (GPx) assays. The supernatant of the intestinal homogenate was collected to detect MDA and GPx activity. The Lipid Peroxidation (MDA) assay kit (Sigma-Aldrich; Merck KGaA) was used to detect MDA activity. GPx activity was detected using the Glutathione Peroxidase assay kit (Cayman Chemical Company). The procedures were conducted according to the manufacturers' instructions.

Cell culture and grouping. Rat epithelial IEC-6 cells were used for the establishment of the NEC cell model *in vitro* based on previous studies (23–25). IEC-6 cells were purchased from Shanghai Kanglang Biotechnology Co., Ltd. (Shanghai, China) and cultured on 96-well plates in DMEM (Sigma-Aldrich; Merck KGaA) supplemented with 10% FBS (Sigma-Aldrich; Merck KGaA). IEC-6 cells were divided into four groups: i) Control; ii) lipopolysaccharide (LPS); iii) vitamin D + LPS; and iv) vitamin D + PD98059 + LPS. Control cells underwent no treatment. LPS cells were treated with LPS at a concentration of 50 μ g/ml for 6 h at room temperature. Vitamin D + LPS cells were first treated with LPS at a concentration of 50 μ g/ml for 6 h and then with vitamin D at a concentration of 50 nM for 48 h at room temperature. Vitamin D + PD98059 + LPS cells were treated first with LPS at a concentration of 50 μ g/ml for 6 h, then with vitamin D at a concentration of 50 nM and the

Table I. Sequences of primers used for reverse transcription quantitative-PCR.

Primers	Sequence (5'-3')	
	Forward	Reverse
IL-6	GATACCACTCCCAACAGAC	CTTTTCTCATTTCCACGAT
IL-1 β	GTGGTGTGTGACGTTCCCATTA	CCGACAGCACGAGGCTTT
TNF- α	TGCAGCAGGACATCAAGTTC	TACGCCTCAGCAGTCTCCTT
GAPDH	GCTTCGGCAGCACATATACTAAAAT	CGCTTCACGAATTTGCGTGTTCAT

IL, interleukin; TNF, tumour necrosis factor.

MEK inhibitor PD98059 (Shanghai BeiZhuo Biotech Co., Ltd.) at a concentration of 5 μ M for 48 h at room temperature.

Reverse transcription-quantitative (RT-q)PCR. TRIzol[®] reagent (Thermo Fisher Scientific, Inc.) was used to extract total RNA from intestinal tissues and cells. cDNA synthesis was conducted at 55°C using the PrimeScript RT reagent kit (Takara Bio, Inc.) according to the manufacturer's protocols. qPCR was performed with an ABI 7500 quantitative PCR instrument (Applied Biosystems; Thermo Fisher Scientific, Inc.) using SYBR Green Realtime PCR Master mix (Toyobo Life Science). The qPCR thermocycling conditions were as follows: Pre-denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 1 min and 55°C for 30 sec. The qPCR primers (Table I) were synthesized by Sangon Biotech Co., Ltd. Relative mRNA expression was calculated using the $2^{-\Delta\Delta C_q}$ method and GAPDH served as the internal control (26).

Western blotting. Intestinal tissue was homogenized in RIPA protein lysis buffer (Beyotime Institute of Biotechnology) and centrifuged at 4°C for 15 min at 16,000 x g. A BCA Protein assay kit (Thermo Fisher Scientific, Inc.) was used to detect protein concentrations. In total, 50 μ g protein was loaded on a 10% gel, resolved using SDS-PAGE and subsequently transferred to PVDF membranes (Thermo Fisher Scientific, Inc.). The membranes were blocked with 5% BSA for 1 h at 37°C and were then incubated with the primary antibodies against ERK1/2 (1:1,000; cat. no. ab17942; Abcam), Ki67 (1:1,000; cat. no. MA5-14520; Thermo Fisher Scientific, Inc.), p-ERK1/2 (1:1,000; cat. no. 4376; Cell Signalling Technology, Inc.), cleaved caspase-3 (1:1,000; cat. no. 9661; Cell Signalling Technology, Inc.), Bcl-2 (1:1,000; cat. no. 3498; Cell Signalling Technology, Inc.) and GAPDH (1:1,000; cat. no. 5174, Cell Signalling Technology, Inc.) at 4°C overnight. The next day, the membranes were washed three times with 0.2% TBS-Tween-20 solution and incubated with HRP-conjugated goat anti-rabbit immunoglobulin G (1:2,000; cat. no. 31460; Thermo Fisher Scientific, Inc.) for 2 h at room temperature. An enhanced chemiluminescence detection kit (Beijing Solarbio Science & Technology Co., Ltd) was used to visualize the protein bands. The intensity of these bands was quantified via Image Lab[™] 3.0 software (version 3.0; Bio-Rad Laboratories, Inc.)

MTT assay. An MTT assay was used to determine cell proliferation. Briefly, cells were cultured in an incubator with

5% CO₂ at 37°C for 48 h and then the cells were incubated with 20 μ l MTT solution (Shanghai Jianglai Biotechnology Co., Ltd.) for 4 h at 37°C. Next, medium was replaced with 150 μ l DMSO for 10 min to promote crystal dissolution. The absorbance of cell suspensions was measured at 570 nm using a spectrophotometer (Shanghai Onlab Instrument Co., Ltd.). Cell viability was normalized to that of the control group.

Apoptosis assay. An Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis kit (Invitrogen; Thermo Fisher Scientific, Inc.) was used to determine apoptosis of the aforementioned cell groups. Briefly, cells were resuspended in binding buffer and incubated with Annexin V-FITC (5 μ l) and PI (5 μ l) for 10 min at 25°C. A flow cytometer (DLK0002095; BD Biosciences) was used to determine the apoptosis rate. CellQuest software (version 3.1; BD Biosciences) was used to analyze the data.

Statistical analysis. SPSS version 22.0 statistical software (IBM, Corp.) was used to analyse the experimental data. The data are expressed as mean \pm standard deviation of three independent repeats. One-way ANOVA followed by Tukey's post hoc test was used for comparisons among multiple groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Vitamin D reduces damage and oxidative stress in intestinal tissues of NEC mice. To determine whether vitamin D has a protective effect on NEC, the body weight of newborn mice was monitored. No significant difference in body weight was found between the control and vitamin D + control group (Fig. 1A). The body weight of mice in the NEC group was lower compared with that of control mice 48 h after establishing the NEC model ($P < 0.01$). Notably, compared with the NEC group, the body weight of mice in the vitamin D + NEC group was higher after 72 and 96 h of modelling ($P < 0.05$). H&E staining (Fig. 1B) demonstrated that the intestinal tissue structure of mice was normal in the control and vitamin D + control group. Mice in the NEC group exhibited severe necrosis and the infiltration of a number of inflammatory cells in intestinal tissues. Notably, necrosis and inflammation in the vitamin D + NEC group were less compared with the NEC group. In NEC group, separation and necrosis of the villous center, submucosal

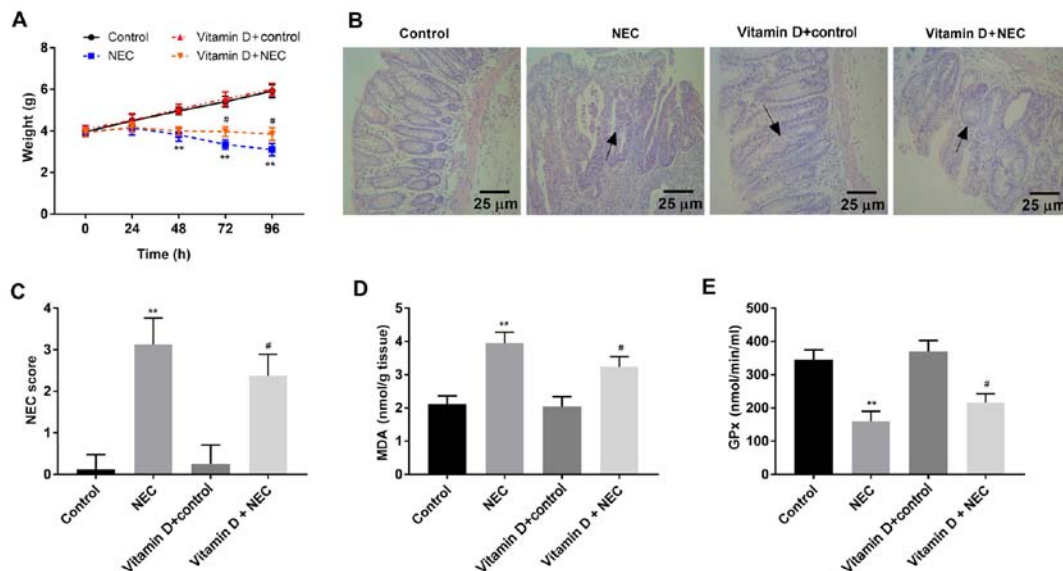


Figure 1. Vitamin D reduces intestinal tissue damage and oxidative stress in NEC mice. (A) Body weight (g) of mice. (B) Histopathological changes were observed by haematoxylin and eosin staining. Arrows indicate necrosis. Scale bar, 25 μ m. (C) Intestinal tissue damage was assessed by NEC score. (D) Levels of MDA in intestinal tissues. (E) Levels of GPx in intestinal tissues. Control mice were given normal saline by gavage under normal environmental conditions (n=8). NEC mice were given normal saline by gavage and treated with hypoxia-cold stimulation (n=8). Vitamin D + control mice were given vitamin D by gavage under normal environmental conditions (n=8). Vitamin D + NEC mice were given vitamin D by gavage and treated with hypoxia-cold stimulation (n=8). *P<0.01 vs. control group. #P<0.05 vs. the NEC group. NEC, necrotising enterocolitis; MDA, malondialdehyde; GPx, glutathione peroxidase.

edema and epithelial cell beating down were observed; in the vitamin D + NEC group, this phenomenon was significantly improved. Furthermore, the vitamin D + NEC group had a significantly lower NEC score compared with the NEC group (P<0.05; Fig. 1C). As presented in Fig. 1D and E, the levels of MDA were significantly higher, while the GPx levels were significantly lower in the NEC group compared with the control group (both P<0.01). Furthermore, vitamin D intervention in the vitamin D + NEC group significantly decreased the levels of MDA and significantly increased the levels of GPx compared the NEC group (both P<0.05).

Vitamin D reduces intestinal inflammatory cytokine levels in NEC mice. Proinflammatory cytokines play important roles in NEC-induced intestinal damage (27). Inflammatory cytokines (IL-6, IL-1 β and TNF- α) in the intestinal tissues of mice were analysed using ELISA and RT-qPCR assays. According to ELISA, no significant differences were observed in the levels of IL-6, IL-1 β and TNF- α between the control and vitamin D + control groups (Fig. 2A-C). Notably, the levels of IL-6, IL-1 β and TNF- α in the NEC group were significantly higher compared with those in the control group (all P<0.01). Furthermore, the levels of IL-6, IL-1 β and TNF- α were significantly lower in the vitamin D + NEC group compared with the NEC group (all P<0.01). The results of RT-qPCR were consistent with those of ELISA (Fig. 2D-F).

Vitamin D increases the proliferation and decreases the apoptosis of intestinal cells and activates the ERK signalling pathway in NEC mice. The expression levels of ERK signalling pathway- (p-ERK1/2 and ERK1/2), proliferation (Ki67) and apoptosis-associated proteins (cleaved caspase-3 and Bcl-2) in the intestinal tissues of mice were analysed using western blotting. As presented in Fig. 3A-E, there

were no significant differences in the protein expression levels of p-ERK1/2/ERK1/2, Ki67, cleaved caspase-3 and Bcl-2 between the control and vitamin D + control groups. Notably, the protein expression levels of Ki67, Bcl-2 and p-ERK1/2/ERK1/2 were significantly lower (P<0.01), and the protein expression of cleaved caspase-3 was significantly higher (P<0.01) in the NEC group compared with the control group. Furthermore, the protein expression levels of Ki67, Bcl-2 and p-ERK1/2/ERK1/2 in the vitamin D + NEC group were significantly higher, but the protein expression of cleaved caspase-3 was significantly lower compared with that in the NEC group (all P<0.05).

Blocking the ERK signalling pathway reverses the protective effect of vitamin D on LPS-treated IEC-6 cells. IEC-6 cells were treated with LPS to establish an *in vitro* NEC model. As presented in Fig. 4A, the ratio of p-ERK1/2/ERK1/2 was significantly lower in the LPS group compared with the control group (P<0.01), however the ratio of p-ERK1/2/ERK1/2 was significantly higher in the vitamin D + LPS group compared with the LPS group (P<0.01). Subsequently, the proliferation and apoptosis of IEC-6 cells were determined using an MTT assay and flow cytometry, respectively. As presented in Fig. 4B and C, cell viability was significantly decreased and the apoptosis rate was significantly increased in the LPS group compared with the control group (both P<0.01). Vitamin D treatment in the vitamin D + LPS group significantly increased the viability and significantly decreased the rate of apoptosis of IEC-6 cells compared with the LPS group (both P<0.01). Furthermore, RT-qPCR revealed that the mRNA expression levels of IL-6, IL-1 β and TNF- α were significantly increased in the LPS group compared the control group, and vitamin D treatment significantly reversed the effect of LPS

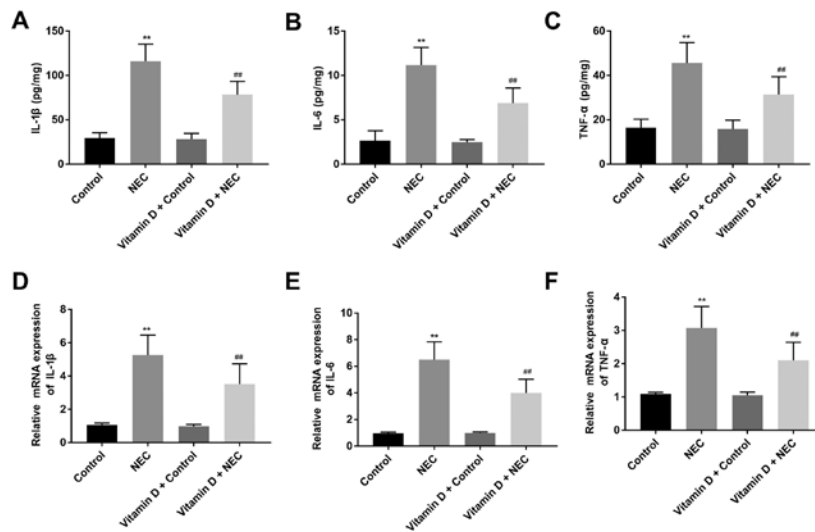


Figure 2. Vitamin D reduces intestinal inflammation in NEC mice. Levels of (A) IL-1 β , (B) IL-6 and (C) TNF- α in intestinal tissues were detected by ELISA. The mRNA expression levels of (D) IL-1 β , (E) IL-6 and (F) TNF- α in intestinal tissues were detected by reverse transcription quantitative-PCR. Control mice were given normal saline by gavage under normal environmental conditions (n=8). NEC mice were given normal saline by gavage and treated with hypoxia-cold stimulation (n=8). Vitamin D + control mice were given vitamin D by gavage under normal environmental conditions (n=8). Vitamin D + NEC mice were given vitamin D by gavage and treated with hypoxia-cold stimulation (n=8). **P<0.01 vs. control group. #P<0.01 vs. NEC group. NEC, necrotising enterocolitis; IL, interleukin; TNF, tumour necrosis factor.

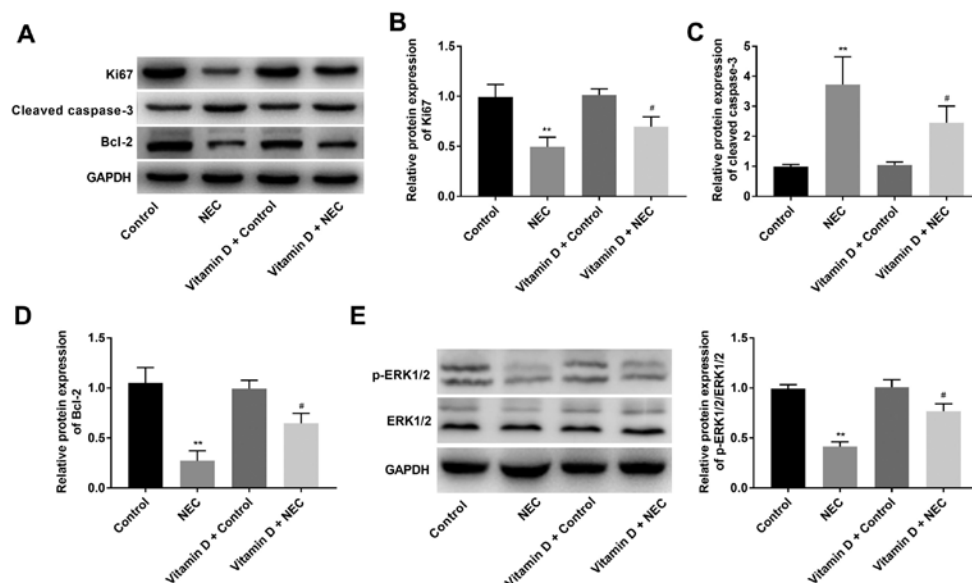


Figure 3. Vitamin D promotes cell growth and activates the ERK signalling pathway in NEC mice. (A) Protein levels of Ki67, cleaved caspase-3 and Bcl-2 in intestinal tissues were detected using western blotting. Protein expression levels of (B) Ki67, (C) cleaved caspase-3, (D) Bcl-2, and (E) p-ERK1/2 and ERK1/2 in intestinal tissues were detected using western blotting. Control mice were given normal saline by gavage under normal environmental conditions (n=8). NEC mice were given normal saline by gavage and treated with hypoxia-cold stimulation (n=8). Vitamin D + control mice were given vitamin D by gavage under normal environmental conditions (n=8). Vitamin D + NEC mice were given vitamin D by gavage and treated with hypoxia-cold stimulation (n=8). **P<0.01 vs. control group. #P<0.05 vs. NEC group. NEC, necrotising enterocolitis; p-, phosphorylated.

(all P<0.01; Fig. 4D-F). Notably, the MEK inhibitor PD98059 significantly reversed the effects of vitamin D on p-ERK1/2 level, proliferation, apoptosis and inflammatory-associated proteins in IEC-6 cells (Fig. 4A-F).

Discussion

NEC is a serious intestinal disease that occurs in the neonatal period and it is a common cause of early death in

neonates (28). At present, NEC is considered to be caused by premature birth, hypoxia, enteral feeding, bacterial infection and intestinal ischemia (29); however, its pathogenesis is still not completely clear. The results of the present study demonstrated that vitamin D reduced intestinal tissue damage, oxidative stress and inflammation in NEC mice. Furthermore, vitamin D promoted cell viability, inhibited apoptosis and inflammation, and activated the ERK signalling pathway in LPS-treated IEC-6 cells.

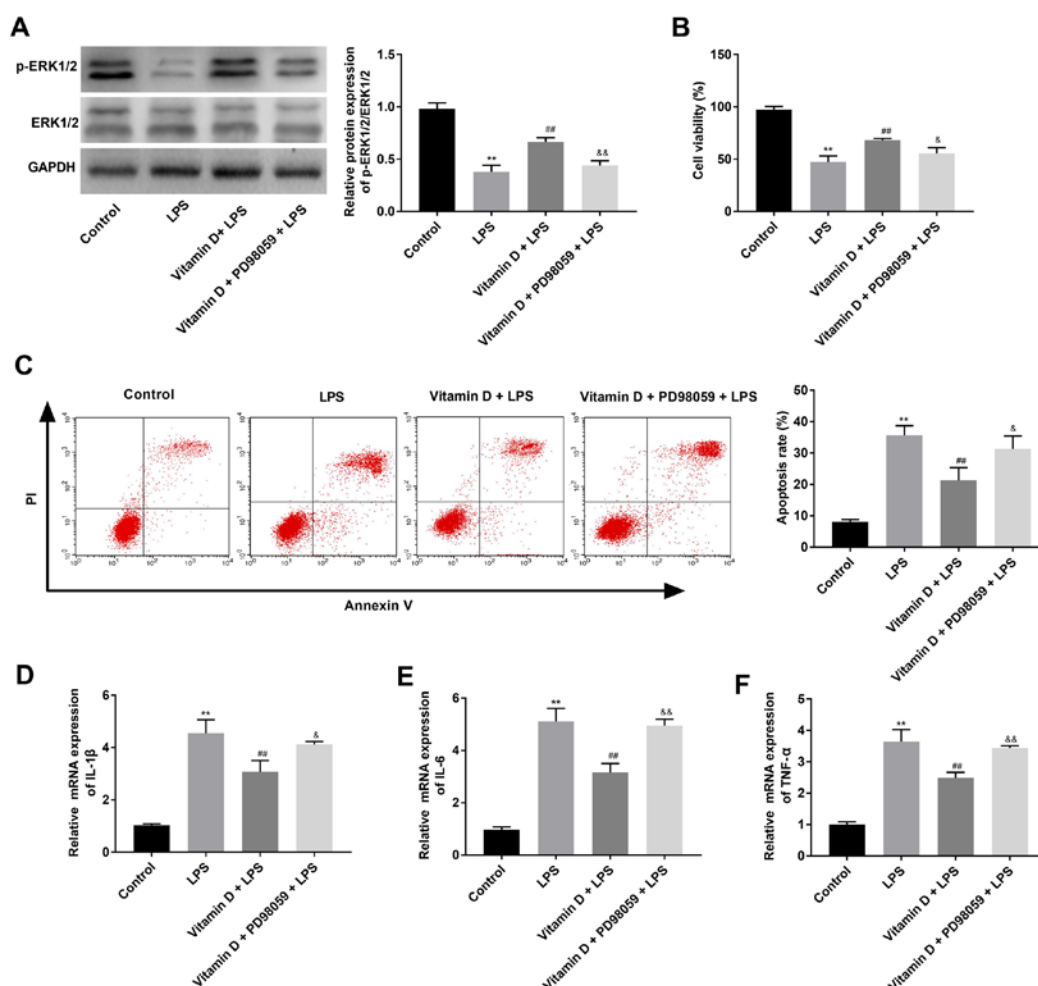


Figure 4. Inhibiting the ERK signalling pathway reverses the protective effect of vitamin D on LPS-induced IEC-6 cells. (A) Protein expression levels of p-ERK1/2 and ERK1/2 in IEC-6 cells were detected using western blotting. (B) Viability of IEC-6 cells was detected using an MTT assay. (C) Apoptosis of IEC-6 cells was assessed by flow cytometry. mRNA expression of (D) IL-1 β , (E) IL-6 and (F) TNF- α in IEC-6 cells was analysed using reverse transcription quantitative-PCR. Control cells were not treated. LPS cells were treated with LPS only. Vitamin D + LPS cells treated with LPS and vitamin D. Vitamin D + PD98059 + LPS cells treated with LPS, vitamin D and PD98059 (an inhibitor of dual specificity mitogen-activated protein kinase). ** $P < 0.01$ vs. control group. ## $P < 0.01$ vs. the LPS group. & $P < 0.05$, && $P < 0.01$ vs. the vitamin D + LPS group. LPS, lipopolysaccharide; p-, phosphorylated; IL, interleukin; TNF, tumour necrosis factor; FITC, fluorescein isothiocyanate; PI, propidium iodide.

Reactive oxygen species produced by oxidative stress can directly or indirectly damage the physiological functions of proteins, lipids, nucleic acids and other biological macromolecules in cells, which is the pathophysiological basis for the occurrence and development of NEC (30). MDA is a marker of lipid oxidation and an indicator of the degree of membrane damage (31). GPx is a marker of anti-oxidative capacity (32). A prior study revealed that vitamin D3 reduces serum MDA levels in patients with irritable bowel syndrome (33). In an animal model, Farhangi *et al* (34) reported that vitamin D treatment leads to a significant reduction in GPx concentrations in the high-fat diet + vitamin D group. The present study demonstrated that vitamin D treatment decreased MDA and increased GPx levels in the intestinal tissues of NEC mice. These results suggest that vitamin D reduced oxidative stress in the intestinal tissue of NEC mice.

Vitamin D plays a key regulatory role in calcium and phosphorus metabolism and anti-inflammation (35-37). For example, Golan *et al* (38) conducted experiments using VDR transgenic mice and revealed that overexpression of VDR in the intestinal epithelium inhibits the occurrence of spontaneous

enteritis caused by IL-10 deficiency. Liu *et al* (39) reported that 1,25(OH) $_2$ D $_3$ (an active form of vitamin D)-deficient mice exhibit notable colon inflammation phenotypes, and high expression of inflammatory cytokines. Mousavi *et al* (40) confirmed that 25-OH-cholecalciferol (a circulating form of vitamin D)-treated mice display low compromised colonic epithelial barrier function and low levels of pro-inflammatory mediators including IL-6, MCP1 and IFN- γ . Consistent with previous results, the present study demonstrated that vitamin D treatment reduced the levels of inflammatory cytokines (IL-6, IL-1 β and TNF- α) in the intestinal tissue of mice and LPS-treated IEC-6 cells. In brief, the present results suggest that vitamin D reduces inflammation in NEC.

Apoptosis is one of the important pathological changes in intestinal injury during NEC. As reported previously, VDR signalling leads to a reduction in IEC apoptosis by blocking NF- κ B activation (41). Zhu *et al* (42) reported that vitamin D deficiency is common in patients with IBD and that vitamin D decreases the expression levels of cleaved caspase-3 in mice with 2,4,6-trinitrobenzene sulfonic acid-induced colitis. He *et al* (43) found that the absence of gut epithelial VDR

promotes the apoptosis of epithelial cells and increases the permeability of the mucosal barrier. In the present study, vitamin D treatment increased the protein expression levels of Bcl-2, decreased the protein expression levels of cleaved caspase-3 in the intestinal tissue of mice and inhibited the apoptosis of LPS-treated IEC-6 cells. The aforementioned results suggest that vitamin D inhibits the apoptosis of intestinal cells in NEC mice.

Previous studies have reported that the ERK1/2 signalling pathway is involved in the genesis and development of intestinal diseases. For example, Ban *et al* (44) demonstrated that treatment with U0126 (an ERK1/2 inhibitor) leads to an increase in intestinal permeability, intestinal injury and inflammatory cytokines in mice with intestinal ischemia-reperfusion. Deng *et al* (45) suggested that leptin reduces intestinal histological alterations and MDA and IL-6 levels by enhancing ERK1/2 phosphorylation and promoting the NO synthesis signalling pathway. Jeong *et al* (46) revealed that spirulina crude protein treatment promotes cell migration and proliferation, and dysregulates the protein expression of H-Ras, p-Raf-1, p-MEK-1 and p-ERK-1/2 in IEC-6 cells. The present study demonstrated that vitamin D increased the protein expression levels of p-ERK1/2 in NEC intestinal tissue and IEC-6 cells. Notably, the MEK inhibitor PD98059 reversed the effects of vitamin D on the proliferation, apoptosis and inflammation of LPS-treated IEC-6 cells. The aforementioned findings demonstrate that vitamin D improved cell viability and inhibited cell apoptosis and inflammation in intestinal cells of NEC mice by activating the ERK signalling pathway.

The present study has some limitations. First, the histopathological change of NEC was only observed in the mucosa layer. The observations of different layers of the intestine may better reflect the therapeutic effect of vitamin D on NEC, as each layer exhibits different characteristics. Second, the potential mechanisms underlying the action of vitamin D still need to be further investigated. For example, the genes and signaling pathways through which vitamin D improves NEC should be elucidated.

In conclusion, vitamin D reduced intestinal tissue damage, oxidative stress and inflammation, and activated the ERK signalling pathway in NEC mice in the present study. Furthermore, vitamin D promoted the viability, and inhibited the apoptosis and inflammation of LPS-treated IEC-6 cells by activating the ERK signalling pathway. The present findings may provide a new theoretical basis for the treatment of NEC.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CL and SJ were mainly responsible for conceptualization, data analysis and interpretation. SJ was responsible for drafting and revising the manuscript. MK performed the experiments. XC and LZ were responsible for software management and visualization. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments were approved by The Institutional Animal Care and Use Ethics Committee (approval no. L2019-4; Foshan, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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