Short communication: Menaquinone-4 (vitamin K₂) induces proliferation responses in bovine peripheral blood mononuclear cells

H. Bai,^{1*} H. Hiura,² Y. Obara,³ M. Kawahara,¹ and M. Takahashi^{1,4}

¹Laboratory of Animal Breeding and Reproduction, Research Faculty of Agriculture, Hokkaido University, Kita-ku Kita 9 Nishi 9, Sapporo 060-8589, Japan

²Department of Bioscience, Tokyo University of Agriculture, Tokyo 156-8502, Japan

³Mito Research Center, Meiji Feed Co., Wakamiya 870, Ibaraki-Cho, Higashi-Ibaraki-Gun, Ibaraki-Prefecture, 311-3123, Japan

⁴Global Station for Food, Land and Water Resources, Global Institution for Collaborative Research and Education, Hokkaido University, Sapporo 060-0815, Japan

ABSTRACT

The effects of vitamin K (VK) on immune cells in ruminants are yet to be fully investigated. The objective of this study was to examine the effects of VK on peripheral blood mononuclear cells (PBMC) in Holstein dairy cows. A cell proliferation assay was performed to evaluate the effect of menaquinone-4 (MK-4, the biologically active form of VK) on immune response of PBMC. The proliferation of PBMC stimulated by MK-4 was significantly higher than that of nonstimulated controls. The expression of T cell-related genes in PBMC, stimulated with MK-4, was assessed by quantitative PCR. No significant changes were observed in the mRNA expression levels of both *CD4* and *CD8* as helper T cell and cytotoxic T cell markers, respectively. The present study demonstrated that MK-4 positively influenced cow PBMC proliferation and suggested the possibility of bovine-specific immune cell activation. The present study lays a foundation for understanding the physiological role of VK in cattle.

Key words: cow, menaquinone-4, peripheral blood mononuclear cells

Short Communication

Vitamin K (VK) is a fat-soluble vitamin and an important factor in blood clotting (Dam and Schønheyder, 1936). The VK family consists of phyloquinones (VK₁ in plants), menaquinones (MK or VK₂, produced by bacteria), and menadione (VK₃, chemically produced). Dietary phyloquinones are endogenously converted to menaquinone-4 (MK-4; Okano et al., 2008; Nakagawa

et al., 2010), which is the dominant form of VK in animal tissues (Thijssen and Drittij-Reijnders, 1994; Yamamoto et al., 1997).

In cattle, VK is synthesized by the rumen microbes to meet dietary requirements. Vitamin K is also found in pasture and green roughages (NRC, 2001). Thus, most studies have been conducted on vitamins A, D, and E due to the frequent occurrences of their deficiency (Weiss, 2017), whereas studies on VK in ruminants are limited. Recently, increasing interest has been expressed in the potential roles of VK besides the regulation of blood clotting. Critical benefits of VK have been demonstrated, including positive effects on bone health, aging-related diseases, cancer, and inflammation (Myneni and Mezey, 2018; Xv et al., 2018; Simes et al., 2019). Several studies have shown the effects of VK on human peripheral blood mononuclear cells (PBMC); VK derivatives attenuated T cell-mediated immunity in activated T cells and increased the CD4+CD25+Foxp3+Treg cell population (Hatanaka et al., 2014). Cattle synthesize a sufficient amount of VK and never present with deficiency symptoms. Nevertheless, understanding the physiological functions of VK in cattle would be informative. To date, no study has investigated the effects of VK on bovine immune cells. Hence, the aim of this study was to assess the effects of VK on bovine PBMC proliferation and T cell-related gene expression.

Animal care, sampling, and PBMC preparation were performed as previously described (Bai et al., 2019). Cows were randomly selected, and no diseases were detected before and during the experiment. All study procedures were conducted in accordance with Hokkaido University guidelines regarding the care and use of animals (approval no. 16-0019). Blood samples were collected in 10-mL tubes containing heparin (Terumo, Tokyo, Japan) from 12 Holstein cows (3–7 yr old, 1–5 parity, 24.8 ± 1.0 kg/d milk yield) through the external jugular vein between 0900 and 1000 h at the Hokkaido

Received November 30, 2019.

Accepted March 23, 2020.

*Corresponding author: hbai@anim.agr.hokudai.ac.jp

University farm. The PBMC were prepared on the same day as sampling. The PBMC were isolated from whole-blood samples using Lymphocyte Separation Medium 1077 (TaKaRa Bio Inc., Shiga, Japan) according to the manufacturer's instructions. Blood samples were diluted with an equal volume of PBS, layered onto a separation medium, and centrifuged at $450 \times g$ for 40 min at room temperature. The erythrocytes were then carefully lysed in NH_4Cl^- base lysis buffer, washed twice with PBS, and suspended in RPMI-1640 medium (Wako Pure Chemical Industries Ltd., Osaka, Japan). The separated viable PBMC were counted using the trypan blue test and seeded on a 96-well plate (1.0×10^4 cells/well) for proliferation assays, or on a 6-well plate (3.0×10^5 cells/well) for RNA extraction. The proportions of live cells among all the examined cells representing cell viability were approximately 90% immediately after separation and 60 to 70% after culturing, respectively.

The MK-4 (VK₂, biologically active form of VK; Wako Pure Chemical Industries Ltd.) was dissolved in 99.5% (vol/vol) ethanol (1 $\mu\text{g}/\text{mL}$, as a stock solution) and diluted with the culture medium. The collected PBMC were cultured in RPMI-1640 medium containing 5% fetal bovine serum and an antibiotic-antimycotic solution (Thermo Fisher Scientific K.K., Yokohama, Japan), with (50 ng/mL) or without (cultured medium only, as the control or 1% ethanol only, as the vehicle control) MK-4. The PBMC were cultured for 72 h under a humidified atmosphere of 5% CO_2 at 38.5°C.

Cell proliferation assays were conducted using Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. The PBMC were treated with CCK-8 reagent (1:10, vol/vol) and incubated for 2 h. To estimate

PBMC proliferation, absorbance at 450 nm wavelength was measured using an auto-microplate reader (ARVO X; PerkinElmer Japan Co., Ltd., Kanagawa, Japan). All assays were performed in triplicate. The stimulated index was calculated as the ratio of the average absorbance of 3 wells containing stimulated cells relative to that of 3 wells containing nonstimulated cells.

The RNA was extracted and reverse-transcribed into cDNA for use in quantitative PCR (qPCR) as previously described (Bai et al., 2019). The RNA quality was verified with an Agilent 2100 Bioanalyzer and the RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA; Supplemental Figure S1, <https://doi.org/10.3168/jds.2019-17987>). All experiments were performed according to the minimum information for publication of quantitative real-time PCR experiments guidelines (Bustin et al., 2009). Target gene expression levels were determined by qPCR using LightCycler 96 (Roche Diagnostics, Basel, Switzerland) and Thunderbird SYBR qPCR Mix (Toyobo, Osaka, Japan). Detailed primer information and thermal cycling conditions are shown in Table 1 and Supplemental Table S1 (<https://doi.org/10.3168/jds.2019-17987>). The relative mRNA abundance was calculated on the basis of the geometric mean of the reference gene expression levels.

The results are presented as means $\pm$ standard error of the mean. Data were analyzed using StatView statistical software (version 5.0, SAS Institute Inc., Cary, NC). One-way ANOVA was followed by a Tukey-Kramer test (post-hoc analysis) for the cell proliferation assay data among 3 groups (control, vehicle, and MK-4, $n = 12$). Student's *t*-test (unpaired, 2 tailed) was performed for comparison of the qPCR data between 2 groups. Changes relative to control with *P*-values < 0.05 were considered statistically significant.

Table 1. Primers for quantitative PCR¹

Name (GenBank accession no.)	Sequence (5'–3')	Product length (bp)	Reference
T cell marker gene			
<i>CD4</i> (NM_001103225)	F: AGCAGAAAGTGAACTCGTGG R: ACCAACTTCGGCTGATTTGAG	148	Wang et al. (2013)
<i>CD8</i> (NM_174015)	F: CGCAGACTAGGTCGGTCTCT R: GTTCCGGCGGTAGCAGAT	176	Allan et al. (2015)
Internal control gene			
<i>ACTB</i> (AY141970)	F: TGGACTTCGAGCAGGAGATG R: GTAGAGGTCCTTGCGGATGT	221	Bai et al. (2019)
<i>GAPDH</i> (NM_001034034)	F: CACCCTCAAGATTGTCAGCA R: GGTCTAAGTCCCTCCACGA	103	
<i>H2AFZ</i> (NM_174809)	F: AGAGCCGGTTTGCAGTTCCCG R: TACTCCAGGATGGCTGCGCTGT	116	

¹F = forward; R = reverse. Cycling conditions: 1 cycle at 95°C for 30 s, followed by 50 cycles at 95°C for 10 s, 60°C for 15 s, and then 72°C for 30 s. Each run was completed with a melting curve analysis to confirm the specificity of amplification and the absence of primer dimer formation. The concentration of RNA and cDNA was assessed using the NanoDrop 2000c spectrophotometer (NanoDrop/ThermoFisher Scientific, Waltham, MA). The RNA quality was checked using the Agilent 2100 Bioanalyzer and the RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA).

Figure 1. Measurement of the proliferation of bovine peripheral blood mononuclear cells in the presence (50 ng/mL) or absence [culture medium only, as control (Cont.), or 1% ethanol, as the vehicle control] of menaquinone-4 (MK-4). Stimulation index (absorbance of stimulated wells/absorbance of nonstimulated wells; the value of non-stimulated cells in each group was considered Cont. = 1.0). Data are presented as mean ± SEM. Asterisks indicate significant differences in absorbance levels: ** $P < 0.01$, 1-way ANOVA with Tukey-Kramer post-hoc test (3 groups: Cont., vehicle, and MK-4, $n = 12$ per group).

The proliferation of the MK-4 treated PBMC significantly increased (1.36 ± 0.11) compared with those of the nonstimulated controls ($P < 0.01$, Figure 1). The MK-4 treatment was not significantly cytotoxic (Supplemental Figure S2, <https://doi.org/10.3168/jds.2019-17987>). To examine whether the T cell population changed with the increase in cell proliferations, the expression of T cell-related genes in PBMC after MK-4 treatment was observed using qPCR (Figure 2). No differences were noted in the mRNA expression of *CD4* ($P = 0.92$) and *CD8* ($P = 0.34$) with the MK-4 treatment, which is a marker of the helper T and cy-

Figure 2. Expression of T cell subset marker genes (*CD4* and *CD8*) in bovine peripheral blood mononuclear cells. Data represent expression levels relative to those of 3 internal control genes (i.e., geometric means of *ACTB*, *GAPDH*, and *H2AFZ*). Data are presented as mean ± SEM. No significant differences were observed after menaquinone-4 (MK-4) treatment; both *CD4* (0.99 ± 0.11 , $P = 0.92$) and *CD8* (0.86 ± 0.16 , $P = 0.34$) expression based on the Student's *t*-test (unpaired, 2 tailed, $n = 12$ per group).

totoxic T cells, respectively. In addition, no difference was observed in the T cell lineage (Th1, Th2, Th17, and Treg cells)-related genes or inflammatory cytokine genes with and without MK-4 treatment (Supplemental Figure S3, <https://doi.org/10.3168/jds.2019-17987>).

The T cell proliferation was reported to be involved in CD4 and CD8 balance in lactating cows (Mehrzaad and Zhao, 2008). In addition, VK₂ was reported to alter the T cell population in mitogen-activated human PBMC (Hatanaka et al., 2014). By contrast, we did not observe a change in the expression of *CD4* and *CD8*, their population, or producing cytokine genes. However, we only assessed the effect of single MK-4 treatment on mRNA expression levels. To fully understand the effect of VK on cow PBMC, combined assays involving both MK-4 and mitogen are required. Furthermore, cell surface protein expression should be measured by flow cytometry.

In conclusion, we found that a single MK-4 treatment positively influenced bovine PBMC proliferation but had no apparent effect on T cell-related gene expression, which is in contrast to previous results in humans (Hatanaka et al., 2014). To the best of our knowledge, this is the first study to examine the effects of VK on bovine PBMC. It was reported that VK-dependent protein S and co-factor-activated protein C of bovines and humans display species-specific activities (He et al., 1995). Although the detailed mechanism of the effect of MK-4 on bovine PBMC remains unclear, a species-specific immune cell activation mechanism may exist. To reveal the mechanism of the immune regulatory functions of VK, further studies are required to examine the differences in T cell populations as well as gene expression differences between cattle and humans.

ACKNOWLEDGMENTS

We are grateful to the members of the Field Science Center for Northern Biosphere Experimental Farm in Hokkaido University for their assistance with animal handling and sampling. We express our gratitude to Kohei Oikawa (Hokkaido University) for his comments and suggestions on our manuscript. This work was supported by Japan Society for the Promotion of Science (JSPS), KAKENHI Grant Number 17K1535707. We thank Editage (www.editage.com) for English language editing. The authors have no conflicts of interest.

REFERENCES

- Allan, A. J., N. D. Sanderson, S. Gubbins, S. A. Ellis, and J. A. Hammond. 2015. Cattle NK cell heterogeneity and the influence of MHC class I. *J. Immunol.* 195:2199–2206. <https://doi.org/10.4049/jimmunol.1500227>.

- Bai, H., T. M. A. Shabur, H. Kunii, T. Itoh, M. Kawahara, and M. Takahashi. 2019. Evaluation of the immune status of peripheral blood monocytes from dairy cows during the periparturition period. *J. Reprod. Dev.* 65:313–318. <https://doi.org/10.1262/jrd.2018-150>.
- Bustin, S. A., V. Benes, J. A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M. W. Pfaffl, G. L. Shipley, J. Vandesompele, and C. T. Wittwer. 2009. The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.* 55:611–622. <https://doi.org/10.1373/clinchem.2008.112797>.
- Dam, H., and F. Schönheyder. 1936. The occurrence and chemical nature of vitamin K. *Biochem. J.* 30:897–901. <https://doi.org/10.1042/bj0300897>.
- Hatanaka, H., H. Ishizawa, Y. Nakamura, H. Tadokoro, S. Tanaka, K. Onda, K. Sugiyama, and T. Hirano. 2014. Effects of vitamin K3 and K5 on proliferation, cytokine production, and regulatory T cell-frequency in human peripheral-blood mononuclear cells. *Life Sci.* 99:61–68. <https://doi.org/10.1016/j.lfs.2014.01.068>.
- He, X., L. Shen, and B. Dahlbäck. 1995. Expression and functional characterization of chimeras between human and bovine vitamin-K-dependent protein-S-defining modules important for the species specificity of the activated protein C cofactor activity. *Eur. J. Biochem.* 227:433–440. <https://doi.org/10.1111/j.1432-1033.1995.tb20406.x>.
- Mehrzhad, J., and X. Zhao. 2008. T lymphocyte proliferative capacity and CD4+/CD8+ ratio in primiparous and pluriparous lactating cows. *J. Dairy Res.* 75:457–465. <https://doi.org/10.1017/S0022029908003439>.
- Myneni, V. D., and E. Mezey. 2018. Immunomodulatory effect of vitamin K2: Implications for bone health. *Oral Dis.* 24:67–71. <https://doi.org/10.1111/odi.12759>.
- Nakagawa, K., Y. Hirota, N. Sawada, N. Yuge, M. Watanabe, Y. Uchino, N. Okuda, Y. Shimomura, Y. Suhara, and T. Okano. 2010. Identification of UBIAD1 as a novel human menaquinone-4 biosynthetic enzyme. *Nature* 468:117–121. <https://doi.org/10.1038/nature09464>.
- NRC. 2001. Nutrient Requirements of Dairy Cattle. 7th rev. ed. Natl. Acad. Press, Washington, DC.
- Okano, T., Y. Shimomura, M. Yamane, Y. Suhara, M. Kamao, M. Sugiura, and K. Nakagawa. 2008. Conversion of phylloquinone (vitamin K1) into menaquinone-4 (vitamin K2) in mice: Two possible routes for menaquinone-4 accumulation in cerebra of mice. *J. Biol. Chem.* 283:11270–11279. <https://doi.org/10.1074/jbc.M702971200>.
- Simes, D. C., C. S. B. Viegas, N. Araújo, and C. Marreiros. 2019. Vitamin K as a powerful micronutrient in aging and age-related diseases: Pros and cons from clinical studies. *Int. J. Mol. Sci.* 20:4150. <https://doi.org/10.3390/ijms20174150>.
- Thijssen, H. H., and M. J. Drittij-Reijnders. 1994. Vitamin K distribution in rat tissues: Dietary phylloquinone is a source of tissue menaquinone-4. *Br. J. Nutr.* 72:415–425. <https://doi.org/10.1079/BJN19940043>.
- Wang, X. S., Y. Zhang, Y. H. He, P. P. Ma, L. J. Fan, Y. C. Wang, Y. I. Zhang, D. X. Sun, S. L. Zhang, C. D. Wang, J. Z. Song, and Y. Yu. 2013. Aberrant promoter methylation of the CD4 gene in peripheral blood cells of mastitic dairy cows. *Genet. Mol. Res.* 12:6228–6239. <https://doi.org/10.4238/2013.December.4.10>.
- Weiss, W. P. 2017. A 100-year review: From ascorbic acid to zinc-mineral and vitamin nutrition of dairy cows. *J. Dairy Sci.* 100:10045–10060. <https://doi.org/10.3168/jds.2017-12935>.
- Xv, F., J. Chen, L. Duan, and S. Li. 2018. Research progress on the anticancer effects of vitamin K2. *Oncol. Lett.* 15:8926–8934. <https://doi.org/10.3892/ol.2018.8502>.
- Yamamoto, R., M. Komai, K. Kojima, Y. Furukawa, and S. Kimura. 1997. Menaquinone-4 accumulation in various tissues after and oral administration of phylloquinone in Wistar rats. *J. Nutr. Sci. Vitaminol. (Tokyo)* 43:133–143. <https://doi.org/10.3177/jnsv.43.133>.

ORCIDS

- H. Bai <https://orcid.org/0000-0001-9940-1880>
H. Hiura <https://orcid.org/0000-0002-1493-2306>
Y. Obara <https://orcid.org/0000-0002-5866-8420>
M. Kawahara <https://orcid.org/0000-0002-7172-6082>
M. Takahashi <https://orcid.org/0000-0002-2448-915X>