Difference in the metabolism of vitamin K between liver and bone in vitamin K-deficient rats

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The difference between vitamin K metabolism in the liver and that in the bone of vitamin K-deficient rats was examined. After 17 d administration of vitamin K-deficient food, vitamin K in the liver was almost depleted, and prothrombin time (PT) was prolonged. Serum total osteocalcin level was slightly decreased by vitamin K deficiency, whereas serum undercarboxylated osteocalcin level did not change. The level of menaquinone (MK)-4 as well as that of phylloquinone was decreased, but approximately 40 % of the initial level still existed in the femur after the 17 d period. A single-dose administration of vitamin K (250 nmol/kg body weight) markedly increased vitamin K level in the liver but not in the femur. These results suggest that the turnover of vitamin K in the bone is slower than that in the liver, and bone metabolism may be little affected by the short period of intake of vitamin K-deficient food. However, intake of a larger amount of vitamin K is required for its accumulation in the bone than in the liver. Furthermore, the counteracting effect of MK-7 on prolonged PT in vitamin K-deficient rats was found to be higher than phylloquinone or MK-4.

Menaquinone: Phylloquinone: Vitamin K: Coagulation: Osteocalcin

Vitamin K is a cofactor required for the post-translational conversion of glutamic acid residues of specific proteins into γ-carboxyglutamic acid (Gla) to form Gla-containing proteins via γ-glutamylcarboxylase (Shearer, 1990). A number of blood coagulation factors including coagulation factors II (prothrombin), VII, IX and X are Gla-containing proteins, and are synthesized in the liver. Osteocalcin (OC), a bone-specific protein synthesized by osteoblasts, is also a Gla-containing protein. Noncarboxylated or undercarboxylated (uc) OC cannot bind to hydroxyapatite in mineralized tissues (Price, 1985). The nutritional requirement of vitamin K for the complete γ-carboxylation of OC is thought to be higher than that for the maintenance of normal blood coagulation in human subjects (Booth & Suttie, 1998). Hoffa et al. (2000) have shown that a low intake of vitamin K for 80 d increased in serum ucOC level but did not affect prothrombin time (PT) in rats.

There are two naturally occurring forms of vitamin K: phylloquinone (K1) and menaquinone (MK). K1 is synthesized by plants and is present in large amounts in various green vegetables, which are the major source of dietary vitamin K (Booth & Suttie, 1998). MK has a variable side-chain length of four to thirteen isoprene units. They are referred to as MK-n, where n denotes the number of isoprenoid residues. MK-n are bacterial products that are found in fermented foods (Sakano et al. 1988, Hirauchi et al. 1989), as well as in intestinal flora in the colon (Collins & Jones, 1981; Conly & Stein, 1992). The extent to which MK-n derived from intestinal bacteria contribute to the daily requirement of vitamin K remains a matter of debate at the present time (Uchida et al. 1986; Conly & Stein, 1992; Suttie, 1995). In addition, in rats coprophagy is a rich dietary source of MK-n because faeces contain substantial amounts of MK-n (Vermeer et al. 1995). Whether hepatic MK-n in the rat is derived from direct intestinal absorption or by coprophagy is also still not known. Mathers et al. (1990) reported that a white rice-based diet reduced hepatic K1 and longer-chain MK levels. They showed that the low-fibre content of the diet resulted in caecal metabolism

Abbreviations: APTT, activated partial thromboplastin time; Gla, γ-carboxyglutamic acid; K1, phylloquinone (vitamin K1); MK, menaquinone (vitamin K2); OC, osteocalcin; PT, prothrombin time; uc, undercarboxylated.

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including reduced number of MK-producing bacterial species in the intestine. Groenen-van Dooren et al. (1995) developed an experimental animal model that exhibits severe vitamin K deficiency, in which rats equipped with anal cups to prevent coprophagy are fed vitamin K-deficient white-rice diet. In the present study, using the model of Groenen-van Dooren et al. (1995), comparative tissue measurement of K₁ and MK-n in liver and bone was carried out in vitamin K-deficient rats. Then, the effect of acute vitamin K deficiency on blood coagulation and serum total OC or ucOC level was examined. Furthermore, the relative effects of K₁, MK-4 and MK-7 on blood coagulation and serum OC levels were investigated.

Materials and methods

Chemicals

K₁ and MK-4 were purchased from Wako Pure Chemicals Industries (Osaka, Japan). MK-6, -7 and -8 were purified from Bacillus subtilis in our laboratory (Sato et al. 2001). 2,3′-Dihydrophylloquinone was prepared in our laboratory. K₁, MK-4 and MK-7, dissolved in ethanol solution (995 ml/l) to make a concentration of 2 mM, were suspended in gum arabic (50 g/l) while adjusting the final concentration of each K-vitamer to 40 or 200 nmol/ml. Rat OC was purchased from Biomedical Technologies Inc. (Stoughton, MA, USA). Hydroxyapatite and other chemicals from Wako Pure Chemicals Industries were of reagent grade. Warfarin sodium was purchased from Sigma (St Louis, MO, USA).

Animals and diet

The study was conducted in accordance with current legislation on animal experimentation in Japan. Male Sprague-Dawley rats (Charles River Co., Yokohama, Japan) were housed in individual stainless-steel, wire-bottomed cages. They were kept in an air-conditioned room at 24°C with a 12 h light–dark cycle. They were 11-weeks-old when the experiment started. The rats had free access to the AIN93 formula diet (Oriental Yeast, Tokyo, Japan) and radiated (50 kGy) vitamin K-deficient diet (Oriental Yeast) and the experiment started. The rats had free access to the AIN93 formula diet (Oriental Yeast, Tokyo, Japan) and radiated (50 kGy) vitamin K-deficient diet (Oriental Yeast) and the experiment started. The rats had free access to the AIN93 formula diet (Oriental Yeast, Tokyo, Japan) and radiated (50 kGy) vitamin K-deficient diet (Oriental Yeast) and the experiment started.

PT and activated partial thromboplastin time (APTT) were determined to study blood coagulation using plasma by Thromboplastin C Plus and Symex CA-500 (Symex Co., Kobe, Japan) respectively. Before starting an experiment, blood was collected from each rat to determine the initial values.

Total serum OC level was determined by ELISA using a commercially available kit (Biomedical Technologies Inc.). We prepared thermally decarboxylated OC according to the method of Poser & Price (1978) and confirmed that the kit detected decarboxylated OC with the same sensitivity as that for γ-carboxylated OC detection.

ucOC was measured using the method that is based on the difference in affinity between carboxylated OC and decarboxylated OC for hydroxyapatite (Merle & Delmas, 1990). Briefly, 50 μl samples were incubated with 3 mg hydroxyapatite suspended in 30 μl 10 mM-borate buffer (pH 7-3) containing 2 mM-CaCl₂ in an Eppendorf tube at 4°C for 4 h and then centrifuged. This concentration of hydroxyapatite was chosen because it gave the best discrimination between the hydroxyapatite-binding ability of γ-carboxylated OC and that of thermally decarboxylated OC. To confirm the adequacy of this assay, three rats were treated with sodium warfarin (1-5 mg/kg diet) for 7 d and their serum ucOC and total OC levels were measured. The percentage of ucOC in total OC was determined to be 69–87 %, indicating that the assay is sufficiently sensitive to detect an increase in ucOC.

Determination of concentration of vitamin K

K₁ and MK-n concentrations in the plasma, liver and femur were measured by HPLC as follows. Plasma (500 μl), 2-propanol (650 μl) and 1.3 ml n-hexane containing 5 ng 2′,3′-dihydrophylloquinone as an internal standard were added, mixed vigorously, and centrifuged. A portion of the n-hexane layer (1 ml) was removed and evaporated. To the residue obtained, 200 μl 2-propanol was added and a 50 μl aliquot was injected into the HPLC column. Femurs were cleaned of soft tissue and marrow, and added to 5 ml water, 20 ml 2-propanol and 20 ml sodium citrate (38 g/l). The tube was centrifuged at 3000 rpm at 4°C for 15 min and the plasma obtained was used for the examination of coagulation. Another portion of the blood collected was centrifuged at 3000 rpm at 4°C for 15 min and the resultant serum was used for the determination of serum OC level.

In order to analyse the change in concentration of vitamin K after administration, some animals were killed under light anaesthesia with diethyl ether, and the blood, liver and femur were removed immediately. Blood (1.8 ml) was collected from the abdominal aorta using a syringe containing 0.2 ml sodium citrate (38 g/l). The blood collected was centrifuged at 3000 rpm for 15 min and a plasma sample was obtained. The liver was washed with saline to remove blood. The concentrations of vitamin K in the plasma, liver and femur were determined as described later.

Chemical analyses

The study was conducted in accordance with current legislation on animal experimentation in Japan. Male Sprague-Dawley rats (Charles River Co., Yokohama, Japan) were housed in individual stainless-steel, wire-bottomed cages. They were kept in an air-conditioned room at 24°C with a 12 h light–dark cycle. They were 11-weeks-old when the experiment started. The rats had free access to the AIN93 formula diet (Oriental Yeast, Tokyo, Japan) and radiated (50 kGy) vitamin K-deficient diet (Oriental Yeast) and the experiment started. The rats had free access to the AIN93 formula diet (Oriental Yeast, Tokyo, Japan) and radiated (50 kGy) vitamin K-deficient diet (Oriental Yeast) and the experiment started. The rats had free access to the AIN93 formula diet (Oriental Yeast, Tokyo, Japan) and radiated (50 kGy) vitamin K-deficient diet (Oriental Yeast) and the experiment started.
n-hexane containing 10 ng 2',3'-dihydrophylloquinone. Samples of livers (1 g) were added to 3 ml water, 20 ml 2-propanol and 20 ml n-hexane containing 2',3'-dihydrophylloquinone (10 ng). They were homogenized using a blender for 2 min and then centrifuged. The n-hexane layer was isolated and dried. The resultant pellet was dissolved in n-hexane, and then eluted through Sep-Pak silica (Milford, MA, USA) using toluene–hexane (3:2, v/v). The eluted samples were dried and dissolved in n-hexane. The samples were purified by HPLC using n-hexane-ether (30:1, v/v) and a silica-gel column (Unisil Q, 300 × 16-7 mm; GL Sciences, Tokyo, Japan), and elution was monitored at 253 nm. The solvent was evaporated, and the samples were re-dissolved in 2-propanol and analysed by HPLC. HPLC evaluation was carried out using a Shimadzu LC-10A system equipped with a fluorescence detector. The sample was separated using an ODS column (L-column, 250 mm £ 4·6 mm; Chemical Inspection and Testing Institute, Tokyo, Japan) and peaks were detected using a fluorescence detector after reduction by Pt black (Reduction column, 10 £ 4·6 mm; Toa, Tokyo, Japan). Detection of vitamin K level was carried out at an excitation wavelength of 320 nm for serum and bone or 244 nm for liver; and an emission wavelength of 430 nm. The mobile phase contained 200 ml 2-propanol and 800 ml methanol. The flow rate was 1·2 ml/min. Detection limits of K1 and MK-n were 1·0 pmol/ml plasma or /g liver or femur.

**Statistical analysis**

All data were expressed as mean values with their standard errors. Differences between groups were compared using Dunnet’s multiple comparison tests, and were considered to be significant when P values were <0·05.

**Results**

**Effect of vitamin K deficiency on blood coagulation and serum osteocalcin**

Rats equipped with anal cups were given the vitamin K-deficient diet ad libitum throughout this study. On day 10 or 17 after the experiment was started, PT, APTT, serum total OC level and serum ucOC level (ucOC:total OC) were determined (Table 1). PT and APTT were prolonged in vitamin K-deficient rats, but not in the normal control group fed 1 mg K1/kg diet. Vitamin K deficiency caused a slight decrease in the total OC level. However, the serum ucOC level did not change after the 17 d administration of vitamin K-deficient diet (Table 1).

Levels of K1 and MK-n in the plasma, the liver and the femur before or after 17 d administration of the vitamin K-deficient diet or the same diet supplemented with 1 mg K1/kg diet were determined by HPLC (Table 2). K1 and a small amount of MK-n were detected in the liver of the normal control group, while both K1 and MK-n were almost depleted in the liver of the vitamin K-deficient group. In the femur, more MK-4 than K1 existed on day 0 or day 17 in both groups. The levels of MK-4 and K1 were decreased in the femur of vitamin K-deficient rats, although approximately 40 % of the initial values (day 0) remained.

**Changes in vitamin K levels in plasma, liver and bone after administration of K-vitamers**

Vitamin K-deficient rats were treated with a single dose of K1, MK-4 or MK-7 (250 nmol/kg body weight). Three samples each of blood, liver and femur were collected from three rats per time point, before the experiment and at 6 and 24 h after administration of vitamin K, and five samples were collected from five different rats at 72 h. Then, the levels of vitamin K were determined (Fig. 1). The levels of the three K vitamers, K1, MK-4 and MK-7, in the plasma at 6 h were almost the same. In the liver, all K vitamins were detected throughout the experiment in each group, while the turnover of MK-7 was slower than that of K1 or MK-4. No significant increase in vitamin K was found in the femur for all groups. The vitamin K level in the femurs of rats that received administered a single dose of K-vitamer (250 nmol/kg body weight) was the same as that of the vitamin K-deficient control group (Table 2 and Fig. 1).

**Effect of K-vitamers on blood coagulation in vitamin K-deficient rats**

PT was measured to study blood coagulation in vitamin K-deficient rats. It was found to be significantly prolonged after 14 d vitamin K deficiency (Table 1). The effects of

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<th>Table 1. Effects of vitamin K deficiency on blood coagulation and serum osteocalcin in rats†</th>
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<td>(Mean values with their standard errors for five rats per group)</td>
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PT, prothrombin time; APTT, activated partial thromboplastin time; OC, osteocalcin; uc, undercarboxylated.

Mean values were significantly different from those of the control group: *P<0·05, **P<0·01.

† For details of diets and procedures, see p. 308.
K₁, MK-4 and MK-7 on blood coagulation were compared on a molar basis, 50 nmol/kg body weight, which was equivalent to 22.5 (K₁), 22.2 (MK-4) or 32.5 (MK-7) μg/kg, and 250 nmol/kg body weight, which was equivalent to 112.5 (K₁), 111.0 (MK-4) or 162.5 (MK-7) μg/kg (Fig. 2). On day 14, the 50 or 250 nmol vitamin K/kg body weight was administered to vitamin K-deficient rats (t = 0).

Administration of vitamin K shortened the prolonged PT induced by vitamin K deficiency. These effects continued over 72 h in all groups that received K-vitamers (250 nmol/kg), whereas in the low-dose case (50 nmol/kg), the effect of MK-7 lasted longer than that of K₁ or MK-4.

The counteracting effects of a single dose of K₁, MK-4 or MK-7 (50 or 250 nmol/kg body weight) on the prolonged APTT in vitamin K-deficient rats were also examined (results not shown). The effect of MK-7 on shortening APTT lasted longer than that of K₁ or MK-4. The effect of MK-7 (50 nmol/kg) on APTT was greater than that of K₁ (250 nmol/kg) or MK-4 (250 nmol/kg).

**Effect of K-vitamers on osteocalcin in vitamin K-deficient rats**

Serum total OC level slightly decreased after 10 or 17 d of vitamin K deficiency (Table 1). The effect of K-vitamers (250 nmol/kg body weight) on the level of serum total OC was examined (Fig. 3). A low dose of vitamin K (50 nmol/kg) had no effect on the serum total OC level (results not shown). Serum ucOC level was not elevated by 17 d of vitamin K deficiency (Table 1) and the administration of vitamin K (250 nmol/kg body weight) did not affect serum ucOC level in all groups (results not shown).

**Discussion**

Many of the factors involved in blood coagulation are vitamin K-dependent Gla-containing proteins. With regard to blood coagulation, vitamin K deficiency is rare in adult man (Sokoll *et al.* 1997). Recent studies, however, revealed that the current daily intake of vitamin K is not sufficient to maximally γ-carboxylate OC. Low intake of vitamin K causes an increase in the serum ucOC level, which is correlated with hip-bone mineral density in elderly women (Szułc *et al.* 1994) and with hip fracture in elderly women (Szułc *et al.* 1993). Supplementation of K-vitamers, K₁ (Sokoll *et al.* 1997), MK-4 (Shiraki *et al.* 2000) and MK-7 (Tsukamoto *et al.* 2000), decreased serum ucOC level in human subjects. Furthermore, insufficient intake of vitamin K₁ (20 ng/g diet) over an 80 d period caused an increase in serum ucOC level but did not affect PT in rats (Haffa *et al.* 2000). These observations have led to the conclusion that more vitamin K is necessary for the γ-carboxylation of OC than the amount required for the maintenance of plasma prothrombin levels (Booth & Suttie, 1998). In contrast to these reports, however, we obtained an apparently conflicting result in the present study. An acute vitamin K deficiency for 14–17 d made blood coagulation abnormal, i.e. prolonged PT and APTT, without affecting serum ucOC level. K₁ and MK-ₙ were almost depleted in the liver by the 17 d vitamin K deficiency, whereas K₁ as well as MK-4 decreased by approximately 60 % from their initial values and considerable amounts of K₁ and MK-4 still remained in the femur. These results suggested that the turnover of vitamin K in the femur is slower than that in the liver.

In rats, coprophagy, as well as absorption of MK-ₙ produced in intestinal flora, is thought to be an important source of longer-chain MK-ₙ, although the extent to which coprophagy or absorption contributes to the synthesis of various Gla-containing proteins remains under debate. In the present study, rats were fed a low-fibre diet based on white rice. The rice diet reduced hepatic longer-chain MK-ₙ by changing the number of MK-producing bacterial species in the intestine (Mathers *et al.* 1990). Barns & Fiala (1959) reported that rats that were fed a vitamin K-deficient feed and whose coprophagy was
effectively prevented, developed hypoprothrombinemia despite the presence of bacteria in their colon. In our present study, the feeding of the rice diet together with the vigorous prevention of coprophagy was expected to drastically reduce hepatic \( \text{K}_1 \) and long-chain \( \text{MK}-n \) levels. We found that longer \( \text{K} \)-vitamers were almost depleted and only low levels of \( \text{MK}-7 \) and \( \text{K}_1 \) were detected after 17 d of vitamin \( \text{K} \) deficiency. On the other hand, the study of Haffa et al. (2000) did not use a low-fibre diet or any specific coprophagy-preventing apparatus. Therefore, it was thought that chronic insufficient \( \text{K}_1 \) intake in their model could cause an increase in ucOC level, while the long-chain \( \text{MK}-n \) derived from coprophagy protects \( \gamma \)-carboxylation of the hepatic coagulation proteins.

When a single dose of \( \text{MK}-4, \text{MK}-7 \) or \( \text{K}_1 \) (250 nmol/kg body weight) was administered to vitamin \( \text{K} \)-deficient rats, the level of every \( \text{K} \)-vitamer, \( \text{K}_1, \text{MK}-4 \) or \( \text{MK}-7 \), markedly increased in the liver but not in the femur. This suggests that intake of a larger amount of vitamin \( \text{K} \) is required for its accumulation in the femur than in the liver of rats. This is consistent with current views of different vitamin \( \text{K} \) requirements of liver and bone (Vermeer et al. 1995; Booth & Suttie 1998). Since a smaller amount of vitamin \( \text{K} \) is sufficient for its accumulation in the liver than in the femur, abnormal blood coagulation hardly occurs. Therefore, it is possible that insufficient intake of vitamin \( \text{K} \) for longer periods will influence bone metabolism without affecting blood coagulation. Other explanations are also possible: (1) in the vitamin \( \text{K} \)-deficiency state, the liver is capable of extracting vitamin \( \text{K} \) from the circulation at very high efficacy; or (2) bone tissue needs longer exposure to circulating vitamin \( \text{K} \) to be able to accumulate the vitamin in significant amounts.

We did not observe any changes in serum ucOC level in rats that were under strict vitamin \( \text{K} \)-deficient conditions for 17 d. In human subjects, serum ucOC level is influenced by the intake of vitamin \( \text{K} \) for relatively short periods (Sokoll et al. 1997; Tsukamoto et al. 2000). Haffa et al. (2000) suggested that rats may be an inappropriate model for studying the effect of vitamin \( \text{K} \) on bone metabolism in human subjects, because they found that warfarin reduced bone OC level to only 0·65 mg/mg, which was still much higher than that in healthy human subjects. Although serum ucOC level did not change, serum total OC level appeared to be slightly decreased by vitamin \( \text{K} \) deficiency (Fig. 3); this must be clarified in future studies.

Several researchers revealed that \( \text{MK}-4 \) is converted from \( \text{K}_1 \) in liver, pancreas, salivary gland, kidney, brain, testis, heart and spleen (Thijssen & Drittij-Reijnders, 1994; Yamamoto et al. 1997; Davidson et al. 1998; Ronden et al. 1998). The results obtained in the current study suggest that the conversion of \( \text{K}_1 \) into \( \text{MK}-4 \) occurred also in the femur of rat because \( \text{MK}-4 \) was more abundant than \( \text{K}_1 \) in the femur of the group administered \( \text{K}_1 \) (1 mg/kg diet) for 17 d and \( \text{MK}-4 \) level was higher than that in vitamin \( \text{K} \)-deficient rats. Yamaguchi et al. (1999) reported that feeding of \( \text{MK}-7 \) to rats increased \( \text{MK}-4 \) level in the femur, indicating that \( \text{MK}-7 \) was converted into \( \text{MK}-4 \) in the femur. \( \text{MK}-4 \) enhances mineralization in human osteoblasts (Koshihara et al. 1992) and increases OC level in human osteoblasts in the presence of 1\( \alpha \),25-dihydroxycholecalciferol (Koshihara et al. 1996); it also inhibits bone resorption in vitro (Hara et al. 1995). However, therapeutic treatment with \( \text{MK}-4 \) in osteoporotic patients did not have any effects on bone resorption markers (Shiraki et al. 2000). \( \text{MK}-4 \) may have some specific but as yet unknown functions in bone as well as other tissues.

PT was prolonged in vitamin \( \text{K} \)-deficient rats. A single dose of a \( \text{K} \)-vitamer, \( \text{K}_1, \text{MK}-4 \) or \( \text{MK}-7 \), shortened the
prolonged PT and APTT. The effect of MK-7 lasted longer than that of MK-4 and K₁ on a molar basis. The longer acting effect of MK-7 was supported by the measurement of K-vitamers in the liver, in which the turnover of MK-7 was found to be delayed. The longer acting effect of MK-7 on blood coagulation corresponded well with the report of Groenen-van Dooren et al. (1995), who showed that the stimulation of prothrombin synthesis by MK-9 lasts longer than that by MK-4 or K₁. Several other comparative studies on vitamin K activity, i.e. the ability to promote the γ-carboxylation of Gla-containing proteins in the liver by MK homologues, have been reported. MK-4, MK-5 and MK-6 have been shown to be highly effective in improving hypoprothrombinaemia in vitamin K-deficient rats induced by an anticoagulant (Akiyama et al. 1995). On the other hand, MK-7 was shown to have a much stronger effect on decreasing PIVKA-II (protein induced by vitamin K absence or antagonist II) than MK-4 in vitro (Nakanishi et al. 1989). Recently, Craciun et al. (1998) demonstrated that these discrepancies were due to the difference in the experimental model used. They suggested that the strong activity of MK-4 is only valid in rats treated with an anticoagulant. Although only limited amounts of MK-n have been found in food, the most abundant homologues are MK-7, MK-8, and MK-9 (Sakano et al. 1988; Hirauchi et al. 1998).

![Fig. 2. Effect of a single dose of vitamin K on prothrombin time in vitamin K-deficient rats. For details of diets and procedures, see p. 308. (a) ■ vitamin-K-deficient control; □, normal control fed 1 mg phylloquinone (K₁)/kg of diet. At t₀, the following doses of vitamin K were given to vitamin K-deficient rats: (b) 50 nmol each vitamer/kg body weight (∗, K₁; ○, menaquinone (MK)-4; △, MK-7); and (c) 250 nmol each vitamer/kg body weight (●, K₁; ●, MK-4; ●, MK-7). Values are means for five rats with standard errors shown by vertical bars.](https://www.cambridge.org/core).
1989; Shearer et al. 1996). The Japanese traditional food, natto, contains MK-7 at a high level and is a major source of vitamin K for the Japanese (Kaneki et al. 1995). In European studies, MK-7 and MK-8 were reported to be the predominant homologues of MK in human serum (Hodges et al. 1993a,b). It was thought that the bioavailability and the nutritional importance of K1 and various MK homologues with different side-chains should be evaluated in studies on human subjects.

In summary, we have shown that: (1) the turnover of vitamin K in the femur is slower than that in the liver; and (2) a larger amount of vitamin K is required for its absorption in the femur than in the liver. Furthermore, the counteracting effect of MK-7 on the impaired blood coagulation mechanism in vitamin K-deficient rats is much stronger than that of K1 or MK-4.

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References


