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Effects of dietary vitamin K₃ supplementation on vitamin K₁ and K₂ (menaquinone) dynamics in dairy cows

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Running head: Dietary vitamin K₃ for cows

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ABSTRACT

The effect of dietary vitamin K₃ (VK3) on ruminant animals is not fully investigated. The aim of this study was to examine the effects of dietary VK3 on lactation performance, rumen characteristics, and VK1 and menaquinone (MK, or VK2) dynamics in the rumen, plasma, and milk of dairy cows. Eight Holstein dairy cows in late lactation periods were used in two crossover trials including a control (non-treatment), and a 50 or 200 mg/day (d) VK3 supplementation group. After 14 days, plasma, ruminal fluid, and milk were sampled and their VK1 and MKs contents were measured using fluorescence-HPLC. Milk production was unchanged after feeding 50 mg/d VK3, but marginally decreased after feeding 200 mg/d VK3. The molar ratio of propionate in ruminal fluid was significantly increased on feeding 200 mg/d VK3. Additionally, MK-4 concentrations significantly increased in both plasma and milk after VK3 feeding (50 and 200 mg/d). In ruminal fluid, MK-4 concentrations increased after 200 mg/d VK3 feeding. These results suggest that VK3 may be a good source of MK-4, the biologically active form of VK, in Holstein dairy cows during their late lactation periods. This study provides a basis for understanding the physiological role of VK in dairy cows.

Keywords: dairy cow, menaquinone-4, milk production, rumen fermentation, vitamin K₃

1 INTRODUCTION

2
3 Vitamin K (VK), a fat-soluble vitamin, is an important factor for blood
4 coagulation (Dam & Schönheyder, 1936). The VK family consists of phyloquinone
5 (VK1), menaquinones (MKs, or VK2), and menadione (VK3, chemically synthesized or
6 intermediate of MK-4 synthesis). VK1 is found in pasture grasses and green roughages.
7 VK2 refers to a group of MKs, characterized by different side chain lengths. The different
8 forms of MK are represented as MK-n, where “n” indicates the number of isoprenoid
9 residues with the side chain (Walther et al., 2013). MK-4, the most common form of short-
10 chain MK, is found in animal tissues, whereas long-chain MKs (MK-5 to MK-13) are
11 synthesized by bacteria (Walther et al., 2013). Dietary VK1 is endogenously converted to
12 predominantly MK-4 (Okano et al., 2008; Nakagawa et al., 2010), which is the dominant
13 and functional form of VK in animal tissues (Thijssen & Drittij-Reijnders 1994;
14 Yamamoto et al., 1997). The accumulation of MK-4 in tissues suggests that it plays
15 specific physiological roles in the body. In addition to regulating blood coagulation, VKs
16 play roles in a range of other processes, such as bone metabolism, prevention of
17 atherosclerosis, and regulation of cell proliferation, via the VK-dependent Gla protein
18 (Shearer, 1995; Vermeer, 2012; Simes et al., 2019). VK3 is also a good source of VK for
19 bone health in horses (Terachi et al., 2011). Furthermore, we have previously reported
20 various novel functions of VKs, including anti-inflammatory effects (Ohsaki et al., 2006,
21 2010, Saputra et al., 2019), enhancement of testosterone production (Ito et al., 2011),
22 amplification of insulin secretion (Ho et al., 2019), and regulation of bile acid synthesis
23 (Sultana et al., 2018).

24
25 Several studies have examined the role of dietary VK in the gastrointestinal tract
26 of monogastric animals. In humans, dietary VK deficiency alters the composition of the
27 gut microbiome (Ellis et al., 2021). In rats, dietary VK may alter the supply of VK derived
28 from MKs in intestinal bacteria by altering the density of MK-producing bacteria
29 (Mathers et al., 1990). In Japan, VK3 has been prohibited to use for the treatment of the
30 vitamin K-deficient bleeding in human because of its high chemical reactivity which
31 causes various side effects such as jaundice and so on. In rodents and domestic animals,

1 however, VK3 is useful feed ingredient; for example, in chickens it has been reported that
2 VK3 feeding has a great advantage for the tissues' and eggs' MK-4 enrichment which
3 benefits not only animals health condition but also egg quality (O'Sullivan et al., 2020).
4 However, these findings are limited to monogastric animals. In cows, VK2 (MK-n) is
5 synthesized by rumen microbes to meet the dietary requirements. Although extensive
6 studies have been conducted on vitamins A, D, and E, because of their frequent deficiency
7 in dairy cows (Weiss, 2017), comparatively few studies have assessed VK and its
8 functions. Recently, we demonstrated that MK-4 positively influences the activation of
9 peripheral blood mononuclear cells and potentially activates immune functions in dairy
10 cows (Bai et al., 2020). Cows generally produce sufficient amounts of MKs, however, it
11 might be beneficial to increase the amounts of functional VK in cattle for healthier feeding.
12 To the best of our knowledge, this is the first to investigate the effects of dietary VK3
13 supplementation on VK dynamics in ruminant animals, including dairy cows. The aim of
14 this study was to assess the effects of dietary supplementation of VK3 on the lactation
15 performance, ruminal characteristics, and VK1 and MK dynamics in the rumen, plasma,
16 and milk of lactating cows.

MATERIALS AND METHODS

Animals and Diet

All animals were cared for according to the Guide for the Care and Use of Experimental Animals of Hyogo Prefectural Technology Center of Agriculture, Forestry and Fisheries (Hyogo Prefecture, Japan).

Eight Holstein dairy cows in late lactation periods were used in the study. We conducted two crossover trials using VK doses of 50 or 200 mg/day (d). Four cows were subjected to each trial (50 mg/d VK3 feeding; parity: 1–3, days in milk: 196 ± 27 and 200 mg/d VK3 feeding; parity: 2–3, days in milk: 301 ± 13). The cattle were housed in tie-stalls, and were individually fed total mixed rations (TMRs) *ad libitum* (Table. 1). The diet was designed to meet their energy requirements according to the Japanese Feeding Standard for Dairy Cattle 2006 (National Agriculture and Food Research Organization, 2006). Water was provided *ad libitum* throughout the study period. The cattle were fed and milked twice a day at 0830 h and 1530 h, and at 0800 h and 1630 h, respectively. In each trial, the cows were divided into two groups and over a 14-day period, were daily fed two meals of TMR with or without the addition of VK3 in a crossover design. During the VK3 feeding period, 5 or 20 g of menadione sodium hydrogen sulfite (containing 1% VK3; Kohkin Chemical Co., Ltd., Osaka, Japan) was top-dressed into the diet for 14 days. Blood, ruminal fluid, and milk samples were collected on the final day of the feeding period.

Sample collection

Prior to morning feeding (08:00 h), 9 mL of blood samples were collected from the jugular vein using heparin-sodium tubes. Plasma was immediately separated by centrifugation at $1,660 \times g$ for 15 min at 4 °C, and was stored at -30 °C until analysis.

Ruminal fluids were collected orally using an esophageal tube (Lumenar stomach evacuator outfit; Fujihira Industry Co. Ltd., Tokyo Japan) before the morning feeding, and were immediately filtered through two layers of gauze. Each sample was stored at -30 °C until the measurement of ammonia-nitrogen (NH₃-N) concentrations. For volatile fatty acid (VFA) concentration measurement, 5 mL aliquot of ruminal fluid was mixed with 1.0 mL of 3N-H₂SO₄ containing 24% metaphosphoric acid and stored at room

temperature for 18 h. The supernatant was separated following centrifugation at $1,660 \times g$ for 30 min at 4 °C and stored at -30 °C until further use. Total VFA, acetate, propionate, and butyrate were separated and quantified by gas chromatography (GC2014, Shimadzu Corporation, Kyoto, Japan) using a packed glass column [Thermon-3000 (3%)] on a Shimalite TPA 60-80 support (Shinwa Chemical Industries Ltd., Kyoto, Japan).

Milk yield was measured daily using a milk meter (F series; TRU-Test Distributors, Auckland, New Zealand). Average milk yield over the 14 days of experimental period, including the day of blood sampling, was calculated. Milk samples were collected during the morning and afternoon milking times. Milk composition (fat, protein, lactose, and somatic cell count) was calculated from weighted average based on the milk yields obtained from morning and afternoon milking, using Fourier transform infrared spectroscopy (CombiFoss FC; Foss Electric, Denmark).

High-performance liquid chromatography (HPLC) analysis of VK1 and MKs

VK was extracted from the samples collected morning feeding and the amounts of VK1, MK-4, and MK-5 to MK-13 were determined using a fluorescence-HPLC system (Agilent Technologies, Inc., CA, US), as previously described (Sultana et al., 2018).

Statistical analysis

Data were analyzed using the mixed procedure of JMP (Ver16.1.0; SAS Institute Inc., Cary, NC, USA). The presence or absence of VK3 supplemented diet was used as a fixed effect. The herd, animal, and period were used as random effects. Differences were considered statistically significant at $P < 0.05$. Results are expressed as the least-squares means and standard error (SE).

RESULTS

Effects of dietary VK3 on lactation performance

The lactation performance of cows fed 50 and 200 mg/d VK3 is shown in Tables 2 and 3, respectively. Dietary VK3 feeding did not significantly affect milk yield, 4% fat corrected milk (FCM), milk composition, or linear score of somatic cell counts. However, cows fed a diet containing 200 mg/d VK3 caused a marginal decrease in milk yield (25.1 kg) compared with that obtained in the control group (25.6 kg) ($P < 0.05$).

Effects of dietary VK3 on rumen fluid characteristics

Tables 4 and 5 show the effect of dietary VK3 on the ruminal fluid characteristics at 50 and 200 mg/d, respectively. At both supplementary concentrations, no significant changes were observed in rumen pH, concentrations of $\text{NH}_3\text{-N}$, total VFA, or molar ratios of acetate and butyrate. The molar percentage of propionate significantly increased in cows receiving the 200 mg/d VK3 supplemented diet (15.7%) compared with that recorded in control cows (14.7%) ($P < 0.05$).

Effects of dietary VK3 on plasma concentrations of VK1 and MK-4

Among the VKs assessed in the present study, only VK1 and MK-4 were detected in plasma samples. No significant changes in VK1 concentration were detected in the plasma of cows fed diets supplemented with 50 or 200 mg/d VK3 (Figure 1A). In contrast, however, we detected a significant increase in the plasma concentration of MK-4, from 0.21 ng/mL in the control group to 0.33 ng/mL in the VK3 group (1.5-fold) fed the 50 mg/d VK3 supplemented diet ($P < 0.05$) (Figure 1B). Similarly, a significant increase in MK-4 concentration from 0.20 ng/mL in the control group to 0.49 ng/mL in the VK3 group (2.4-fold) was detected in the plasma of cows fed a diet supplemented with 200 mg/d VK3 ($P < 0.05$) (Figure 1B).

Effects of dietary VK3 on milk concentrations of VK1 and MK-4

Similar to plasma, among the assessed VKs, only VK1 and MK-4 were detectable in milk. VK1 concentrations remained unchanged in the milk produced by cows fed diets supplemented with both 50 and 200 mg/d VK3 (Figure 2A). In contrast,

1 the MK-4 concentration significantly increased from 7.09 to 12.82 ng/mL (2.4-fold) in
2 response to dietary supplementation with 50 mg/d VK3 ($P < 0.01$) (Figure 2B).
3 Furthermore, a significant increase in MK-4 concentration from 5.89 to 23.68 ng/mL (4.4-
4 fold) was observed in cows fed a diet supplemented with 200 mg/d VK3 ($P < 0.01$)
5 (Figure 2B).

6 7 *Effects of dietary VK3 on the dynamics of VK1 and MKs in ruminal fluid*

8 The VK1 and MK-n concentrations in the ruminal fluid collected before the
9 morning feeding from cows in the 50 and 200 mg/d of VK3 group are shown in Tables 6
10 and 7, respectively. The VK1 concentration in the ruminal fluid was relatively high,
11 although remained unchanged in cow fed the VK3 supplemented diet. The MK-4
12 concentration in ruminal fluid was relatively lower than those of other MKs. MK-4
13 concentrations remained unchanged in the ruminal fluids of cows in the 50 mg/d VK3
14 feeding group, but significantly increased in cows in the 200 mg/d VK3 feeding group
15 (1.55 ng/mL), compared with that in the control group (0.64 ng/mL) ($P < 0.05$). In
16 contrast to plasma and milk samples, in which only VK1 and MK-4 were detected, a range
17 of other MKs, from MK-5 to MK-13, were detectable in the ruminal fluid. The
18 concentrations of MK-5 to MK-9 remained relatively low, ranging from 2.37 to 29.96
19 ng/mL, those of MK-11 and MK-12 were found to be relatively high in the rumen, ranging
20 from 90.79 to 137.36 ng/mL. The MK-12 concentration increased in response to dietary
21 supplementation with 50 mg/d VK3, but no similar effect was observed when using 200
22 mg/d VK3.

DISCUSSION

In this study, we examined the effects of VK3 feeding for 14 days on the lactation performance, ruminal fermentation, and VK1 and MK dynamics in the rumen, plasma, and milk of Holstein dairy cows in their late lactation periods. To the best of our knowledge, the present study is the first to report the effects of dietary VK3 on the dynamics of menaquinones in the rumen, plasma, and milk in dairy cows, a ruminant animal. Milk yield marginally decreased by feeding 200 mg/d of VK3. However, no significant changes were observed for FCM or other milk components (Tables 2 and 3). The concentrations of MK-4, the functional form of VK, significantly increased in both the plasma and milk of cows fed diets containing 50 and 200 mg/d VK3 (Figure 1, 2). Therefore, a daily supplementation of 50 mg/d VK3 would be sufficient to enhance MK-4 levels in the body fluids of dairy cows in late lactation periods. However, further studies are required to determine accurate amounts of VK3 needed for improving the health of dairy cows.

In the rumen contents, the molar ratio of propionate significantly increased in response to dietary supplementation with 200 mg/d VK3 (Table 5). In this regard, the main component of the growth stimulator produced by *Propionibacterium* is 1,4-dihydroxy-2-naphthoic acid, an intermediate metabolite in MK biosynthesis (Isawa et al., 2002). Therefore, VK3 could activate *Propionibacterium* in the rumen and increase the propionate production.

In this study, the composition of MK-n (MK-4 to MK-13) in the rumen of dairy cows during their late lactation periods was determined. To the best of our knowledge, this is the first study to report rumen concentrations of VK1 and MK-4 to MK-13. The concentrations of MK-4 were relatively low compared to that of other MKs in the rumen fluid. There were no significant changes in the MK-4 levels in the rumen of cows fed a diet supplemented with 50 mg/d VK3 (Table 6). Therefore, the increase in MK-4 concentrations detected in the plasma and milk, in response to dietary VK3 supplementation could be due to the absorption of VK3 from the gastrointestinal tract, and its subsequent predominantly conversion to MK-4 in the mammary glands and other

tissues. Moreover, our detection of a significant increase in the concentration of MK-4 in the ruminal fluid of cows fed a diet containing 200 mg/d VK3 (Table 7) indicates that the conversion of VK3 to MK-4 would occur in body fluids if the level of VK3 supplementation is sufficiently high. Conversely, we observed that the concentration of MK-12 in ruminal fluid increased significantly only in those cows receiving the 50 mg/d VK3 (Table 6). Additionally, the concentrations of long side chain MKs, MK-11, and MK-13, showed an increasing trend. It is suggested that dietary supplementation with 50 mg/d VK3 stimulates rumen bacteria to produce long side chain MKs. Mathers et al. (1990) reported that, dietary modification in rats, may alter the VK supply from intestinal bacteria-derived MKs by changing the density of MK-producing bacteria. Further studies would therefore be desirable to establish the significance of the bacteria involved and the long-chain MKs implicated in this change.

In the biosynthesis of MK-4, the phytyl and prenyl groups of VK1 and VK2 dissociate from the naphthoquinone ring to form VK3, which has no side chain structures. Subsequently, geranylgeranyl diphosphate derived from the mevalonate pathway is covalently bound to VK3 by the prenyltransferase enzyme, *UbiA* prenyltransferase domain-containing 1 (UBIAD1) to predominantly produce MK-4 (Nakagawa et al., 2010, Hirota et al., 2013). UBIAD1 is expressed in several organs, including the mammary glands. Therefore, an increase in the concentration of only MK-4 in the plasma and milk observed in response to dietary supplementation of VK3 could be attributed to the conversion of the absorbed VK3 to MK-4 via UBIAD1. There are no reports regarding the effects of VK3 in ruminants; however, the conversion of VK3 to MK-4 could be similar to that in monogastric animals. In addition, in ruminants, VK3 feeding may not affect the composition of other MKs in plasma and milk. In this study, because we measured VK at only one-time point, it is necessary to analyze the changes in sequential time to better understand the metabolism and absorption dynamics of VK in ruminants.

It has been reported that VKs are converted to MK-4, in multiple organs, including the liver, bone, brain, gonads, and salivary glands (Huber et al., 1999). As the MK-4 concentration in milk significantly increased in response to VK3 feeding, it is clear that there is also an increase in the conversion of VK3 to MK-4 in mammary gland of lactating

1 cows. VK3 is one of the VKs that is readily converted to MK-4, and our observation
2 suggests that the mammary glands may be the primary site of MK-4 conversion,
3 especially in lactating cows. Moreover, MK-4 accumulation in tissues indicates that it
4 plays an important physiological role in the body. Indeed, several findings have indicated
5 that MK-4 may play an immunological role, such as in the anti-inflammatory effects of
6 human macrophage-like cells (Ohsaki et al., 2010). In addition, we have also reported
7 that MK-4 positively influences peripheral blood mononuclear cell proliferation and
8 activates immune functions in dairy cows (Bai et al., 2020).

9 In conclusion, dietary VK3 supplementation increases MK-4, the biologically
10 active form of VK, in the body fluids of dairy cows in their late lactation period. Further
11 studies are needed to determine the appropriate amount of VK3 supplementation and MK-
12 4 concentration in the body for improving the health of dairy cows.

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21 **Conflict of interests:** The authors declare no conflicts of interest for this study.

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Figure legends

Figure 1. Effects of dietary vitamin K₃ (VK3) feeding on vitamin K₁ (VK1) and menaquinone-4 (MK-4) concentrations in plasma.

(A) VK1 and (B) MK-4 concentrations in plasma collected from cows after feeding for 14 days. Cont.: control group. VK3: VK3 feeding group. * $P < 0.05$.

Figure 2. Effects of dietary VK3 feeding on VK1 and MK-4 concentrations in milk.

(A) VK1 and (B) MK-4 concentrations in milk collected from cows after feeding for 14 days. Cont.: control group. VK3: VK3 feeding group. ** $P < 0.01$.

Table 1. Ingredient, chemical compositions, and nutritional contents of total mixed rations fed during the post-parturition period.

Ingredient	(% of dry matter)
Sorghum silage	13.7
Klein grass hay	5.3
Sudan hay	6.5
Alfalfa hay	12.8
Beet pulp	10.2
Flaked corn	26.7
Barley	6.9
Soybean meal	10.4
Cotton seed	3.5
Corn gluten meal	0.4
Heated soybean (Soy plus)	0.3
Energy supplement	1.8
Calcium carbonate	0.2
Calcium secondary phosphate	0.5
Sodium chloride	0.2
Sodium hydrogen carbonate	0.5
Vitamins premix ¹⁾	0.2

Chemical composition and nutritive value ²⁾	
Dry matter (DM) (%)	55.1
Total digestible nutrients (% of DM)	72.8
Crude protein (% of DM)	15.4
Neutral detergent fiber (% of DM)	34.3

¹⁾ Contained 4000 IU/g of vitamin A, 600 IU/g of vitamin D3, and 10 IU of vitamin E.

²⁾ Calculated according to the analytical value of individual ingredient except for minerals.

Table 2. Effect of 50 mg VK3 feeding on lactation performance.

	Cont.	VK3 (50 mg)	SE	<i>P</i> value
Milk yield (kg)	26.4	26.1	2.7	0.364
4% Fat corrected milk (kg)	28.6	27.8	2.5	0.152
Milk fat (%)	4.59	4.48	0.22	0.522
Milk protein (%)	3.86	3.83	0.12	0.388
Lactose (%)	4.31	4.24	0.08	0.127
Linear score	3.00	3.75	1.29	0.312

Results are expressed as the least squares mean.

Table 3. Effect of 200 mg VK3 feeding on lactation performance.

	Cont.	VK3 (200 mg)	SE	<i>P</i> value
Milk yield (kg)	25.6	25.1	1.7	0.005
4% Fat corrected milk (kg)	28.1	27.3	1.1	0.210
Milk fat (%)	4.69	4.63	0.25	0.576
Milk protein (%)	3.53	3.58	0.16	0.365
Lactose (%)	4.34	4.30	0.09	0.402
Linear score	3.75	3.50	0.40	0.363

Results are expressed as the least squares mean.

Table 4. Effect of 50 mg VK3 feeding on ruminal characteristics.

	Cont.	VK3 (50 mg)	SE	<i>P</i> value
pH	7.12	7.05	0.06	0.079
NH ₃ -N (mg/dL)	13.3	13.5	1.2	0.651
Total VFA (mol/L)	7.97	7.87	0.62	0.637
Acetate (%)	67.5	67.7	0.4	0.430
Propionate (%)	16.5	17.4	0.6	0.327
Butyrate (%)	13.0	11.9	0.6	0.209

Results are expressed as the least squares mean.

Table 5. Effect of 200 mg VK3 feeding on ruminal characteristics.

	Cont.	VK3 (200 mg)	SE	<i>P</i> value
pH	7.40	7.30	0.07	0.222
NH ₃ -N (mg/dL)	12.4	12.1	1.7	0.835
Total VFA (mol/L)	9.25	8.32	1.41	0.410
Acetate (%)	68.1	69.9	2.1	0.360
Propionate (%)	14.7	15.7	0.3	0.029
Butyrate (%)	12.7	11.70	1.2	0.154

Results are expressed as the least squares mean.

Table 6. Effect of 50 mg VK3 feeding on levels of ruminal phyloquinone (VK1) and menaquinones (MKs)

	VK1	MK-4	MK-5	MK-6	MK-7	MK-8	MK-9	MK-10	MK-11	MK-12	MK-13 (ng/mL)
Cont.	163.11	1.26	2.51	5.91	24.45	2.37	6.77	53.32	90.79	95.17	40.98
VK3 (50 mg)	140.68	2.11	2.33	5.26	19.31	4.36	6.73	47.04	76.41	83.35	32.94
SE	49.26	0.92	0.28	1.00	6.38	1.26	1.32	8.87	16.29	15.97	7.27
<i>P</i> value	0.457	0.260	0.345	0.208	0.369	0.291	0.940	0.165	0.084	0.017	0.087

Results are expressed as the least squares mean.

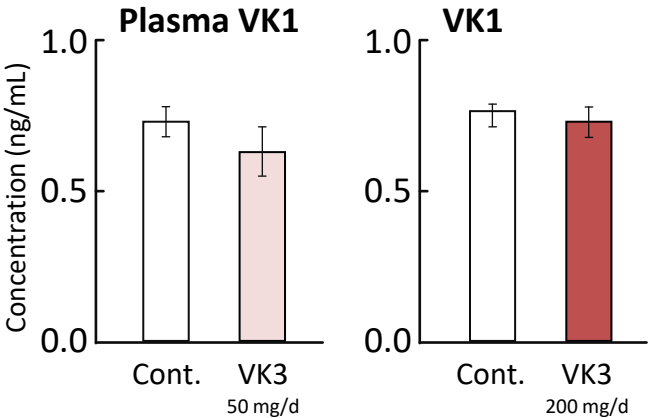
Table 7. Effect of 200 mg VK3 feeding on levels of ruminal VK1 and MKs

	VK1	MK-4	MK-5	MK-6	MK-7	MK-8	MK-9	MK-10	MK-11	MK-12	MK-13 (ng/mL)
Cont.	128.27	0.64	4.74	6.40	29.96	7.06	9.85	65.18	119.62	137.36	53.21
VK3 (200 mg)	163.93	1.55	5.88	6.21	33.03	5.61	11.57	74.63	117.70	139.13	60.04
SE	55.25	0.28	1.79	1.26	3.82	0.88	3.15	11.53	22.75	30.81	11.88
<i>P</i> value	0.277	0.032	0.293	0.833	0.508	0.290	0.518	0.334	0.901	0.917	0.220

Results are expressed as the least squares mean.

Figure 1.

A



B

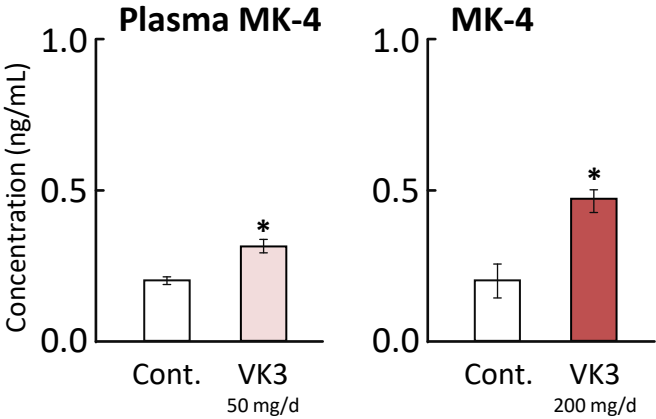
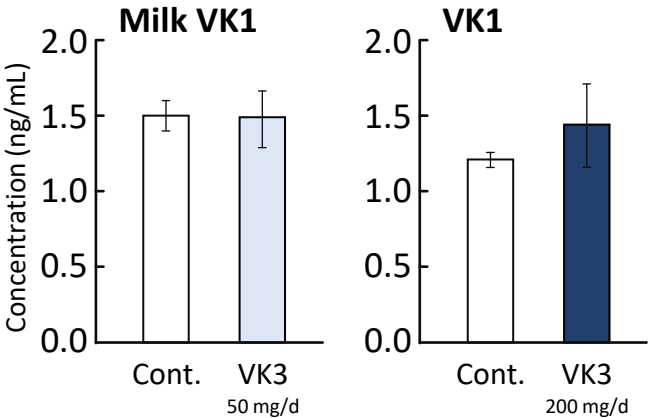


Figure 2.

A



B

