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Short communication
Σύντομη ανακοίνωση

Effect of 1α,25-dihydroxyvitamin D_3 supplementation on peripheral blood polynuclear cells in Japanese Black cattle stimulated with lipopolysaccharide

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ABSTRACT: In recent years, use of the antimicrobial agents has been restricted in livestock animals due to the concern of increasing antibiotic-resistant bacteria. The peripheral blood polymorphonuclear cells (PMNs), most of which are neutrophils, are important innate immune cells that are the first line of defense against pathogens. Many recent papers have reported that vitamin D plays an important role in regulating innate immune responses in humans. Thus, this study investigated the effects of vitamin D on PMNs in cattle. Blood samples were collected from ten Japanese Black cattle for the isolation of PMNs. These PMNs were divided into two groups: one group was cultured with 1α, 25-dihydroxyvitamin D_3 (vitamin D group) and the other without 1α, 25-dihydroxyvitamin D_3 (control group). These groups were further divided into two groups by with or without lipopolysaccharide (LPS) stimulation. The PMN viability in the vitamin D group was significantly higher than that in the control group with LPS stimulation ($p < 0.05$). The gene expression of lingual antimicrobial peptide (LAP) and bovine beta-defensin 3 (DEFB3) in the vitamin D group were significantly higher than those in the control group with LPS stimulation, respectively ($p < 0.01$, $p < 0.01$). The gene expression of nitric oxide synthase 2 (NOS2) in the vitamin D group was significantly higher than that in the control group with LPS stimulation ($p < 0.01$). These results suggested that 1α, 25-dihydroxyvitamin D_3 supplementation to PMNs from cattle might upregulate several innate immunities.

Keyword: Antimicrobial peptide; innate immunity; Japanese Black cattle; peripheral blood polymorphonuclear cells; vitamin D

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INTRODUCTION

Vitamin D is an essential nutrient classified as one of fat-soluble vitamins, and is critical for normal development and growth of cattle (Eder and Grundmann, 2022). Vitamin D can be obtained through dietary intake and ultraviolet radiation (UVR) exposure (Kulling et al., 2017). UVR catalyzes the conversion to pre-vitamin D_3 in the skin, which further undergoes modification to vitamin D_3 (Kulling et al., 2017). Vitamin D_3 is transported through the bloodstream to the liver through interaction with the vitamin D binding protein. The 25-hydroxyvitamin D_3 (25(OH)D_3) is synthesized from vitamin D_3 in the liver. After that, the 25(OH)D_3 is transported to the kidney and converts to the 1α, 25-dihydroxyvitamin D_3 (1,25(OH)$_2$D$_3$), which is active form of the vitamin D (Kulling et al., 2017). In recent years, many reports have shown that vitamin D plays an important role in the regulation of innate immunity in human (Almeida Moreira Leal et al., 2020; Colotta et al., 2017; Khoo et al., 2011). In particular, 1,25(OH)$_2$D$_3$ supplementation to cells increased the gene expression of antimicrobial peptides (Agraz-Cibrian et al., 2019; Lowry et al., 2014; Wang et al., 2004). Antimicrobial peptides, which are formed from a series of several dozen amino acids, are produced in almost all cells in the body and are one of the innate immune systems that functions to protect the body from pathogens (Pazgier et al., 2006).

Japanese Black cattle is a domestic breed that accounts approximately 95% of the beef cattle in Japan (Gotoh et al., 2009). In general, antimicrobial agents are used to treat infectious deceases in cattle. However, using the antimicrobial agents are restricted in livestock animals due to the concern of increasing antibiotic-resistant bacteria (Schrijver et al., 2018). Therefore, measures to prevent infectious diseases in cattle have greater importance.

Regarding the relationship between the vitamin D and immunity in cattle, oral administration of vitamin D increased IgA concentrations in serum after vaccination (Reinhardt et al., 1999), and intramammary administration of vitamin D activated innate immunity of somatic cells in milk. (Merriman et al., 2017). Therefore, supplementing vitamin D in cattle might be one of the measures to reduce the use of antimicrobial agents by enhancing immunity and preventing infectious diseases.

The peripheral blood polymorphonuclear cells (PMNs), most of which are neutrophils, are important innate immune cells that are the first line of defense against pathogens (Kantari et al., 2008; Singh et al., 2017).

However, to the best of the authors' knowledge, the effect of vitamin D on PMNs has not been investigated in cattle. This study investigated the effect of 1,25(OH)$_2$D$_3$ on PMNs in Japanese Black cattle.

MATERIALS AND METHODS

Sampling

This study used ten Japanese black cattle between 28 and 32 weeks of age. The cattle were raised on a farm in Kagoshima Prefecture, Japan. All the cattle received the feeds, which met the nutritional criteria of the Japanese beef cattle feeding standard (National Agriculture and Food Research Organization, 2008). All cattle were clinically healthy at sampling times and had not received any medical treatment. The experimental procedures were reviewed and approved by the Kagoshima University of Veterinary Medicine Ethics Committee on Veterinary Medical Clinical Research (Approval number: KVM220004). Blood samples were collected from the jugular vein into heparin containing tubes.

PMNs isolation and culture

PMNs were isolated as previously described (Agraz-Cibrian et al., 2019; Higuchi et al., 2013). Briefly, heparinized blood was diluted two-fold in phosphate-buffered saline (PBS), placed above the separation medium solution (Lymphocyte Separation Medium 1077, Immuno-Biological Laboratories, Fujioka, Japan; specific gravity 1.077), and subsequently centrifuged at 500 × g for 30 min at 15 °C. After removing the mononuclear cells, PMNs treated with 0.83% ammonium chloride and gently agitated for 2 min to eliminate contaminating erythrocytes. The isolated cells were suspended in RPMI 1640 medium (Invitrogen, Tokyo, Japan) and were confirmed to be polymorphonuclear cells by May-Grunwald-Giemsa staining. Each PMN samples isolated from 10 cattle, were divided into four groups. The PMNs were cultured with 10 nM 1,25(OH)$_2$D$_3$ (Fujifilm Wako, Osaka, Japan) (vitamin D group) or without 1,25(OH)$_2$D$_3$ (control group) and both groups were further treated with or without 1 μg/mL lipopolysaccharide (LPS; *Escherichia coli* O111, Sigma Aldrich, St. Louis, MO, USA) at 37 °C for 4 h in a humidified 5% CO_2 atmosphere. The concentration of 1,25(OH)$_2$D$_3$ was determined according to the previous studies that examined 1,25(OH)$_2$D$_3$ supplementation to bovine peripheral blood mononuclear cells (Oyamada et al., 2023; Waters et al., 2003; Wherry et al., 2022). The concentration of LPS was determined according to the previous studies in which human or bovine peripheral blood cells were treated with LPS (Leelarungrayub et al., 2019; Otomaru et al., 2022; Oyamada et al., 2023).

The cell viability

Examination of cell viability in culture was conducted following previous reports (De Abreu Costa et al., 2017; Yang et al., 1995). Briefly, 5×10^5 cells/mL in RPMI 1640 medium with 10% heat-inactivated fetal bovine serum (FBS) were each seeded into 48-well plates (Greiner Bio-One, Tokyo, Japan). After that, the PMNs were incubated at 37 °C in a humidified 5% CO_2 atmosphere, and live cells were counted with trypan blue (Nacalai Tesque, Kyoto, Japan) after 4 h incubation. The cell viability was calculated by dividing the number of live cells after 4 h by the number of 0 h (i.e. prior to incubation).

The mRNA gene expression of antimicrobial peptides and nitric oxide synthase

To investigate the gene expression in PMNs, PMN samples obtained from each cattle were divided into four groups, and 2.5×10^6 cells/mL were incubated as mentioned above. After 4 h incubation, the cells were harvested and suspended in Trizol (Thermo Fisher Scientific, Tokyo, Japan) for RNA extraction. Quantitative real-time PCR (qRT-PCR) was performed with THUNDERBIRD Next SYBR qPCR Mix (TOYOBO, Osaka, Japan) after reverse-transcribed with ReverTra Ace qPCR RT Master mix (TOYOBO) as previously described (Oyamada et al., 2023). The primer sets used in the analysis were listed in Table 1 (Oyamada et al., 2023; Merriman et al., 2015; Yamane et al., 2008). After comparing the expression of internal control candidate genes, such as β2-microglobulin (B2M) and β-actin (ACTB), B2M with least variation between the groups was selected for internal control. Relative gene expression was calculated by the ΔΔCt method.

Statistical analysis

The normality of the data was confirmed using the Kolmogorov-Smirnov test. When the data followed a normal distribution, the data were expressed as means ± deviation (SD), and Student's *T*-test was performed for comparisons between the groups. When the data did not follow a normal distribution, the data were expressed as medians ± SD, and Mann-Whitney U test was performed for compar-

Table 1. Primer sets for the gene expression of antimicrobial peptides and nitric oxide synthase 2 in this study

Gene	Accession number	Forward	Reverse	Reference
β2-microglobulin (B2M)	NM_173893	AAGGATCAGTACAGCTGCCG	TGGACATGTAGCACCCAAGG	Oyamada *et al.*, 2023
β-actin (ACTB)	NM_173979	GCAGGAGTACGATGAGTCCG	TGTCACCTTCACCGTTCCAG	Oyamada *et al.*, 2023
Lingual antimicrobial peptide (LAP)	NM_203435	ACAGCATGAGGCTCCATCAC	ACCTGATCGGCACACAGATG	Merriman *et al.*, 2015
Bovine beta-defensin 3 (DEFB3)	NM_001282581	CTCCTCGCACTCCTCTTCCT	GCATCTTCGCCTTCTCTACCACGA	Merriman *et al.*, 2015
Nitric oxide synthase 2 (NOS2)	NM_001076799	TCACACAGCTGTGCATCGAC	TTCCATGGGCACCTCGAGAA	Yamane *et al.*, 2008

isons between the groups. *P* values less than 0.05 were considered statistically significant. All statistical analyses were performed with EZR (Kanda, 2013).

RESULTS

Regarding the cell viability, vitamin D group was significantly higher than that in the control group with LPS stimulation ($p = 0.041$) (Figure 1). Regarding the gene expression, the lingual antimicrobial peptide (LAP) in the vitamin D group was significantly higher than that in the control group with LPS stimulation ($p = 0.004$) (Figure 2). The bovine beta-defensin 3 (DEFB3) in the vitamin D group was significantly higher than that in the control group with LPS stimulation ($p = 0.045$) (Figure 3). The nitric oxide synthase 2 (NOS2) in the vitamin D group was significantly higher than that in the control group with LPS stimulation ($p = 0.002$) (Figure 4). This study did not perform statistical sample size calculations. However, a sample size of ten cattle each group gave post hoc power analysis of 0.32, 0.89, 0.34 and 0.65 to detect differences for the cell viability, the gene expressions of LAP, DEFB3 and NOS2 under LPS stimulation with a two-sided significance level of $p < 0{\cdot}05$ between the control group and vitamin D group.

DISCUSSION

In this study, LPS was used as an immunostimulant. LPS is a key component of the outer membrane in Gram-negative bacteria and is widely used for *in vitro* experiments. (Otomaru et al., 2022). The LPS is recognized by toll-like receptor 4 (TLR4) on the cell surface and stimulates immune cells (Adams et al., 2009).

Vitamin D binds to vitamin D receptors on each cell and exerts multiple functions in the cells, including anti-inflammatory effects and activating the transcription of antimicrobial peptides (Dai et al., 2019; Daryabor et al., 2023; Quesada-Gomez et al., 2022). In particular, it has been reported that vitamin D promotes the activity of nuclear factor-erythroid 2-related factor 2 (Nrf2) (Dai et al., 2019; Yu et al., 2022). Nrf2 is a transcription factor, which activity induces anti-inflammatory effects in cells, and inhibits caspase activity in cells (Kaspar et al., 2009; Mo et al., 2014). Apoptosis is a complex pathway induced by various factors, and caspase is one of the key enzymes involved in the apoptosis cascade (Favaloro et al., 2012). It has been reported that $1{,}25(OH)_2D_3$ supplementation to prostate epithelial cells or corneal epithelial cells improve the cell viability (Bao et al., 2008; Dai et al., 2019). In our study, the cell viabil-

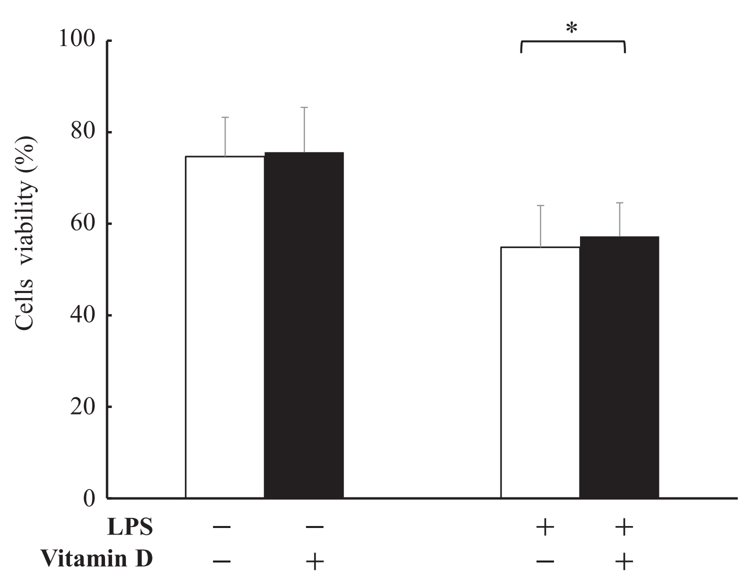

Figure 1. The effect of $1{,}25(OH)_2D_3$ on the cell viability of PMNs. PMNs were treated with $1{,}25(OH)_2D_3$ (10 nM) or without $1{,}25(OH)_2D_3$ under non-stimulated or LPS (1 μg/mL) stimulated conditions for 4 h. The cell viability was calculated by dividing the number of cells after 4 h by that of 0 h. 1α,25-dihydroxyvitamin D_3 supplementation group (dark bars), control group (empty bars). Data are presented as mean ± SD. The asterisk indicates a significant difference between the groups (*: $p < 0.05$).

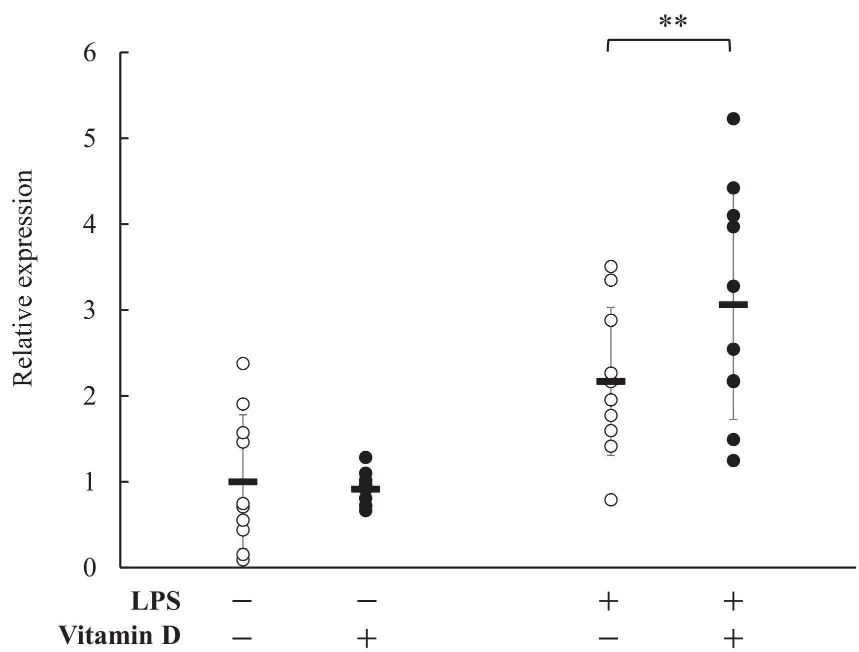

Figure 2. The effect of $1,25(OH)_2D_3$ on the gene expression of lingual antimicrobial peptide (LAP) in PMNs. PMNs were stimulated by LPS (1 μg/mL) with or without $1,25(OH)_2D_3$ (10 nM) for 4 h. Relative gene expression of LAP was determined by qRT-PCR. 1α,25-dihydroxyvitamin D_3 supplementation group (dark circle), control group (empty circle). Data are presented as mean ± SD. The asterisk indicates a significant difference between the groups (**: $p < 0.01$).

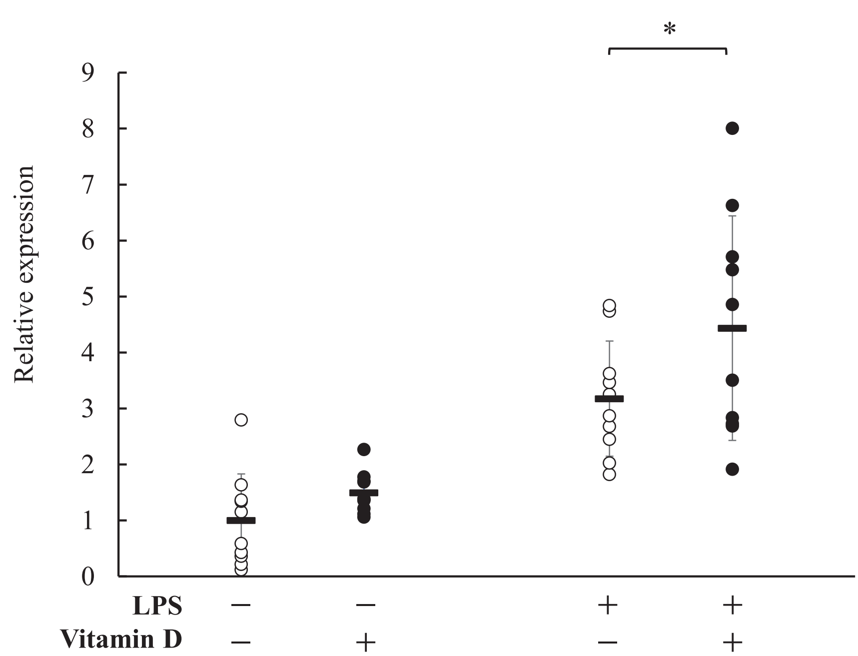

Figure 3. The effect of $1,25(OH)_2D_3$ on the gene expression of beta-defensin 3 (DEFB3) in PMNs. PMNs were stimulated by LPS (1 μg/mL) with or without $1,25(OH)_2D_3$ (10 nM) for 4 h. Relative gene expression of DEFB3 was determined by qRT-PCR. 1α,25-dihydroxyvitamin D_3 supplementation group (dark circle), control group (empty circle). Data are presented as mean ± SD. The asterisk indicates a significant difference between the groups (*: $p < 0.05$).

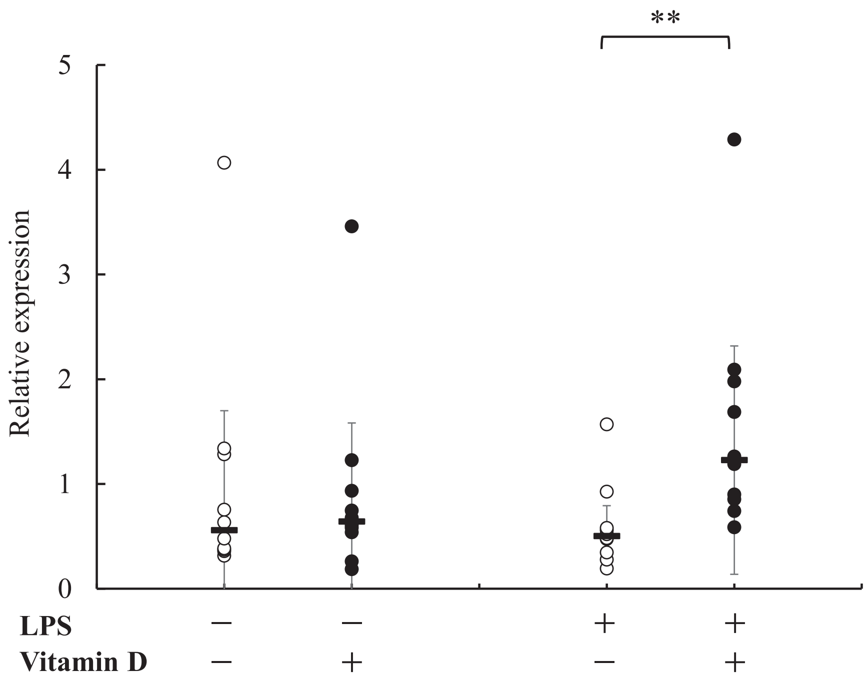

Figure 4. The effect of 1,25(OH)$_2$D$_3$ on the gene expression of nitric oxide synthase 2 (NOS2) in PMNs. PMNs were stimulated by LPS (1 μg/mL) with or without 1,25(OH)$_2$D$_3$ (10 nM) for 4 h. Relative gene expression of NOS2 was determined by qRT-PCR. 1α,25-dihydroxyvitamin D$_3$ supplementation group (dark circle), control group (empty circle). Data are presented as median ± SD. The asterisk indicates a significant difference between the groups (**: $p < 0.01$).

ity in the vitamin D group was significantly higher than that in the control group with LPS stimulation. Therefore, 1,25(OH)$_2$D$_3$ might have improved the Nrf2 activity and reduced the caspase activity in the cells, thereby affecting PMNs survival.

Antibacterial peptides not only act as antibacterial substances, but also, act as bridges between innate immunity and adaptive immunity by activating cells and inducing the production of chemokines (Meade et al., 2014; Park et al., 2018). In cattle, neutrophils produce several antimicrobial peptides, including LAP and DEFB3 (Merriman et al., 2019; Mackenzie-Dyck et al., 2011; Mackenzie-Dyck et al., 2014; Morimoto et al., 2012; Rieg et al., 2010; Tomasinsig et al., 2010). In human, 1,25(OH)$_2$D$_3$ supplementation to PMNs increased the production of antimicrobial peptides (Agraz-Cibria et al., 2019; Lowry et al., 2014; Wang et al., 2004). In cattle, intramammary administration of 1,25(OH)$_2$D$_3$ promoted the gene expression of antimicrobial peptides in somatic cells in milk *in vivo* study (Merriman et al., 2017), and 1,25(OH)$_2$D$_3$ supplementation to peripheral blood mononuclear cells promoted the gene expression of antimicrobial peptides *in vitro* study (Oyamada et al., 2023).

In our study, the gene expression of LAP and DEFB3 in the vitamin D group were significantly higher than those in the control group with LPS stimulation. Therefore, 1,25(OH)$_2$D$_3$ might upregulate the production of the antibacterial peptides in the PMNs.

Next the impact of 1,25(OH)$_2$D$_3$ on gene expression of the NOS2 in PMNs was examined. The NOS2 gene encodes inducible nitric oxide synthase (iNOS) and generates nitric oxide (NO). The NO is a reactive free radical which acts as a biological mediator in several processes, including antimicrobial activities within phagocytes (Boylan et al., 2020). Previous studies in cattle reported that 1,25(OH)$_2$D$_3$ upregulated the gene expression of NOS2 in salivary gland cells, dermal fibroblasts, somatic cells in milk and peripheral blood mononuclear cells (Boylan et al., 2020; Nelson et al., 2010; O'Brien et al., 2021; Merriman et al., 2017; Oyamada et al., 2023; Wells et al., 2023).

In our study, the gene expression of NOS2 in the vitamin D group was significantly higher than that in the control group with LPS stimulation. Therefore, $1,25(OH)_2D_3$ might upregulate the NO production and enhance the antibacterial activity in the PMNs.

Although this study involved small sample size and had limited number of measurements, careful interpretation of the results suggested that $1,25(OH)_2D_3$ supplementation to PMNs in cattle upregulate the several innate immunities. Therefore, vitamin D might help the health maintenance of cattle and contribute to reducing the use of antibiotics agents in cattle. However, further *in vivo* studies, including sunbathing or oral administration of vitamin D, are needed to clarify the effects of vitamin D on innate immunity in cattle.

CONCLUSIONS

This study demonstrated that $1,25(OH)_2D_3$ supplementation to PMNs in cattle upregulated the gene expression of antimicrobial peptides, nitric oxide synthase and cell viability with LPS stimulation. However, further *in vivo* studies are needed to clarify the effect of vitamin D on innate immunity in PMNs of cattle.

CONFLICT OF INTEREST

The authors declare no conflicts of interest relating to this work.

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