

Original Research Article

Investigating the role of vitamin D in reducing norovirus load and diarrhea severity in mice

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Abstract

Purpose: To examine the potential of vitamin D supplementation in reducing norovirus replication and alleviating diarrhea severity in a murine model.

Methods: This study involved three groups of mice ($n = 7$ mice/group): Group K (-), Group K (+) and Group P. Mice in the K (+) and P groups were intraperitoneally inoculated with 1 mL of Human Norovirus (HuNoV) suspension, containing approximately 10^5 genome copies per mL. Thereafter, mice in the treatment group received oral vitamin D3 once daily for five consecutive days, at a dose of 0.75 $\mu\text{g/kg}$ body weight. Diarrhea severity was assessed daily starting from day 1 post-infection using the paper towel method by scoring fecal moisture and consistency. On day 8 post-infection, mice were sacrificed using an overdose of anaesthetic agents and colonic fecal samples were subsequently collected for RT-qPCR analysis to quantify the viral load.

Results: Mice receiving vitamin D3 supplementation exhibited a significant reduction in viral load compared to the untreated group ($p = 0.000$). Additionally, treatment groups showed improved fecal consistency, indicating a protective effect on intestinal tissue.

Conclusion: Vitamin D3 administration reduces norovirus viral load and mitigates diarrhea severity in BALB/c mice infected with HuNoV. Therefore, vitamin D3 could be used as an adjunctive therapy for viral gastroenteritis.

Keywords: Cholecalciferol, Diarrhea, Mice, Norovirus, Viral load

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INTRODUCTION

Infectious diarrheal disease has been an unresolved urgent public health challenge worldwide. It is fatal to young children and the elderly, as they are more likely to develop serious health complications due to their weak

immune system. The global prevalence of diarrhea reached 1.8 billion cases per year, with case distribution mainly concentrated in developing countries, where medical resources and health infrastructure are underdeveloped [1]. A 2017 report revealed that approximately 533,768 children under the age of five died from

diarrhea worldwide, with a mortality rate of 78.4 per 100,000 children [2]. Besides the high morbidity rates, infectious diarrhea impacts children's growth and development [3,4].

Norovirus has been extensively discussed among researchers in recent years for its significant impact on public health, particularly as the most common cause of acute gastroenteritis worldwide. Norovirus is highly contagious and could lead to outbreaks in crowded settings such as schools. In 2018, 4.2 million children suffered from Norovirus diarrhea in the USA [5]. In Indonesia, an epidemiological study revealed that from 2015 to 2019, the prevalence rate of norovirus accounted for 12.3 % of diarrheal cases and making it the second leading cause of acute gastroenteritis after rotavirus [6]. This situation demands comprehensive treatment and preventive measures against norovirus infection in global setting. Therefore, it is necessary to determine specific and expedited pathways of disease transmission [7]. In addition to a clean and healthy lifestyle, nutritional adequacy also contributes to the prevention of the disease and the reduction of its prevalence. Vitamin D deficiency has been a major factor contributing to the increase in the incidence and frequency of diarrhea in vulnerable populations. Vitamin D maintains overall health and also the immune system [8]. Deficiency of vitamin D3, in particular, is known as a risk factor for diarrhea, especially in children [9]. In this study, the effects of vitamin D supplementation on diarrhea intensity and viral titers associated with diarrhea from norovirus infection were examined.

METHODS

Design

Three groups of seven (7) mice per group were used in this study. Group K (-) served as the negative control group and was neither injected with human norovirus (HuNoV) nor given any intervention. On the other hand, Group K (+) served as the infected control group (mice infected with HuNoV but without vitamin D3). The treatment group (P) was also infected with HuNoV and subsequently administered oral vitamin D3 at a dosage of 0.75 µg/kg body weight once daily over five days. This design allowed for a comparative analysis of the effects of vitamin D3 on HuNoV infection across the experimental groups.

Animal and ethical clearance

A total of 21 BALB/c strain male mice (15 – 20 g; 2 – 3 months old), provided by the Pharmacology

Laboratory of Universitas Airlangga, were used as the test animals. All animals were clinically healthy, with no visible physical abnormalities. Mice were maintained in polypropylene cages with stable environmental conditions (± 29.3 °C; 12:12 h light cycle). Animals were provided with a standard laboratory diet and water *ad libitum*. All treatments given to mice in this study were approved by the UNUSA Medical Research Ethics Committee (approval no. 0207/EC/KEPK/UNUSA/2022).

Preparation and administration of HuNoV suspension

Human norovirus suspension was prepared as previously reported [10]. A total of 25 mL of fecal samples contaminated with HuNoV genogroup II (HuNoV GII) strain were obtained from the Tropical Diseases Center, Universitas Airlangga, Indonesia. The concentration of viral particles was determined through quantitative Polymerase Chain Reaction (qPCR) analysis, resulting in a significant titer of 10^5 genome copies per mL. For further analysis, fecal samples were dissolved with 10 % phosphate-buffered saline (PBS) solution and centrifuged at $15,000 \times g$ to ensure thorough homogenization and even distribution. Subsequently, the resulting viral suspension was carefully stored in Eppendorf tubes and placed in a -80 °C freezer to maintain the integrity of the sample. Finally, the entire viral suspension was transferred to a designated laboratory for storage in preparation for subsequent experimental procedures.

Prior to infection, all mice were tested for Norovirus infection using QuickNavi™-Noro2 (Denka Co., Ltd, Japan) as rapid diagnostic test (RDT) to ensure negative infection and each mouse was placed in separate treatment cage. Inoculation of HuNoV suspension was adapted from a previously established protocol [10]. The volume and viral doses were meticulously calculated to align with the experimental design and minimize distress to the animal. Norovirus was administered intraperitoneally to the posterior portion of the abdomen with 1 mL of HuNoV suspension, containing approximately 10^5 genome copies per mL in all experimental groups. This route was selected to ensure efficient viral delivery and systemic distribution while minimizing variability in infection outcomes. After injection, the mice were closely observed for any indications of distress or negative reactions. All procedures performed in this study adhered to both institutional and national guidelines for animal care and use. A follow-up examination was performed 48 hours post-

infection (hpi) to see the HuNoV infection in the K (+) and P groups.

Vitamin D preparation and administration

All mice from the P group received commercial vitamin D3 supplementation (L-Vit D3 400 IU, PT. Lapi Laboratories, Indonesia) at a dose of 0.75 µg/kg body weight per day (≈30 IU/kg) for five days. The supplementation began 48 hpi to evaluate the therapeutic effect of vitamin D3 after viral exposure. The required concentration was calculated based on the intended dosage per body weight of the mice. Liquid vitamin D3 (0.00896 mL) was dissolved in 0.5 mL of olive oil to form a carrier that enhanced absorption. Before each use, the mixture was gently stirred to maintain its consistency and then administered to the mice by gavage.

RT-qPCR examination

The RT-qPCR procedure was carried out according to standard protocols described in prior studies [10]. Fecal specimens were collected from each experimental group at predetermined intervals and promptly frozen at -80 °C to ensure the integrity of viral RNA. Samples were subsequently homogenized in phosphate-buffered saline (PBS) and centrifuged at 5,000 g for 10 min to remove debris. Viral RNA was isolated using the QIAamp Viral RNA Mini Kit (Qiagen, Germany) following the manufacturer's procedures and the purified RNA was then analyzed using a one-step RT-qPCR with specific primers and probes targeting the HuNoV ORF1-ORF2 junction of the HuNoV genome (Table 1). COG2F and COG2R primers were used for amplification, while the RING-TP probe was employed for sequence-specific detection of the viral genome.

Diarrhea degree severity assessment

The examination protocol was conducted according to a validated protocol described in earlier studies [11]. Each mouse was placed in a cage with the bottom of the cage replaced with accordion wire and tissue placed on the bottom of the wire as a base to obtain clear images of the shape and consistency of the mouse feces. The parameters used to classify diarrhea severity

were systematically divided into four distinct classifications, each based on measurable characteristics of fecal consistency: wet fecal, pasty fecal, semi-watery fecal or watery diarrhea.

Fecal extraction

On the last day of the study (day 8), all mice were anesthetized by intraperitoneal injection of 0.05 mL of ketamine. The procedure was conducted via a mid-sagittal incision in the lower and middle abdomen that reached the top of the abdomen. Collateral organs were separated from their attachments, and then the ileum was separated proximally along 2 – 3 cm from the ileocolic junction. Fecal samples were gathered from the descending segment and cross-section of the colon [10]. The ileum and collateral tissues were placed in Eppendorf bottles filled with 10 % formalin solution, while the colon tissues were placed in bottles filled with Dulbecco's Modified Eagle Medium (DMEM) solution. All the collected tissues were stored at -80 °C.

Statistical analysis

Statistical analysis was performed using the standard function of GraphPad Prism software version 9 (GraphPad Software, San Diego, CA, USA). Comparisons were only made between the K (+) group (infected control) and P group (vitamin D3-treated group) using two-way ANOVA. A *p*-value < 0.05 was considered statistically significant.

RESULTS

Confirmation of HuNoV infection/viral load

The results indicate that both the K(+) and P groups tested positive for HuNoV. To confirm these findings, RT-qPCR analysis was performed, revealing an increase in the viral load (Table 2). Following successful infection, vitamin D3 intervention was administered at 0.75 µg/kg body weight per day for five consecutive days. On day 8, the mice were euthanized, and colon samples were extracted for further analysis. Overall, a summary of RDT and RT-qPCR results is provided in Table 2.

Table 1: Sequences of primers and probe used for detection of HuNoV genogroup II (GII) via RT-qPCR

Gene group	Primer of probe	Sequence (5'→3') ^a	Polarity ^b	Location
GII	Primer COG2F	CARGARBCNATGTTYAGRTGGATGAG	+	5003
	Primer COG2R	TCGACGCCATCTTCATTCACA	-	5100
	Primer RING-TP	FAM-TGGGAGGGCGATCGCAATCT-TAMRA	+	5048

^aSequence shown in the 5'–3' orientation; ^bPolarity indicates the sense (+) or antisense (–) strand targeted.

Table 2: Rapid diagnostic test (RDT) and RT-qPCR outcomes for Norovirus detection across experimental groups at 48 hpi and day 8

Group	Sample ID	48 hpi			Day 8		
		RDT	RT-qPCR		RDT	RT-qPCR	
			Results	Viral load		Results	Viral load
Positive control group	K+1	+	+	10^5	+	+	10^6
	K+2	+	+	10^5	+	+	10^7
	K+3	+	+	10^5	+	+	10^6
	K+4	+	+	10^5	+	+	10^7
	K+5	+	+	10^5	+	+	10^6
	K+6	+	+	10^5	+	+	10^7
	K+7	+	+	10^7	-	-	-
Treatment group	P1.1	+	+	10^7	-	-	ND
	P1.2	+	+	10^6	-	-	ND
	P1.3	+	+	10^7	-	-	ND
	P1.4	+	+	10^7	-	-	ND
	P1.5	+	+	10^6	-	-	ND
	P1.6	+	+	10^6	-	-	ND
	P1.7	+	+	10^7	-	-	ND

The viral load in mice was determined at 48 hours post-infection (hpi) and Day 8 to evaluate the antiviral efficacy of the treatment. As shown in Figure 1, statistical analysis using a mixed-effects model demonstrated a highly significant interaction between treatment group and time point ($p < 0.0001$), indicating that the change in viral load over time differed significantly between groups. At 48 hpi, the treatment group (P) exhibited a slightly higher mean viral load (6.57 ± 0.53) compared to the positive control group (K+) (5.29 ± 0.75). However, by Day 8, the viral load in the treatment group was completely undetectable (0.00 ± 0.00), while that in the control group remained high (6.50 ± 0.55), demonstrating a pronounced divergence in viral dynamics between the groups.

The main effect of time was statistically significant ($p < 0.0001$), suggesting a substantial change in viral load from 48 hpi to Day 8. Additionally, the main effect of treatment group was also significant ($p < 0.0001$), indicating a clear difference in overall viral load between the treated and control animals. These results collectively suggest that the administered treatment effectively suppressed viral replication, leading to complete viral clearance by Day 8 post-infection in the treated group, whereas the control group maintained high viral titers throughout the study period.

Severity of diarrhea in infected mice

The severity of diarrhea in mice was monitored over 8 days and is summarized in Figure 2. A mixed effects model with two-way ANOVA was employed to assess the influence of treatment group, time and their interaction on diarrhea severity scores. The analysis revealed a statistically significant interaction between time

and subject ($p < 0.0001$), indicating that progression of diarrhea severity over time differed significantly between groups. A significant main effect of time was also observed ($p = 0.0087$), suggesting that diarrhea severity changed significantly across the observation days regardless of the group.

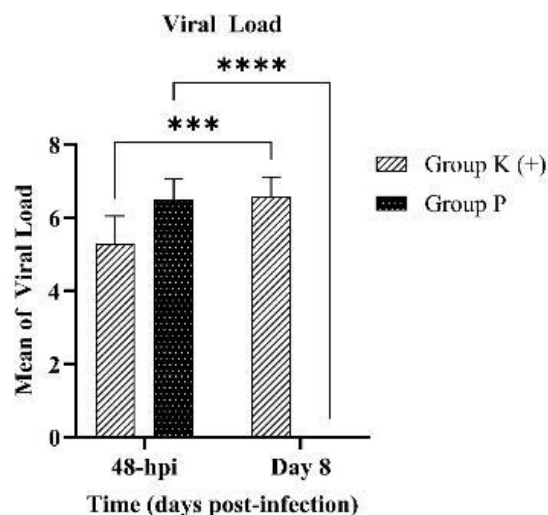


Figure 1: Comparison of viral load in mice between the control group (K+) and treatment group (P) at 48 hpi and Day 8. Data are presented as mean $\pm$ SD. *** $p < 0.0005$; **** $p < 0.0001$ vs control

However, the main effect of treatment group was not statistically significant ($p = 0.1132$), indicating that, when averaged over time, there was no significant overall difference in diarrhea severity between the control (K+) and treatment groups. Descriptive data indicated that the K(+) group showed higher median diarrhea severity ($1.87 \mu\text{M}/\text{mg}$) compared to the P group ($1.37 \mu\text{M}/\text{mg}$). As shown in Figure 1, diarrhea scores in the K(+) group continued to increase toward the end of

the observation period, whereas scores in the P group peaked around day 5 and then declined steadily until day 8 (Figure 2). These findings suggest that while the overall severity between groups was not significantly different, the temporal pattern of diarrhea progression was substantially altered by the treatment, with the treated group showing an earlier resolution of symptoms (Table 3).

DISCUSSION

The global burden of many health challenges shows an uptrend, with digestive system disorders, such as diarrhea, remaining prevalent, particularly in densely populated areas. Human norovirus is classified as a positive-sense, single-stranded, non-enveloped virus belonging to the Caliciviridae family. This virus is the most common cause of acute gastroenteritis cases worldwide, particularly affecting children [12]. In response, therapies and preventive measures are being developed to mitigate its severity and transmission. Vitamin D3 has emerged as a

potential immunomodulatory agent, demonstrating antiviral properties and enhancing digestive immunity.

This study investigated the effects of vitamin D3 on HuNoV infection using 21 mice, 14 of which were infected and confirmed via RDT Norovirus Kit and RT-qPCR. Analysis revealed that vitamin D3 supplementation significantly reduced HuNoV viral titers. The K(+) group exhibited a higher viral titer, indicating higher viral replication and shedding compared to the vitamin D3-treated group. Immunomodulatory effects of vitamin D3 were evident in this study, as RT-qPCR results demonstrated a reduced viral load in the vitamin D3-treated group compared to the untreated infected group (K+). These findings suggest that vitamin D3 may suppress norovirus replication and reduce fecal viral shedding. These observations are consistent with those reported in previous studies, where vitamin D3, through AMP activation via cathelicidin (LL-37), exhibited antiviral activity against HSV-1, significantly reducing viral titers at specific doses [13].

Table 3: Results of observations on the severity of diarrhea in mice based on minimum, maximum, and median values of scoring during the experimental period

Group	N	Min (μM/mg)	Max (μM/mg)	Median (μM/mg)	*P-value
K (+)	6	1.25	2.25	1.87	<0.0001 ^x
P	7	1.25	1.50	1.37	0.0087 ^y
					0.1132 ^z

*Statistical analysis was conducted using a mixed effects model with two-way ANOVA. The *p*-values are annotated as follows: ^x indicates a significant interaction between time and subject ($p < 0.0001$), ^y indicates a significant main effect of time, and ^z indicates a non-significant main effect of group.

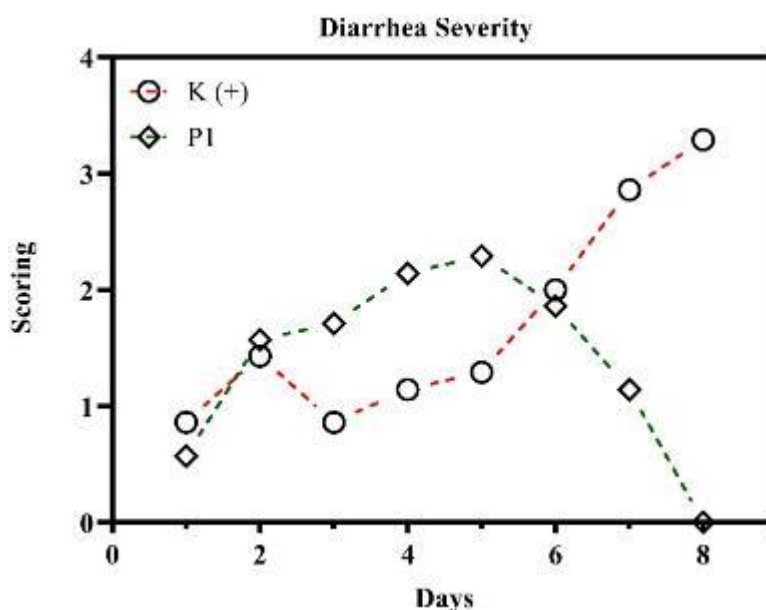


Figure 2: Severity of diarrhea observed in mice over 8 days. Data are presented as mean severity scores per group per day. The red dashed line represents the positive control group (K+), and the green dashed line represents the treatment group (P1)

Another study confirmed that vitamin D deficiency impairs intestinal barrier function, reducing colonic mucosal thickness and increasing pro-inflammatory cytokines, which may heighten susceptibility to diarrhea-causing infections [14]. The severity assessments also indicated that diarrhea improved in the P group from days 5 to 6 post-infection, while the K(+) group experienced severe diarrhea until day 8. This observation is also consistent with results reported in previous studies, which showed that active vitamin D3 supplementation increased expression of antimicrobial molecules and suppressed excessive inflammation in the intestines of BALB/c mice with *Salmonella* colitis [15].

Norovirus infection disrupts gut barrier function and triggers inflammation, leading to diarrhea and intestinal damage [16]. The notable decrease in norovirus titers in mouse feces indicates that vitamin D3 could potentially demonstrate antiviral effects by interfering with viral replication in the gastrointestinal tract. Previous studies have highlighted vitamin D3's essential role in immune function, particularly its antiviral and anti-inflammatory properties. Vitamin D enhances innate immunity by stimulating pattern recognition receptors (PRRs), antimicrobial peptides and cytokines [17]. Its antibacterial and antiviral mechanisms involve the conversion of 25(OH)D3 to $1\alpha,25(\text{OH})_2\text{D}_3$ during infection, which activates antimicrobial responses, including cathelicidins and defensins [18]. Cathelicidin, an antimicrobial peptide, is induced by vitamin D3 via direct stimulation of cathelicidin (cAMP) gene expression in immune cells. Defensins, another group of antimicrobial peptides, function as innate effectors and immune modulators in microbial defense [19]. By reducing viral replication and shedding, vitamin D3 may help limit norovirus persistence and severity in the gut.

Limitations of this study

The observed reduction in norovirus titers in mouse feces suggests that vitamin D3 exerts an antiviral effect by interfering with viral replication in the gastrointestinal tract. However, this Study has several limitations. Notably, a mechanistic analysis was not conducted to elucidate the specific pathways through which vitamin D3 mediates its antiviral effects. Histopathological analysis of intestinal tissue, which could have provided further insights into the protective role of vitamin D3 in maintaining gut integrity during norovirus infection, was not performed. Given its safety and efficacy, vitamin D3 supplementation, particularly for children and the elderly, may be a

viable preventive and treatment strategy for norovirus-induced diarrhea. However, clinical trials are needed to establish its effectiveness in managing norovirus infections.

Future studies should include dose-dependent studies, extended observation periods and additional immune response biomarkers to comprehensively assess vitamin D3's therapeutic potential against norovirus. Molecular analyses, such as evaluating viral RNA levels in intestinal tissues and assessing immune markers, are necessary to confirm its antiviral mechanism. Further investigations into immune regulation and intestinal barrier maintenance will deepen an understanding of vitamin D3's efficacy in mitigating norovirus infection.

CONCLUSION

Vitamin D3 administration reduces HuNoV viral load, alleviates disease severity, contributes to intestinal wall recovery and reduces inflammation. These findings highlight the potential of vitamin D3 as an adjuvant therapy for viral diarrhea.

DECLARATIONS

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Ethical approval

All procedures involving animals in this study were reviewed and approved by the Animal Ethics Committee of Universitas Nahdlatul Ulama Surabaya (no. 0207/EC/KEPK/UNUSA/2022), in accordance with the Indonesian national guidelines on the ethical use of animals.

Use of Artificial intelligence/Large language models

We also declare that we did not use Generative artificial intelligence (AI) and AI-assisted technologies in writing the manuscript.

Availability of data and materials

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

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the author(s) named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. All authors contributed equally to the conception, design, data collection, analysis, and interpretation of the study. They were all involved in drafting and revising the manuscript critically for important intellectual content and approved the final version for submission. Each author agrees to be accountable for all aspects of the work.

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