Effects of Vitamin K-Deficient Diets and Fasting on Blood Coagulation Factors in Conventional and Germ-Free Rats

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Abstract—Feeding of vitamin K-deficient diets or fasting produced vitamin K deficient syndromes in both conventional and germ-free male rats in 3 days, increasing prothrombin time (PT), activated partial thromboplastin time (APTT), plasma and liver descarboxyprothrombin (PIVKA) levels and liver gamma-glutamylcarboxylase activities, but decreasing plasma clotting factor VII and prothrombin levels. These changes were not found when daily 30 μg/rat of vitamin K₁ was injected during this period. The changes caused by fasting were comparable with those caused by a diet containing 20–30 ng/g of vitamin K₁, while a diet containing less than 5 ng/g caused greater changes in both conventional and germ-free rats. Germ-free rats on a diet containing sufficient amounts of vitamin K₁ showed PT and APTT values similar to those in conventional rats, but lower plasma clotting factor levels and higher PIVKA and microsomal gamma-glutamylcarboxylase activities. The values for PT, APTT, factor VII, prothrombin and PIVKA in the fasted germ-free rats were almost the same as those in the fasted conventional rats. These findings suggest that menaquinones synthesized in the large intestine are not utilized sufficiently to prevent vitamin K deficiency in rats.

Vitamin K deficiency causes hypoprothrombinemia in humans and experimental animals. Vitamin K is usually obtained from green vegetables that contain a large amount of it in the form of phylloquinone. Menaquinones which intestinal bacteria synthesize are also thought to be utilized by humans and animals since they are as effective as phylloquinone for preventing hypoprothrombinemia (1), and Hollander et al. (2, 3) have shown with rat studies that menaquinones are absorbed from the distal ileum and colon by a diffusion process. However, the question remains as to whether menaquinones are actually utilized, since their passive absorption is not as efficient as the active absorption process of phylloquinone that takes place at the jejunum (4).

Germ-free rats have no intestinal bacteria and lack menaquinones in the gut. If menaquinones are utilized by the host animals, then germ-free rats should be more susceptible to vitamin K deficiency than conventional ones. In the present experiments, we compared conventional and germ-free rats in the manifestation of vitamin K deficient syndromes after fasting or vitamin K-deficient diet feeding by measuring prothrombin time (PT), activated partial thromboplastin time (APTT), plasma vitamin K dependent clotting protein levels, and liver microsomal gamma-glutamylcarboxylase activities.

Materials and Methods

Wistar strain conventional and germ-free mature male rats were housed individually in suspended wire-bottomed cages kept in an air-conditioned room (25±1°C, 50–60% humidity) lighted 12 hours a day (8.00 a.m. to 8.00 p.m.). The animals were fed a commercially available rat diet (Oriental CMF containing protein (27.7%), carbohydrate (46.4%) and fats (8.8%); Oriental Kobo Co., Ltd., Tokyo, Japan) and water given ad
Two kinds of vitamin K-deficient diets were used. One of them, obtained from Teklad Co. (Madison, WI, U.S.A.) consisted of protein (19.2%), sucrose (51.7%), corn starch (15.0%) and corn oil (5.0%), and contained less than 5 ng/g of vitamin K$_1$. The other one was prepared in our laboratory from vitamin K-free casein (18.0%), sucrose (67.6%), a mixture of rapeseed and soybean oils (8.0%), a salt mixture (Hegsted salt) (4.0%), fibers (1.5%), and adequate amounts of vitamins except for vitamin K. The vitamin K content of the latter one was 20 to 30 ng/g.

The rats were fed the vitamin K-deficient diets or fasted for 3 days. In another set of experiments, daily 30 ng/rat of vitamin K$_1$ (Eisai Co., Ltd., Tokyo) was injected subcutaneously once a day for 3 days from the first day of the experiment. The animals were then placed under sodium methylhexabital anesthesia (125 mg/kg, i.p.), blood was withdrawn from the abdominal aorta with a disposable injection syringe containing 1/10 volume of 3.8% sodium citrate solution, and the liver immediately removed.

When male rats were fed a vitamin K deficient diet (Teklad), plasma prothrombin time (PT) increased soon after starting the experiment: 13.1 sec on Day 0 (control), 22.9 sec on Day 2, 45.4 sec on Day 4 and 109.5 sec on Day 6. No rats died by Day 6, but two-thirds of the rats died on Day 8. The plasma factor VII level also decreased markedly, to almost the bottom level, on Day 2. Therefore, we limited the experiment period to 3 days.

Prothrombin time (PT), activated partial thromboplastin time (APTT), and plasma clotting factor VII and fibrinogen levels were measured with a photo-optical clot sensing system, COAG-A-MATE-X2 (Warner-Lambert Co. Morris Plains, NJ, U.S.A.). Plasma concentrations of prothrombin and both plasma and liver descarboxyprothrombin (proteins induced by vitamin K absence, PIVKA) levels were determined by the method of Shah et al. (5). The liver microsomes used for the gamma-glutamylcarboxylase assay were prepared according to the procedure reported by Suttie et al. (6). Enzyme assay was performed according to the procedure of Suttie et al. (6, 7). Vitamin K levels in the diets and biological materials were determined by the method reported by Sakano et al. (8). Briefly, the samples were extracted with 75% isopropanol-hexane (5/6), the extract was purified by thinlayer chromatography with a solvent system of petroleum ether-ethyl ether (85/15), and the vitamin K fraction was analyzed by high performance liquid chromatography (column: Nucleosile C$_{18}$, mobile phase: 92.5% ethanol) where the detection was performed by fluorometry. The details of the method will be published elsewhere (Hirauchi et al., to be published), but the minimum detectable amount was 0.5 ng in the case of feces and intestinal contents. Statistical analysis was performed by Student’s $t$-test.

**Results**

The higher grade vitamin K-deficient diet obtained from Teklad produced marked vitamin K deficient syndromes in both conventional and germ-free rats by 3 days, increasing PT and APTT, decreasing plasma clotting factor VII and prothrombin levels, increasing plasma and liver PIVKA levels, and increasing microsomal gamma-glutamylcarboxylase activities (Table 1). The lower grade vitamin K-deficient diet, which contained 20–30 ng/g of vitamin K$_1$, caused similar changes in the conventional rats, but to lesser extents. Fasting for 3 days also increased PT, APTT, PIVKA and the enzyme activity, and decreased plasma factor VII and prothrombin levels in both conventional and germ-free rats to degrees less than those caused by the higher grade vitamin K-deficient diet. In the conventional rats, the changes caused by fasting were rather similar in their degrees to those caused by the lower grade vitamin K-deficient diet, but the decreases in plasma factor VII and prothrombin levels and the increase in plasma PIVKA level were larger than those produced by the lower grade diet ($P<0.05$).

When the germ-free rats were compared with conventional ones, both on the ordinary diet, there was no significant difference in PT and APTT, but the germ-free rats showed lower plasma factor VII and prothrombin levels as well as higher plasma and liver PIVKA levels and microsomal gamma-
### Table 1. Effects of fasting and vitamin K-deficient diets on blood coagulation factors in conventional and germ-free rats

|                      | Conventional rats |                      | Germ-free rats |                      |
|----------------------|-------------------|----------------------|----------------|----------------------|
|                      | Ordinary diet     | Vitamin K-deficient diet | Fasting | Ordinary diet     | Vitamin K-deficient diet | Fasting |
|                      | (Oriental CMF)    | (Teklad)             | (Shionogi)    |                      | (Teklad)             |                      |
| No. of rats          | 5                 | 5                    | 5             | 5                   | 5                    | 5                    |
| Body weight initial (g) | 383±6             | 382±5                | 384±7         | 384±4               | 336±13               | 335±17               | 317±15               |
| Body weight final (g) | 386±7             | 374±5                | 383±7         | 344±4               | 334±12               | 333±15               | 286±15               |
| PT (sec)             | 12.5±0.1          | 35.4±1.6*            | 19.7±0.3*     | 22.3±0.9*           | 12.2±0.1            | 44.2±2.3**†          | 22.9±0.7*            |
| APTT (sec)           | 20.3±0.5          | 43.0±1.1*            | 33.1±0.7*     | 36.6±1.6*           | 19.5±0.5            | 53.2±3.9*            | 35.9±1.0*            |
| Plasma factor VII (%) | 354.3±14.1        | 14.3±2.3*            | 29.7±1.4*     | 17.3±0.8*           | 248.8±4.2†          | 10.6±1.5*            | 23.2±1.0**†          |
| Plasma prothrombin (U/ml) | 277.7±4.3         | 31.6±2.4*            | 65.9±0.8*     | 59.6±2.1*           | 230.8±3.2†          | 15.5±2.5**†          | 47.5±3.4**†          |
| Plasma PIVKA (U/ml)  | 2.6±0.3           | 10.2±0.2*            | 5.6±0.1*      | 8.5±0.2*            | 5.0±0.5†            | 10.0±0.4*            | 8.7±0.4*             |
| Liver PIVKA (U/mg protein) | 0.22±0.00         | 1.01±0.03*           | 0.83±0.02*    | 0.82±0.01*          | 0.29±0.02†          | 1.01±0.03*           | 0.76±0.02*           |
| γ-Glutamylcarboxylase activity (10⁹ dpm/mg protein/30 min) |                      |                      |                |                      |                      |                      |                      |
| Pentapeptide         | 7.2±0.40          | 16.0±1.49*           | 15.0±1.44*    | 15.5±1.22*          | 10.1±0.53†          | 20.1±0.67*           | 13.9±1.56           |
| Endogenous protein   | 0.7±0.05          | 4.5±0.41*            | 4.1±0.45*     | 3.9±0.33*           | 1.6±0.06†           | 5.3±0.26*            | 3.3±0.43*            |

*Statistically significant compared with the ordinary diet group (P<0.05). †Statistically significant compared with the paired conventional group (P<0.05).
Commercially available vitamin K-deficient diet (Teklad) contains less than 5 ng/g of vitamin K₁. The diet we prepared (Shionogi) contains 20–30 ng/g of vitamin K₁. Both the diets were fed for 3 days ad libitum. ‡Rats were fasted for 3 days. §The level was expressed in % compared with normal human plasma (VNC, Warnar Lambert Co.). ‡The precursor activity was defined as NIH thrombin unit. *Substrates for the enzyme assay (7). †Mean±S.E.
**Table 2.** Effects of vitamin K on blood coagulation factors in conventional rats

|                        | Ordinary diet | Vitamin K-deficient diet<sup>a</sup> | Fasting<sup>b</sup> |
|------------------------|---------------|--------------------------------------|--------------------|
|                        | (Oriental CMF)| (Teklad)                             | (Shionogi)         |                     |
|                        | Control       | VK<sup>c</sup>                        | Control            | VK<sup>c</sup>      |
| No. of rats            | 5             | 5                                    | 5                  | 5                   |
| Body weight initial (g)| 241±3         | 242±3                                | 241±2              | 241±3               |
| Body weight final (g)  | 255±3         | 252±2                                | 241±3              | 241±3               |
| PT (sec)               | 12.0±0.1      | 12.3±0.1                             | 20.0±0.9           | 12.8±0.1*           | 14.7±0.5              | 12.5±0.2*              |
| APTT (sec)             | 19.3±0.2      | 19.1±0.1                             | 31.1±1.5           | 17.7±0.1*           | 25.6±0.7              | 17.0±0.3*              |
| Factor VII (%)<sup>d</sup> | 168.0±30.4   | 142.4±26.8                           | 22.3±4.0           | 121.8±23.8*         | 56.2±13.0             | 202.4±34.6*            |
| Plasma prothrombin (U/ml)<sup>e</sup> | 173.4±3.0     | 183.2±1.6                            | 30.7±3.6           | 195.4±1.2*           | 59.4±5.2              | 237.3±2.2*              | 60.6±4.9               | 174.6±1.4*              |
| Plasma PIVKA (U/ml)<sup>e</sup> | 1.9±0.1      | 1.9±0.2                              | 5.8±0.2            | 1.3±0.1*            | 4.1±0.2               | 1.9±0.2*               | 6.6±0.4               | 1.2±0.2*               |
| Liver PIVKA (U/mg protein)<sup>e</sup> | 0.10±0.00    | 0.10±0.00                            | 0.67±0.04          | 0.10±0.01*           | 0.55±0.02             | 0.10±0.00*             | 0.57±0.02             | 0.09±0.00*             |
| γ-Glutamylcarboxylase activity (10<sup>5</sup> dpm/mg protein/30 min) |              |                                      |                    |                     |
| Pentapeptide<sup>f</sup> | 3.4±0.27     | 1.6±0.13*                            | 7.7±0.30           | 1.4±0.06*           | 8.0±0.29              | 2.0±0.12*              | 9.4±0.42             | 1.6±0.16*             |
| Endogenous protein<sup>f</sup> | 0.3±0.01  | 0.1±0.01*                            | 2.0±0.05           | 0.1±0.01*           | 1.9±0.06              | 0.1±0.01*              | 1.7±0.06             | 0.1±0.01*             |

<sup>a</sup>Statistically significant compared with the respective vitamin K nontreated group (P<0.05). <sup>a</sup>Commercially available vitamin K-deficient diet (Teklad) contains less than 5 ng/g of vitamin K<sub>1</sub>. The diet we prepared (Shionogi) contains 20–30 ng/g of vitamin K<sub>1</sub>. Both the diets were fed for 3 days ad libitum. <sup>b</sup>Rats were fasted for 3 days. <sup>c</sup>Daily 30 μg/rat of vitamin K<sub>1</sub> was injected subcutaneously once a day for 3 days. <sup>d</sup>The level was expressed in % compared with normal human plasma (VNC, Warnar Lambert Co.). <sup>e</sup>The precursor activity was defined as a NIH thrombin unit. <sup>f</sup>Substrates for the enzyme assay (7). <sup>g</sup>Mean±S.E.
### Table 3. Effect of vitamin K on blood coagulation factors in germ-free rats

|                      | Ordinary diet | Vitamin K-deficient diet<sup>a</sup> | Fasting<sup>b</sup> |
|----------------------|---------------|--------------------------------------|---------------------|
|                      | Control       | VK<sup>c</sup>                        | Control             | VK<sup>c</sup> | Control | VK<sup>c</sup> |
| No. of rats          | 4             | 3                                    | 4                   | 4               | 4       | 4               |
| Body weight initial (g) | 383±30<sup>f</sup> | 380±33                              | 359±30              | 384±28          | 371±28  | 359±28          |
| Body weight final (g) | 372±27        | 364±27                              | 355±29              | 365±24          | 322±28  | 314±28          |
| PT (sec)            | 11.6±0.1      | 11.8±0.3                            | 28.5±0.7            | 12.3±0.1*       | 20.8±0.4 | 12.5±0.3*       |
| APTT (sec)         | 18.4±0.3      | 17.3±0.1                            | 36.1±0.3            | 17.2±0.2*       | 31.7±0.8 | 17.7±0.3*       |
| Factor VII (%)<sup>d</sup> | 268.2±48.7    | 240.1±70.0                          | 17.3±2.6            | 174.5±33.1*     | 20.1±2.4 | 194.6±52.9*     |
| Plasma prothrombin (U/ml)<sup>e</sup> | 220.2±6.3   | 247.1±13.0                          | 22.9±2.5            | 250.3±10.9*     | 41.6±0.9 | 205.0±11.0*     |
| Plasma PIVKA (U/ml)<sup>e</sup>       | 3.7±0.7      | 0.8±0.2*                            | 9.5±0.3             | 0.5±0.1*        | 8.3±0.3  | 0.3±0.1*        |
| Liver PIVKA (U/mg protein)<sup>e</sup> | 0.26±0.04    | 0.13±0.01*                          | 0.89±0.04           | 0.11±0.01*      | 0.77±0.03 | 0.09±0.00*      |

<sup>*</sup>Statistically significant compared with the respective vitamin K nontreated group (P<0.05). <sup>a</sup>Commercially available vitamin K-deficient diet (Teklad) contains less than 5 ng/g of vitamin K<sub>1</sub>. The diet we prepared (Shionogi) contains 20–30 ng/g of vitamin K<sub>1</sub>. Both the diets were fed for 3 days ad libitum. <sup>b</sup>Rats were fasted for 3 days. <sup>c</sup>Daily 30 μg/rat of vitamin K<sub>1</sub> was injected subcutaneously once a day for 3 days. <sup>d</sup>The level was expressed in % compared with normal human plasma (VNC, Warnar Lambert Co.). <sup>e</sup>The precursor activity was defined as a NIH thrombin unit. <sup>f</sup>Mean±S.E.

### Table 4. Vitamin K<sub>1</sub> and menaquinone contents in the large intestine of conventional and fasted rats

|                  | Conventional | Fasting<sup>a</sup> |
|------------------|--------------|---------------------|
|                  | (ng/rat)     | (ng/rat)            |
| Vitamin K<sub>1</sub> | 1894±272<sup>b</sup> | 112 (53–172)<sup>c</sup> |
| Menaquinone −4     | 22±5         | 26 (22–30)          |
| −5                | 79±14        | 21 (18–23)          |
| −6                | 46±8         | 57 (48–66)          |
| −7                | 110±23       | 220 (197–242)       |
| −8                | 542±103      | 3070 (2361–3779)    |
| −9                | 699±119      | 1506 (1310–1702)    |
| −10               | 4514±592     | 6870 (5613–8126)    |

<sup>a</sup>Rats were fasted for 3 days. <sup>b</sup>Mean±S.E. in 3 rats. <sup>c</sup>Mean in 2 rats.
glutamylcarboxylase activity. The vitamin K-deficient diet produced more significant changes in the germ-free rats, increase in PT and decrease in the plasma prothrombin level. Fasting of germ-free rats, however, caused a smaller decrease in factor VII and a larger decrease in plasma prothrombin level compared with the fasted conventional rats. 

Table 2 shows the effect of vitamin K\textsubscript{1} injection on PT, APTT, plasma clotting factor VII and liver microsomal gamma-glutamylcarboxylase activity in conventional rats. The injection of vitamin K\textsubscript{1} caused no change in rats on the ordinary diet, but normalized the abnormally increased PT, APTT and enzyme activities and the decreased factor VII levels after the vitamin K deficient diet feeding or fasting. 

Table 3 shows the effect of vitamin K\textsubscript{1} injection in germ-free rats. Again, the injection of vitamin K\textsubscript{1} caused no change in the rats on the ordinary diet, but normalized the PT, APTT and factor VII levels in the rats fed the vitamin K-deficient diet or fasted. 

The vitamin K contents in the large intestines of conventional rats on the ordinary diet and of rats fasted for 3 days are given in Table 4. The content of vitamin K\textsubscript{1} markedly decreased in the fasted rats, but that of menaquinones, especially menaquinone-8, -9 and -10, markedly increased. 

**Discussion**

The present experiments demonstrated that feeding of vitamin K-deficient diets for 3 days or fasting for 3 days produced almost the same extent of vitamin K deficient syndromes in both conventional and germ-free rats. Germ-free rats had no menaquinones but conventional rats kept on an ordinary diet had about 6 \( \mu \text{g} \)/rat of menaquinones in the large intestine (Table 4) and excreted 10–20 \( \mu \text{g} \)/rat a day via their feces (8). The large intestine of rats fasted for 3 days contained about 10 \( \mu \text{g} \)/rat of menaquinones, which would be sufficient to prevent vitamin K deficiency if absorbed, since menaquinones are known to be as effective as phyloquinone as a cofactor for gamma-glutamylcarboxylase (1, 9), but these menaquinones did not prevent the manifestation of vitamin K deficient syndromes after fasting for 3 days. 

Germ-free rats on the ordinary diet showed lower plasma factor VII and prothrombin levels and higher PIVKA levels and microsomal gamma-glutamylcarboxylase activity than the corresponding conventional rats. In addition, the vitamin K-deficient diet produced a larger increase in PT and decrease in plasma prothrombin levels in the germ-free rats than in the conventional ones. These data seem to suggest that menaquinones formed in the large intestine are absorbed and used to some extent in vivo, but this is unlikely since the ordinary diet we used contained about 500 \( \mu \text{g} \)/g of vitamin K\textsubscript{1} (our own data) and the dose, which is calculated to be daily 10–13 \( \mu \text{g} \)/rat since the rats eat 20–25 g of diet a day, is enough to prevent vitamin K deficiency in rats (10). In addition, treatment of rats on the ordinary diet with daily 30 \( \mu \text{g} \)/rat of vitamin K\textsubscript{1} caused no change in PT, APTT and factor VII levels of both conventional and germ-free rats (Tables 2 and 3), suggesting that the amount of vitamin K\textsubscript{1} in the diet is enough. We have no data to explain the differences in the basal levels of plasma factor VII and prothrombin and the levels of PIVKA between conventional and germ-free rats. 

Nutritional components of the two diets we used were different. Therefore, the difference in them or the nutritional defect after fasting might affect manifestation of the vitamin K deficient syndromes. However, vitamin K\textsubscript{1} injection at the dose of 30 \( \mu \text{g} \)/rat, daily, completely normalized PT, APTT and plasma factor VII levels of both conventional and germ-free rats. When a dose of 200 \( \mu \text{g} \)/kg of vitamin K\textsubscript{1} was injected once subcutaneously on the second day of fasting, PT, APTT, plasma levels of factor VII, prothrombin and PIVKA and liver levels of PIVKA in the fasted rats were all normalized in the following day (Uchida et al., unpublished data). These data suggest that the changes observed after feeding vitamin K-deficient diets or fasting was due to defect of vitamin K and not of nutritional components. 

The vitamin K deficiency caused by fasting, which should be a condition with no dietary vitamin K, was not so severe as that
caused by the higher grade vitamin K-deficient diet which contained less than 5 ng/g of vitamin K₁, and it roughly corresponded to that caused by the lower grade deficient diet with 20–30 ng/g of the vitamin. This finding, however, does not mean that menaquinones were used or coprophagy was performed, since the germ-free rats showed vitamin K deficient syndromes of almost the same degree as the conventional rats.

Some species of animals including rats perform coprophagy which makes it difficult to estimate the necessary amounts of vitamin K (1). In the present experiments where rats were caged in individual wire-bottomed cages, none showed normal clotting parameters after being fed the vitamin K-deficient diets or fasting, which indicates that coprophagy was not performed to the extent of preventing vitamin K deficiency.

The lower grade vitamin K-deficient diet contained 20–30 ng/g of vitamin K₁ but caused vitamin K deficiency in 3 days. When rats were fed a diet containing 30–50 ng/g of vitamin K₁ for 1 week, no or only a slight hypoprothrombinemia (11) with increased hepatic gamma-glutamylcarboxylase activity (7, 11) was observed. The data on PT of the present (Table 1) and the previous experiments (11) are combined and shown in Fig. 1. These data suggest that the level of about 50 ng/g of vitamin K₁ is the marginal dose for not manifesting hypoprothrombinemia in male rats, but it is not yet sufficient since microsomal gamma-glutamylcarboxylase activity is increased by the dose (7). Bieri (10) also suggested that a sufficient dietary vitamin K level for rats is 500 μg/kg of diet. Although Hollander et al. (2, 3) have shown that menaquinones are absorbed from the colon in rats, our data indicate that menaquinones are not absorbed sufficiently to prevent vitamin K deficiency upon fasting or feeding of a vitamin K-deficient diet. Udall (12) also concluded from human studies that bacterially produced menaquinones in the gut cannot be utilized by humans.

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