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ORIGINAL ARTICLE

Protective effects of vitamin D against injury in intestinal epithelium

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Abstract

Background Vitamin D deficiency is associated with intestinal barrier dysfunction, which contributes to pathogenesis of acute intestinal injury in children. We aim to investigate the effects of vitamin D on intestinal injury in intestinal epithelial cells and organoids.

Methods Lipopolysaccharide (LPS) was used to induce injury in intestinal epithelial cells (IEC-18) and organoids, and the effect of vitamin D was assessed. Cell viability was measured and inflammation cytokines TNFα and IL-8 were quantified. FITC-dextran 4 kDa (FD4) permeability was measured using Transwell while tight junction markers were assessed by immunofluorescence staining in IEC-18 and intestinal organoids. Data were compared using one-way ANOVA with Bonferroni post-test.

Results IEC-18 viability was decreased by LPS treatment, but was prevented by vitamin D. The upregulation of inflammation was inhibited by vitamin D, which also decreased epithelium permeability. Vitamin D restored tight junction ZO-1 and claudin 2. In addition, vitamin D decreased TNFα expression and prevented the disruption of ZO-1 in injured organoids.

Conclusions Vitamin D rescued epithelial barrier function by improving permeability and restoring tight junctions, leading to decrease inflammation. This study confirms the protective effects of vitamin D, which could be used as a treatment strategy for infants at risk of developing intestinal injury.

Keywords Vitamin D · Intestinal epithelial cells · Permeability · Organoids

Introduction

Intestinal epithelium injury in infants and children can occur in various diseases including midgut volvulus, necrotizing enterocolitis (NEC), sepsis and multisystem organ failure, inflammatory bowel disease such as Crohn's disease and ulcerative colitis, and high-grade intestinal obstruction.

There is an association of low maternal serum vitamin D concentrations and preterm delivery [1, 2] in preterm infants [3]. Vitamin D plays an important role in the maintenance of calcium absorption in several organs including the intestine [4]. In addition to its intestinal calcium homeostatic mechanism, vitamin D also regulates intestinal stem cell activity [5], inflammation and immunity in association with gastrointestinal diseases including inflammatory bowel diseases, Crohn's disease [6] and ulcerative colitis. Maternal/neonatal vitamin D deficiency has been shown to link with the development of NEC in preterm infants [7, 8] and more-severe colitis [9]. Studies have revealed vitamin D to be essential for attenuating the progression of gastrointestinal diseases. Shi et al. demonstrated that vitamin D can ameliorate NEC in neonatal rats, while also attenuating apoptosis and suppressing inflammation in NEC rats partly by suppressing the expression of Toll Like Receptor 4 (TLR4) [10]. Vitamin D is known to suppress inflammatory response in vascular smooth muscle cells [11] and ameliorate colitis by inhibiting apoptosis [12]. In active ulcerative colitis, barrier integrity was preserved and tight junction proteins were up-regulated by vitamin D [13].

The aim of this study was to investigate the protective effects of vitamin D on the intestinal injury using intestinal epithelial cell and intestinal organoid models.

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Methods

Cell cultures

Rat IEC-18 cells was obtained from the American-Type Culture Collection (ATCC, Manassas, VA). Cells were maintained in Dulbecco's modified Eagle's medium containing high glucose (DMEM, Gibco BRL), at pH 7.4, supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1% non-essential amino acid solution and 0.1% penicillin–streptomycin solution in a humidified atmosphere (5% CO2, 95% air, 37 °C). The cells were grown under standard conditions until 60–70% confluency prior to experiments.

Cell viability

IEC-18 cell viability was measured utilizing a colorimetric assay that measures formazan, which is a species generated by the conversion of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] into formazan by the mitochondrial enzyme succinate dehydrogenase. Cells were seeded in 96-well plate overnight and then subjected to lipopolysaccharide (LPS) (200 µg/ml) to induce cell injury. Active form of vitamin D (1,25-$(OH)_2D_3$) (Sigma-Aldrich MO, USA) at 10 µM was administered to cells to study the vitamin D effects. MTT reagent (0.5 mg/mL) (ATCC, Manassas, VA) was added to the medium. Following 3 h of incubation, the insoluble formazan crystals within the cells were extracted using DMSO and the absorbance was measured using a microplate reader (Molecular Devices SpectraMax Gemini EM) at a wavelength of 570 nm.

Inflammation

The inflammations markers were evaluated by quantitative gene expression of TNFα and IL-8. Total RNA was isolated using TRIzol reagent (Invitrogen). RNA (1 µg) was reverse transcribed using qScript cDNA supermix (Quanta Biosciences, Gaithersburg, MD). SYBR green-based RT-qPCR was performed in a CF384 C1000 Thermal Cycler (Bio-Rad) using Evagreen Supermix (Bio-Rad) using specific temperatures and cycles as previously described [14]. The primer TNFα and IL-8 sequences were adopted from the previous publications [15]. Results are from three independent experiments performed in triplicate. Data were analyzed using CFX Manager 3.1 (Bio-Rad). Expression levels were calculated using the ΔΔCt method, and data were normalized to reference housekeeping gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

FITC-dextran flux assay

The transport of fluorescein isothiocyanate dextran 4 kDa (FD4, Sigma-Aldrich MO, USA) across the IEC-18 cell monolayer at pH 7.4 was studied. Cells were seeded on tissue culture polycarbonate membrane filters (pore size 0.4 µm) in 24-well Transwell® plates (Corning Life Sciences Massachusetts, USA) at a seeding density of 1×10^5cells/transwell. The culture medium was added to both the apical and the basolateral compartments. To induce cell injury, LPS was added to the apical compartment containing the monolayer either with or without vitamin D (10 µM) as a protective agent. After 3 days, apical chamber of transwell was replaced with medium containing FD4 (1 mg/ml) and basolateral chamber was replaced with PBS. After 30 min incubation, FD4 flux was assessed by taking 100 µl from the basolateral chamber and fluorescent signal was measured in a fluorometer (excitation, 492 nm; emission, 520 nm; BioTek).

Immunofluorescence staining

Cells were seeded in 24-well plate containing three groups, control, LPS and LPS + Vitamin D, similar to above. Cells were fixed in 4% formalin for 1 h at room temperature, washed three times with phosphate buffered saline (PBS) and permeabilized with 0.5% Triton-X100 for 15 min. Cells were then washed with PBS, blocked with 3% bovine serum albumin for 1 h and incubated with primary antibodies. Primary antibodies, zonula occludens-1 (ZO-1) and claudin 2, were diluted to 1:1000 and incubated overnight at 4 °C. Cells were washed with PBS and incubated with secondary antibody for 1 h at room temperature before imaging. The second antibody (Goat anti-Rabbit IgG, Alexa Flour 488) (Thermo Fisher Scientific, Inc., IL, USA) was diluted to 1:1000 and images were captured using Nikon TE-2000 digital microscope (Nikon Instruments Inc., NY, USA) with Hamamatsu C4742–80-12AG camera (Hamamatsu Photonics K.K., Hamamatsu, Japan).

Intestinal organoids

Ex vivo organoid cultures were derived from small intestine of C57BL/6 mice and grown in IntestiCult organoid growth medium for 3 days (StemCell Technologies, Cambridge, MA), as previously reported [15]. Organoids were exposed to LPS and hypoxia for 48 h to induce injury and the active form of vitamin D, 1,25-$(OH)_2D_3$ was used as a protective agent. Gene expression of inflammation marker TNFα was measured and immunofluorescence staining was performed as described above. Organoids were fixed, permeabilized and blocked. Organoids were then incubated overnight at

4 °C with primary antibody ZO-1 (dilution 1:50) and incubated with an Alexa Fluor-conjugated secondary antibody (Invitrogen, Carlsbad, CA, USA) and nuclear counterstain DAPI (Vector Laboratories) (dilution 1:100) at room temperature for 2 h.

Statistical analysis

All experiments were performed in triplicates. Data are presented as mean ± SEM, as data were normally distributed (Kolmogorov–Smirnov test). The data were compared using one-way ANOVA with a Bonferroni post-hoc test. Results were considered significant if $p < 0.05$.

Results

Vitamin D increased cell viability and decreased intestinal epithelial cell inflammation

To assess IEC-18 cell viability, MTT assay was used following administration of LPS (200 µg/mL), either with or without vitamin D. There was a decrease in cell viability in the LPS group compared to control (Control: 0.69 ± 0.03; LPS: 0.51 ± 0.03; $p < 0.0001$). Administration of vitamin D increased cell survival back to control level (LPS + Vit D: 0.60 ± 0.02; $p < 0.04$) (Fig. 1a). Exposure of LPS induced intestinal inflammation, as indicated by increased gene expression of pro-inflammation cytokines *TNFα* (Fig. 1b) and *IL-8* (Fig. 1c). However, *TNFα* and *IL-8* expression significantly decreased in vitamin D treated group.

Intestinal permeability and tight junctions were restored by vitamin D

There was an increase in the permeability of FD4 in IEC-18 cells treated with LPS compared to control (Control: 20.89 ± 1.9; LPS: 32.56 ± 2.0; $p < 0.003$) (Fig. 2a). Administration of vitamin D reduced the flux of FD4 similar to control cells (LPS + Vit D: 19.11 ± 1.9; $p < 0.001$) (Fig. 2a). Immunofluorescent images taken with fluorescent microscope revealed that LPS-treated cells showed disrupted tight junction protein ZO-1 (indicated with white arrows) and up-regulated pore-forming protein claudin 2 which were prevented by administration of vitamin D (Fig. 2b).

Organoid inflammation and tight junction disruption were prevented by vitamin D

Intestinal organoids treated with LPS and hypoxia led to increase in inflammation as shown by the increase in the expression of inflammation marker *TNFα* and vitamin D restored this change (Fig. 3a). Similarly, tight junction protein ZO-1 was altered in the injured intestinal organoids induced by LPS and hypoxia (indicated with white arrows), and this change was recovered with the addition of vitamin D (Fig. 3b).

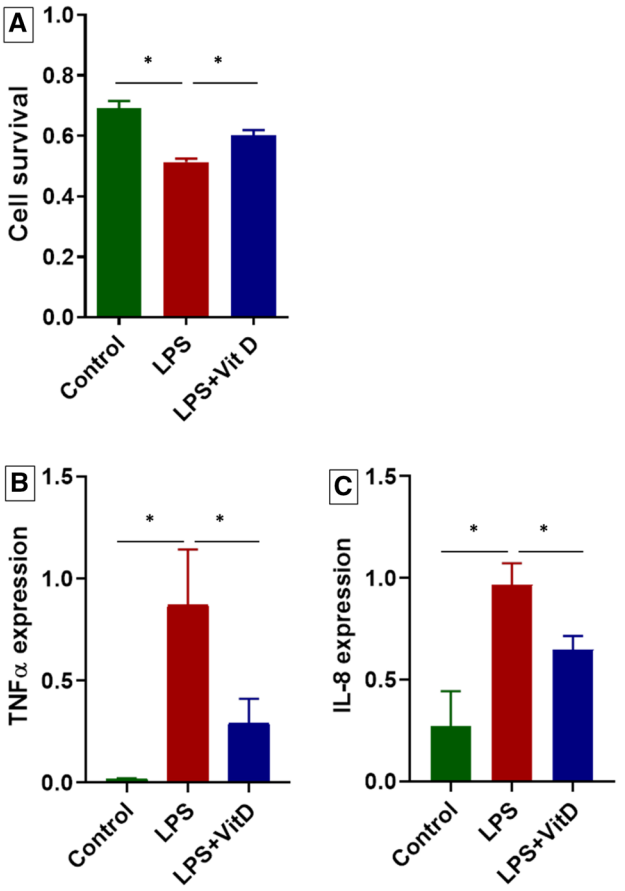

Fig. 1 Vitamin D increased cell viability and decreased intestinal epithelial cell inflammation. **a** Cell viability MTT assay was used to assess cell viability of rat IEC-18 cells following administration of LPS (200 µg/mL) and vitamin D 10 µM). **b**, **c** Gene expression of inflammation markers TNFα and IL-8 in IEC-18 cells Real-time polymerase chain reaction for inflammation markers TNFα and IL-8 in control IEC-18 cells and IEC-18 cells subjected to LPS and LPS + Vit D. Data are presented as means ± SEM from three independent experiments and differences between mean values were assessed by one-way ANOVA. $p < 0.05$ was considered significant

Discussion

This study investigates the effects of the 1,25-$(OH)_2D_3$, the active form of vitamin D, in intestinal epithelial cell IEC-18 and intestinal organoids. Our results show that LPS-induced injury in IEC-18 cells were recovered by administration of vitamin D as shown by the increased cell viability, decreased inflammation markers TNFα and IL-8, decreased permeability of FD4, and restored tight junction proteins

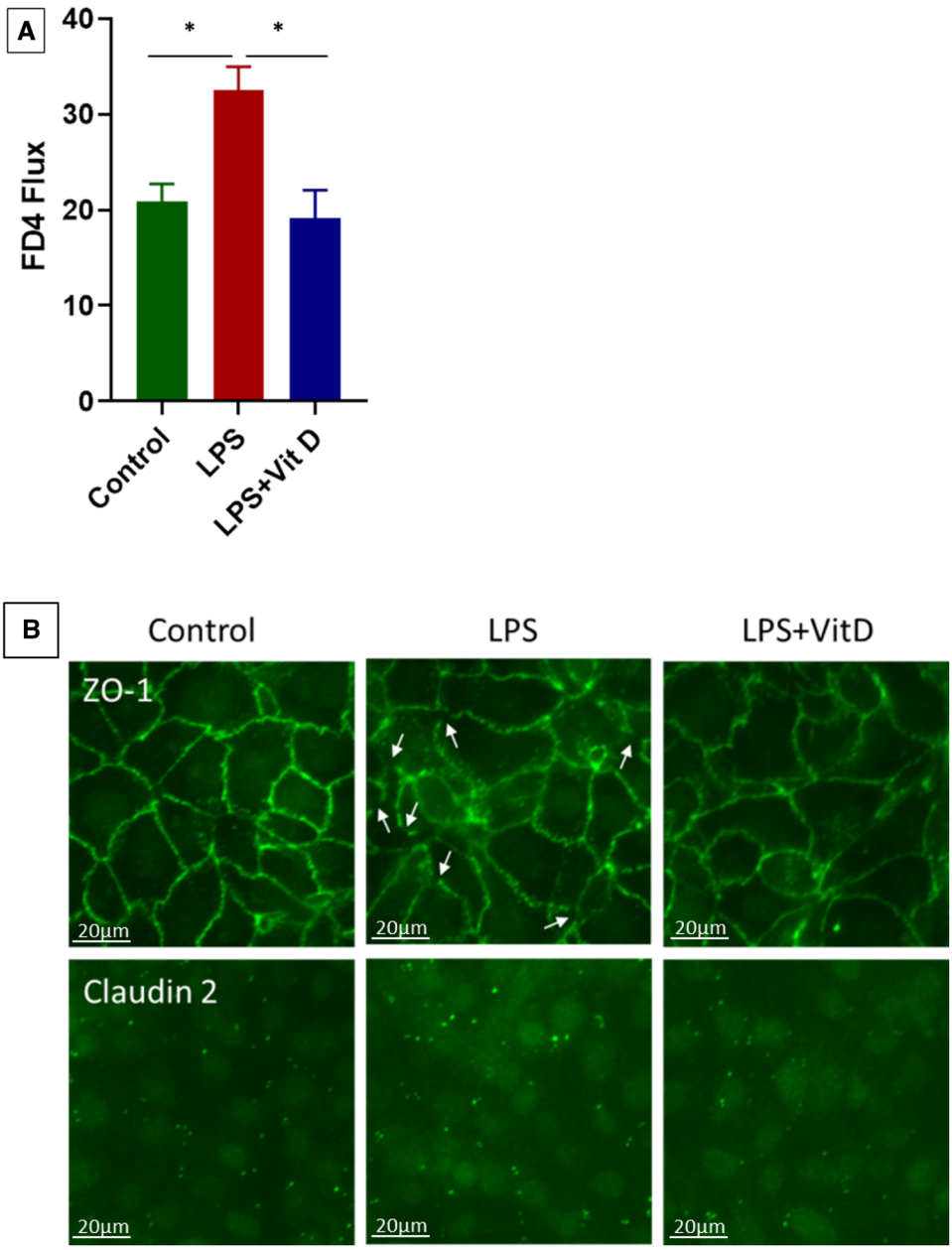

Fig. 2 Intestinal permeability and tight junctions were restored by vitamin D. **a** Permeability of FD4 effects of vitamin D on intestinal permeability in LPS-induced IEC-18 cells. Intestinal permeability was determined based on FD4 flux, measured by fluorescence spectrophotometry. **b** IEC-18 cells tight junction proteins expression Immunofluorescence staining micrographs of tight junction ZO-1 and pore-forming protein claudin 2 in IEC-18 cells. ZO-1 protein was disrupted in LPS-treated cells as indicated by white arrows. Data are presented as means ± SEM from three independent experiments and differences between mean values were assessed by one-way ANOVA. $p < 0.05$ was considered significant

ZO-1 and claudin 2. These findings were also confirmed in injured intestinal organoids as the administration of vitamin D decreased inflammation and restored to normal level the tight junction ZO-1 expression.

In patients with sepsis [16], intestinal obstruction [17], NEC [18, 19] and other inflammatory intestinal diseases, the level of LPS was elevated. LPS is an endotoxin found in the outer membrane of Gram-negative bacteria. It stimulates the release of inflammatory cytokines in various cell types, leading to an acute inflammatory response. In this study, LPS was used to induce intestinal epithelial cell injury which was confirmed by cell viability and inflammation markers TNFα and IL-8. Previous studies have shown that LPS can induce intestinal barrier dysfunction [20, 21], leading to the development of various intestinal disorders such as celiac disease [22], inflammatory bowel disease, colitis-associated cancer [23], sepsis [24] and NEC [25]. Understanding how different factors that regulate gut permeability including diet [26], prebiotics/probiotics [27, 28] and vitamins [29, 30] is essential for disease prevention and therapy.

Vitamin D is known to be protective by reducing intestinal epithelial barrier. FD-40 flux and trans-epithelial electrical resistance, measurements of tight junction dynamics, were increased in LPS-induced injury in Caco-2 monolayers. Administration of 1,25-$(OH)_2D_3$ abolished the effects of LPS by restoring the expression and localization of tight junction

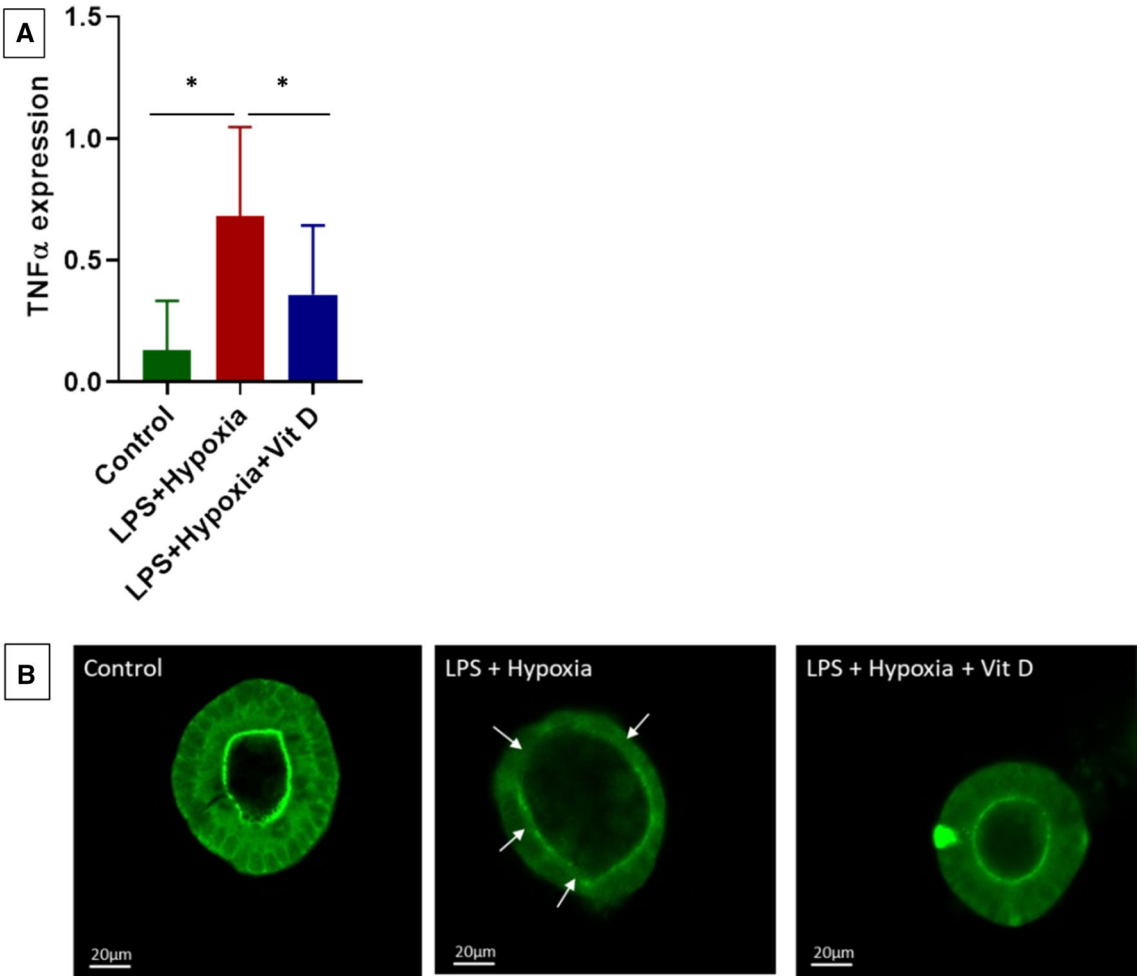

Fig.3 Organoid inflammation and tight junction disruption were prevented by vitamin D. **a** Gene expression of inflammation marker TNFα in intestinal organoids Real-time polymerase chain reaction for inflammation markers TNFα in control intestinal organoids and organoids subjected to LPS + Hypoxia and LPS + Hypoxia + Vit D. **b** Immunofluorescence staining micrographs of tight junction protein in intestinal organoids Immunofluorescence staining micrographs of tight junction ZO-1. ZO-1 protein formed a distinct ring in the control organoid and it was disrupted in LPS and hypoxia-treated organoids as indicated by white arrows. Organoids treated with vitamin D showed similar ring structure as control organoids. Experiments were independently repeated three times. $p < 0.05$ was considered significant

proteins [31]. Vitamin D has been shown to have protective effects against different models of injuries. Vitamin D has protective effects against pepsin–trypsin-resistant gliadin-induced tight junction injuries both in vitro and in vivo [32]. In a mice model of toluene diisocyanate-induced asthma, vitamin D decreased airway hyper-responsiveness, suppressed neutrophil and eosinophil infiltration, as well as an increased in E-cadherin and ZO-1 expression [33]. Sayeed et al. examined the role of vitamin D in a rat model of stroke. The effect of vitamin D on the brain was investigated in animals subjected to stroke by transient middle cerebral artery. 72 h after the stroke, the tight junction proteins occludin and claudin 5 in the brain decreased significantly in the vitamin D deficient group compared to vitamin D sufficient group [34]. Other studies showed that vitamin D promotes intestinal Ca2 + absorption via the up-regulation of tight junction proteins in enterocytes and Caco-2 cell cultures [35, 36]. Conversely, vitamin D deficiency promotes epithelial barrier dysfunction and intestinal inflammation as shown in mice fed with vitamin D-deficient diet challenged with *Citrobacter rodentium* demonstrated increased colonic hyperplasia and epithelial barrier dysfunction which resulted in an altered composition of the fecal microbiome [9]. In line with previous studies, here we have demonstrated that vitamin D improved intestinal epithelial permeability in IEC-18 through the restoration of tight junction ZO-1 and the down-regulation of the expression of pore forming claudin 2. Similar results were observed with decrease inflammation and the restoration of tight junction proteins by vitamin D in intestinal organoids.

Conclusion

We conclude that vitamin D is important in the maintenance of intestinal permeability. Vitamin D attenuates the effects of LPS by decreasing inflammation and restoring intestinal epithelial cells barrier function at cellular level. This experimental evidence supports the protective effects of vitamin D in intestinal injury. Further studies are required to evaluate in vivo the effects of vitamin D in various disease processes.

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Author contributions CL: Conception and design, Collection and/or assembly of data, Data analysis and interpretation, Manuscript writing, Final approval of manuscript. EL, SC, RF, BL, HZ, MY: Collection and/or assembly of data, Data analysis and interpretation, Final approval of manuscript. AP: Conception and design, Financial support, Final approval of manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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