ABSTRACT: The effect of several vitamin K homologs on plasma vitamin K concentration was determined to assess their potential as a vitamin K supplement for adult horses. Sixteen Thoroughbred horses consisting of 8 mares and 8 geldings, aged 8.4 ± 3.6 yr and weighing 520.8 ± 36.1 kg, were allocated to 4 groups (n = 4). Each group was given phylloquinone, menaquinone-4, or menadione at 58 µmol/d, or no vitamin K supplement for 7 d. Plasma samples were collected before feeding, and 2, 4, and 8 h after feeding on d 7, and plasma concentrations of phylloquinone and menaquinone-4 were determined. Plasma phylloquinone concentration was greater in the phylloquinone group than in the other groups (P < 0.001). The phylloquinone concentration quadratically increased (P < 0.001) after feeding in the phylloquinone group but no changes in the plasma phylloquinone concentration were observed after feeding in the other groups. Plasma menaquinone-4 concentration was greater (P < 0.001) in the menadione group than the other groups, including the menaquinone-4 group. Menaquinone-4 concentration did not change (P = 0.192) after feeding in each group. Menaquinone-4 has been considered the most potent vitamin K homolog for bone metabolism; therefore, the present experiment indicates that menadione is a good source of vitamin K for bone health in horses because it is the only vitamin K homolog that increased the plasma concentrations of menaquinone-4.

Key words: horse, menadione, menaquinone-4, phylloquinone, plasma, vitamin K

INTRODUCTION

Vitamin K consists of phylloquinone, menaquinones, and menadione. Vitamin K deficiency induced by warfarin suppresses blood coagulation and decreases bone mass and bone strength in rats (Simon et al., 2002). Craciun et al. (1998) suggested that dietary phylloquinone increased bone formation and decreased bone resorption in long-distance runners. The results of cell culture studies indicated that menaquinone-4 was far more active than phylloquinone and menadione in stimulating bone formation by human osteoblasts (Koshihara et al., 1996) and in suppressing bone resorption by mouse osteoclasts (Takeuchi et al., 2000). These reports indicated that menaquinone-4 is the most potent vitamin K for regulating bone metabolism in humans and rodents. Although there are no direct reports indicating its effects on bone strength, it is assumed that vitamin K has similar effects in horses.

Phylloquinone in forage, along with menaquinones synthesized by intestinal microbes, is assumed to meet the requirement of horses (NRC, 2007). However, the risk of vitamin K deficiency in horses has been also suggested (Siciliano et al., 2000b; Rich and Breuer, 2002). Phylloquinone and menaquinone-4 are often used as vitamin K supplements for humans (Cornelissen et al., 1997; Knapen et al., 2007), and menadione is the major supplement for pigs (NRC, 1998) and poultry (NRC, 1994). The circulating concentration of vitamin K homologs has been used determined to assess their absorption (Schurgers et al., 2007). Our preliminary study indicated that menadione supplementation effectively increased plasma menaquinone-4 concentration in horses (Inoue et al., 2009). In the present study, the effect of several vitamin K homologs on plasma vitamin K concentration was determined to assess their potential as a vitamin K supplement for adult horses.
MATERIALS AND METHODS

The experimental protocols for the study were reviewed and approved by the Ethics Committee for Laboratory Animals of the Japan Racing Association, Equine Research Institute.

Experimental Animals and Diet

Eight mares and 8 geldings (Thoroughbred), aged 8.4 ± 3.6 yr and weighing 520.8 ± 36.1 kg, were used in the present study. All horses were housed in a single barn with each horse occupying a single stall from 1500 to 1200 h and were in a dry lot for spontaneous exercise from 1200 to 1500 h. The horses were allocated to 4 groups of 2 mares and 2 geldings with similar average BW. The horses were individually given 11 kg of diet/d (Table 1). The phylloquinone concentration was 2.71, 0.04, 0.49, 18.80, and 0.09 µmol/kg of timothy hay, oat grain, commercial concentrate, alfalfa hay cube, and wheat bran, respectively. Thus, the dietary phylloquinone concentration was 3.25 µmol/kg. The diet was offered as 2 equal meals at 0800 and 1700 h. After a 7-d adaptation period, each group was given 58 µmol/d of phylloquinone, menaquinone-4, or menadione (Wako Chemicals, Osaka, Japan) for 7 d. The vitamin K homologs (2.9 µmol/g) were mixed with wheat bran as the supplements, which were stored at −20°C under nitrogen gas. Each supplement was replaced and mixed with the same weight of dietary wheat bran just before feeding. The control group was not given any vitamin K supplement. Our preliminary study indicated that plasma menaquinone-4 concentration was increased by menadione supplementation at a similar dose as the present experiment and reached a plateau by 1 wk of supplementation in horses (Inoue et al., 2009), but a half-dose did not affect the plasma menaquinone-4 concentration (Y. Inoue and T. Matsui, unpublished data).

Blood Collection and Plasma Analyses

Blood samples were collected just before the morning feeding (0800 h), and 2, 4, and 8 h after the morning feeding on d 7 by puncturing a jugular vein and collecting blood into a 10-mL sodium heparin-treated tube (VP-H070K, Terumo, Tokyo, Japan). Blood samples were also collected just before the initiation of vitamin K supplementation (0800 h) on d 1. Plasma samples were harvested by centrifugation (2,500 × g for 30 min at 4°C) and stored at −20°C under nitrogen gas until analyzed. The plasma concentrations of phylloquinone and menaquinone-4 were determined by the method of Kamao et al. (2005), with slight modification. Briefly, plasma samples were diluted with distilled water. Ethyl alcohol was added, and then the vitamin K homologs were extracted by n-hexane. Vitamin K homologs were partly purified by a silica cartridge (Sep-Pak Plus Sili-

| Table 1. Composition of experimental diet (as-fed basis) |
|-----------------|-----------------|
| Item            | Content         |
| Ingredient, g/kg|                 |
| Timothy hay     | 546.9           |
| Oat grain       | 227.9           |
| Commercial supplement | 109.4         |
| Alfalfa hay cube| 91.2            |
| Wheat bran      | 22.8            |
| Sodium chloride | 0.9             |
| Calcium carbonate| 0.9            |
| Composition     |                 |
| DE,2 Mcal/kg    | 2.29            |
| CP,2 %          | 10.5            |
| Phylloquinone,3 µmol/kg | 3.25       |

1Power Up Horse II (Keiba Shiryo, Tokyo, Japan; DE, 2.6 Mcal/kg; CP, 14.0%).
2Calculated value.
3Analyzed value.

ca, Waters Japan, Tokyo, Japan). The eluent was evaporated and dissolved in ethyl alcohol. The HPLC system consisted of a solvent delivery module (LC-10AD, Shimadzu, Kyoto, Japan) and a reversed-phase column (250 × 4.6 mm, 5 µm, Capcel Pak UG120, Shiseido, Tokyo, Japan). The eluted vitamin K homologs from the HPLC column were reduced by a platinum column (RC-10, Shiseido) and then determined by a fluorescent detector (RF-10A, Shimadzu). The fluorescence detector was set at an excitation wavelength of 320 nm and emission wavelength of 430 nm. The eluent was 94% methyl alcohol and 6% water for analysis. The HPLC was set at a flow rate of 1.0 mL/min and an injection volume of 100 µL. The minimum detection limits of plasma phylloquinone and menaquinone-4 concentrations were 0.015 and 0.020 nmol/L, respectively. The intra- and interassay CV were 4.0 and 5.2% for phylloquinone analysis and 6.4 and 7.4% for menaquinone-4 analysis, respectively. The recoveries of phylloquinone and menaquinone-4 calculated by measuring plasma spiked at 0.4 ng (0.89 pmol) of phylloquinone and 0.4 ng (0.90 pmol) of menaquinone-4 were approximately 108 and 94%, respectively.

Dietary phylloquinone was extracted according to the method of Kindberg et al. (1987). Briefly, feed samples were added to distilled water, mixed with 33% ethyl alcohol and 66% diethyl ether, and homogenized. Phylloquinone was extracted by n-hexane, and its concentration was determined by the same method as described for the plasma phylloquinone concentration previously.

Statistical Analysis

The data obtained were subject to ANOVA using PROC MIXED (SAS Inst. Inc., Cary, NC). The horse was the experimental unit and measurements at different times on the same horse were considered as repeated measures in the following model:
where $Y_{ijk}$ is the plasma concentration of menaquinone-4 or phylloquinone, $\mu$ is the overall mean, Treat$_i$ is the fixed effect of treatment ($i = 4$), Time$_j$ is the fixed effect of sampling time ($j = 4$), (Treat × Time)$_{ij}$ is the fixed effect of treatment and sampling time interaction, Animal$_{k(i)}$ is the random effect of the horse ($k = 4$) nested within treatment, and $e_{ijk}$ is the residual error. Least squares means over time within the same treatment were compared by linear and quadratic contrasts. Treatment least squares means were calculated using the LSMEANS option of SAS and were separated using Tukey’s test. When the effect of treatment and sampling time interaction was significant, the difference among least squares means in the same sampling time was further analyzed using Tukey’s test. Probability values less than 0.05 were used as the criterion for statistical significance.

### RESULTS

All horses were healthy throughout the experiment and did not show any symptoms of typical vitamin K toxicosis, such as renal colic and hematuria (data not shown). As indicated before, the dietary phylloquinone concentration was 3.25 µmol/kg (Table 1); thus, phylloquinone intake was 35.8 µmol/d in all groups except the phylloquinone group, which corresponded to 60% of supplied phylloquinone in the phylloquinone group.

Plasma menaquinone-4 concentration was $0.059 \pm 0.021$ nmol/L before the supplementation period. The menadione group showed approximately 4-fold greater ($P < 0.001$) plasma menaquinone-4 concentration than the other groups, including the menaquinone-4 group, and there were no differences between the other treatments (Table 2). The plasma menaquinone-4 concentration did not change after feeding in each group; therefore, the menadione group had stable increased plasma menaquinone-4 concentration during the experiment.

Plasma phylloquinone concentration was $0.959 \pm 0.548$ nmol/L before the supplementation period. Plasma phylloquinone concentration quadratically increased ($P < 0.001$) after feeding in the phylloquinone group and peaked at 4 h after feeding (Table 3). On the other hand, plasma phylloquinone concentration did not change after feeding in the other groups. Furthermore, plasma phylloquinone concentration was 2- to 3-fold greater ($P < 0.001$) in the phylloquinone group than the other groups throughout the experimental period, and plasma phylloquinone concentration did not differ among the other groups.

### DISCUSSION

There has been extensive research investigating vitamin K metabolism in humans and rodents. To our

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**Table 2.** Plasma menaquinone-4 concentration (nmol/L) after feeding in horses given the diet supplemented with different vitamin K homologs$^1$

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time after feeding, h</th>
<th>LS mean of treatment$^2$</th>
<th>SEM</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>2</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>Control</td>
<td>0.119</td>
<td>0.073</td>
<td>0.168</td>
<td>0.139</td>
</tr>
<tr>
<td>Phylloquinone$^3$</td>
<td>0.051</td>
<td>0.084</td>
<td>0.118</td>
<td>0.132</td>
</tr>
<tr>
<td>Menaquinone-4$^3$</td>
<td>0.091</td>
<td>0.148</td>
<td>0.158</td>
<td>0.151</td>
</tr>
<tr>
<td>Menadione$^3$</td>
<td>0.528$^6$</td>
<td>0.49</td>
<td>0.496</td>
<td>0.576</td>
</tr>
</tbody>
</table>

$^1$Means of treatment within a column without a common superscript differ ($P < 0.001$).

$^2$Effect of treatment, $P < 0.001$; time, $P = 0.192$; treatment and sampling time interaction, $P = 0.907$; and $n = 4$.

$^3$Wako Chemicals, Osaka, Japan.

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**Table 3.** Plasma phylloquinone concentration (nmol/L) after feeding in horses given the diet supplemented with different vitamin K homologs$^1$

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time after feeding, h</th>
<th>LS mean of treatment$^2$</th>
<th>SEM</th>
<th>Linear</th>
<th>Quadratic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>2</td>
<td>4</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.957$^a$</td>
<td>0.932$^a$</td>
<td>0.961$^a$</td>
<td>0.656$^a$</td>
<td>0.876$^a$</td>
</tr>
<tr>
<td>Phylloquinone$^3$</td>
<td>2.067$^b$</td>
<td>2.863$^b$</td>
<td>4.770$^b$</td>
<td>3.122$^b$</td>
<td>3.268$^b$</td>
</tr>
<tr>
<td>Menaquinone-4$^3$</td>
<td>1.039$^a$</td>
<td>1.008$^a$</td>
<td>1.008$^a$</td>
<td>0.784$^a$</td>
<td>0.960$^a$</td>
</tr>
<tr>
<td>Menadione$^3$</td>
<td>0.958$^a$</td>
<td>0.720$^a$</td>
<td>0.941$^a$</td>
<td>0.780$^a$</td>
<td>0.866$^a$</td>
</tr>
</tbody>
</table>

$^a$Means within a column without a common superscript differ ($P < 0.001$).

$^1$Effects of treatment, $P < 0.001$; effect of time, $P = 0.010$; effect of treatment and sampling time interaction, $P < 0.001$; and $n = 4$.

$^2$LS mean = least squares mean.

$^3$Wako Chemicals, Osaka, Japan.
knowledge, however, vitamin K metabolism has scarcely been investigated in horses and plasma concentration of vitamin K homologs have been reported only by our preliminary study (Inoue et al., 2009). When phylloquinone activates vitamin K-dependent proteins, including blood coagulation factors, phylloquinone is converted to its 2,3-epoxide; then this metabolite is recycled to phylloquinone by epoxide reductase (Cain et al., 1997). Wilson et al. (2003) reported that the Michaelis constant and the maximum velocity of this epoxide reductase differ between horses and other animals such as humans and pigs. Indyk and Woolard (1997) indicated that milk phylloquinone concentration was relatively greater in horses compared with other animals, but milk menaquinone-4 concentration was decreased in horses. Thus, vitamin K metabolism may be different among animal species.

Plasma menaquinone-4 concentration was greater in the menadione group than in the control group, but a postprandial change in plasma menaquinone-4 concentration was not observed in the menadione group. We did not measure plasma menadione concentration in the present experiment; therefore, the change in plasma menadione concentration was not clarified. Thijsen et al. (2006) suggested that phylloquinone is converted to menadione through dephytylation in the intestine, followed by isoprenylation to menaquinone-4 in tissues such as the liver and pancreas. It is likely that menadione is efficiently absorbed in horses, the absorbed menadione is gradually converted to menaquinone-4 in tissues, and the produced menaquinone-4 is secreted into the circulation, resulting in a stable, increased plasma menaquinone-4 concentration in the menadione group. Unexpectedly, supplementation of menaquinone-4 did not affect plasma menaquinone-4 concentration. In view of the greater menaquinone-4 concentration by supplementation of the metabolic precursor, menadione, these results indicate that menaquinone-4 is poorly absorbed in horses. Thijsen et al. (1996) indicated that rats given menadione had 3-fold greater plasma menaquinone-4 concentration and 10-fold greater organ menaquinone-4 concentrations than rats given menaquinone-4, indicating that menaquinone-4 is poorly absorbed in rats.

Plasma phylloquinone concentration was greater in the phylloquinone group than in the control group. Although supplementation of phylloquinone is known to increase plasma menaquinone-4 concentration in rats (Ronden et al., 1998; Koivu-Tikkanen et al., 2000), phylloquinone supplementation did not affect plasma menaquinone-4 concentration in horses. These results indicate that conversion of phylloquinone to menadione is small in horses because absorbed menadione is converted to menaquinone-4. As a result, plasma menaquinone-4 concentration was less even in horses with increased concentration of plasma phylloquinone. Plasma phylloquinone concentration quadratically increased after feeding in the phylloquinone group. On the other hand, plasma phylloquinone concentration was consistently less in the control group than the phylloquinone group, whereas dietary phylloquinone concentration in the control group was corresponded to 38% of the phylloquinone group. Schurgers and Vermeer (2002) reported that phylloquinone was mainly incorporated into chylomicron after ingestion, and subsequently into chylomicron remnants, and then transported the liver. It is likely that the postprandial increase in plasma phylloquinone concentration results from increasing plasma concentration of chylomicron and chylomicron remnants loaded with phylloquinone in the phylloquinone group. We supplied pure phylloquinone with wheat bran in the phylloquinone group. The form of phylloquinone may affect its availability (i.e., phylloquinone contained in plants may be more poorly or slowly absorbed than pure phylloquinone in horses), which results in a stable plasma phylloquinone concentration after feeding in groups without supplemental phylloquinone.

Vitamin K is generally known as a cofactor for $\gamma$-glutamyl carboxylase, which activates vitamin K-dependent proteins such as blood coagulation factors produced in the liver and osteocalcin produced in bone (Vermeer, 1990). In vitamin K homologs, phylloquinone and menaquinones are cofactors of $\gamma$-glutamyl carboxylase, but menadione itself does not have cofactor activity (Buitenhuis et al., 1990). On the other hand, several reports indicated that menaquinone-4 is far more active than phylloquinone and menadione in stimulating differentiation of human osteoblasts (Koshihara et al., 1996), and in suppressing differentiation of mouse osteoclasts (Takeuchi et al., 2000). Hara et al. (1993) suggested that menaquinone-4, but not phylloquinone, suppressed bone resorption in cultured mouse calvaria. Furthermore, Tabb et al. (2003) reported that menaquinone-4 stimulated osteoblast differentiation through its transcriptional regulatory function. Hara et al. (1995) reported that the inhibitory effect of menaquinone-4 on bone resorption was related to its side chain. These reports indicate that menaquinone-4 is the most potent vitamin K for regulating bone metabolism, which is independent of the activity of $\gamma$-glutamyl carboxylase. From the standpoint of the effect on plasma menaquinone-4 concentration, dietary menadione is considered as the most potent vitamin K homolog for bone health in horses.

Phylloquinone and menaquinones are considered to have negligible toxicity, but menadione can be toxic at large doses (Combs, 2008). Rebhun et al. (1984) administered a single dose of menadione to horses at approximately 12 and 48 $\mu$mol/kg of BW via intramuscular or intravenous routes, respectively, which resulted in toxicity such as renal colic, hematuria, and azotemia. Maxie et al. (1992) also reported that menadione induced depression, muscle stiffness, colic, and azotemia when adult horses were intravenously injected vitamin K as menadione sodium bisulfite at 200 mg/animal (720 $\mu$mol/animal). The vitamin K requirement was
reported to be 2.9 µmol (0.5 mg) menadione/kg of diet in both pigs (NRC, 1998) and chicks (NRC, 1994). The NRC (1987) suggests that menadione is tolerated well when fed in excess and that 1,000 times the required amount has no adverse effects in animals. The present study indicated that plasma menaquinone-4 concentration was increased by menadione supplementation at 0.11 µmol·kg BW−1·d−1 or at 5.2 µmol/kg of diet, and the menadione supplementation did not induce symptoms of menadione toxicosis such as renal colic and hematuria. However, the present experiment indicated that the effective dose of menadione for increasing plasma menaquinone-4 concentration is far less than its toxic amount in horses; therefore, menadione is considered to be a useful vitamin K supplement without having toxicity in horses.

The NRC (2007) suggests that phylloquinone in forage along with menaquinones synthesized by intestinal microbes meet the requirement of horses, which is based on the absence of impaired blood coagulation (Siciliano et al., 2000a). Impairment of blood coagulation is the easily diagnosable and typical symptom of vitamin K deficiency (Combs, 2008). On the other hand, the requirement of vitamin K to affect skeletal tissues is greater than that for maintaining normal blood coagulation in humans (Booth and Suttie, 1998). The beneficial effect of vitamin K administration on bone quality has not been reported in horses. Further studies are necessary to identify the effect of menadione treatment on bone health such as prevention of racing injury in horses.

In conclusion, the availability of vitamin K homologs largely differs in horses. The conversion efficiency of phylloquinone to menaquinone-4 and the absorption of menaquinone-4 are probably low in horses, which results in the lack of a response of plasma menaquinone-4 concentration to phylloquinone and menaquinone-4 supplementation. Menadione is converted to menaquinone-4 in horses and increases plasma menaquinone-4 concentration; therefore, menadione is likely the best source of vitamin K for bone health in horses.

**LITERATURE CITED**


