Vitamin K Deficiency of Germfree Mice Caused by Feeding Standard Purified Diet Sterilized by γ -Irradiation

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Abstract: Germfree mice died when they were fed a purified diet of AIN-76 formula sterilized by γ -irradiation. Vitamin K deficiency was suspected and this study was performed to confirm the cause of the death. Germfree mice were fed purified diets of AIN-76 or AIN-93M formula, which were pelleted and sterilized by γ -irradiation at a dose of 50 kGy. One half of the mice fed the AIN-76 diet died within two weeks and the surviving animals were also in poor health, while 91% of mice fed the AIN-93M diet survived. No hemorrhage was observed grossly in any organs of the surviving animals. Histologically, degeneration with inflammatory cell infiltration was observed as well as hemorrhage and fibrosis in the heart muscles of mice fed the AIN-76 diet. No microscopic lesions were observed in the other organs. Prothrombin time (PT) and activated partial thromboplastin time (APTT) were extremely prolonged when mice were fed the AIN-76 diet. The animals totally recovered when they were intragastrically administered 1 μ g/day of vitamin K₃ from the third day of feeding of the AIN-76 diet, except for PT and APTT which were still slightly longer than in mice fed the AIN-93M diet. The concentration of vitamin K₃ supplied in the AIN-76 diet decreased to an undetectable level after γ -irradiation, while the AIN-93M diet contained 240 μ g/kg of vitamin K₁. These results indicate that the deaths of the germfree mice fed the γ -irradiated AIN-76 diet were caused by vitamin K deficiency. Vitamin K deficiency may cause fatal degeneration of cardiac muscle cells.

Key words: germfree mice, myocardium, purified diet, vitamin K deficiency

Introduction

Mortality attributable to spontaneous hemorrhagic diathesis has been reported mainly in hysterectomy-

derived mouse or rat colonies and vitamin K deficiency has been suggested to be the cause of this condition [1, 5]. When we fed the sterile purified diet of AIN-76 formula to a group of germfree mice, acute death was

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observed. Bleeding from injuries was noted in the suffering animals. Although we did not observe gross hemorrhage, including hemothorax, which has been reported to be a common alteration of the affected animals, at necropsy, vitamin K deficiency was suspected to be the cause of death and the present study was conducted to confirm this.

Materials and Methods

Animals

Germfree BALB/cA mice were bred at our laboratory in flexible vinyl isolators in a room at 24°C, relative humidity of 60% and 12 h light/dark. Mice were originally from Central Institute for Experimental Animals (Kanagawa, Japan). Animals were fed CMF pelleted diet (Oriental Yeast Co., Tokyo, Japan) sterilized by γ -irradiation at a dose of 50 kGy. Each group of 10–11 male mice was transferred into an autoclavable stainless steel isolator at 20–24 weeks of age.

Diet and experiment

Purified standard diets of AIN-76 and AIN-93M formula were pelleted and sterilized by γ -irradiation at a dose of 50 kGy by the Oriental Yeast Co., Tokyo, Japan. The compositions of the AIN-76 and AIN-93M diets and vitamin mixtures are shown in Tables 1 and 2. The concentrations of vitamin K₃ in the AIN-76 diet and vitamin K₁ in the AIN-93M diet were measured by the Japan Food Research Laboratories (Tokyo, Japan) using HPLC with a L-column ODS (150 × 4 mm, Chemical Evaluation and Research Institute, Tokyo, Japan). The column was maintained at 40°C. Vitamin K₁ was eluted with 0.5 ml/min of 0.2% sodium perchlorate/acetonitrile (1:1, v/v) and detected with an UV detector (SPD-10AV, Shimadzu, Kyoto, Japan) at wavelength of 254 nm. Vitamin K₃ was eluted with 0.8 ml/min of methanol and detected with a RF-10A_{XL} fluorescence detector (Shimadzu). The excitation wavelength was 320 nm and the emission wavelength was 430 nm. A platinum reduction column (4.0 mm × 15 mm) was located between the HPLC column and the fluorescence detector for post-column reduction. The lipid peroxide value of the diets was also measured by the Japan Food Research Laboratories using the acetate-chloroform method. At the start of the experiment, the diet of each group of mice was changed to the AIN-76 or AIN-93M

Table 1. Compositions of AIN-76 and AIN-93M purified diets (%)

	AIN-76	AIN-93M
Casein	20.0	14.0
DL-Methionine	0.3	
Cornstarch	15.0	46.5692
Dextrinized cornstarch		15.5
Sucrose	50.0	10.0
Cellulose powder	5.0	5.0
Corn oil	5.0	
Soybean oil		4.0
Vitamin mixture	1.0	1.0
Mineral mixture	3.5	3.5
L-Cystine		0.18
Choline bitartrate	0.2	0.25
Tert-butylhydroquinone		0.0008

Table 2. Composition of vitamin mixture for AIN-76 and AIN-93M diets (%)

	AIN-76	AIN-93M
Vitamin A (500,000 IU/g)	0.08	0.08
Vitamin D ₃ (500,000 IU/g)	0.02	0.02
Vitamin E (500 IU/g)	1.00	1.50
Vitamin B ₁	0.06	0.06
Vitamin B ₂	0.06	0.06
Vitamin B ₆	0.07	0.07
Vitamin B ₁₂ (0.1% cyanocobalamin in mannitol)	0.10	0.25
Nicotinic acid	0.30	0.30
D-Calcium pantothenate	0.16	0.16
Folic acid	0.02	0.02
D-Biotin	0.002	0.002
Vitamin K ₁		0.0075
Vitamin K ₃	0.0005	
Sucrose, finely powdered	97.9495	97.2125

diet which the animals accessed freely. One of the two groups fed the AIN-76 diet was administered 1 μ g/day of menadione (vitamin K₃) intragastrically from the third day of the AIN-76 diet. The condition of the animals was observed daily. After 14 days of the experimental diets, the surviving animals were sacrificed and whole blood was withdrawn from the caudal vena cava. One tenth the volume of the whole blood of 3.8% sodium citrate solution was added to the whole blood and plasma was separated. Plasma prothrombin time (PT) and activated partial thromboplastin time (APTT) were measured with commercial kits (Thromborel® S and Pathromtin, Dade Behring Marburg, Marburg, Ger-

many). The brain, heart, lungs, liver, stomach, large and small intestines, spleen, kidneys and testes were collected and fixed in 10% buffered formalin for further histopathological investigation. The tissues were embedded in paraffin, sectioned at 4 μ m and stained with hematoxylin and eosin.

Statistical analysis

Survival rates of the groups were compared by the χ -square test and the mean values were analyzed by Student's *t*-test.

Results

General conditions and survival of the mice

Mice fed only the AIN-76 formula diet without vitamin K treatment exhibited non-specific clinical signs from the third day of feeding. The animals appeared depressed and lethargic, with hunched backs and an ungroomed appearance. Bleeding from injuries was also apparent in some animals. The mice in this group started to die from the eighth day of feeding and only half of the animals had survived at the end of the experiment, after 14 days of feeding (Fig. 1). The surviving animals were also in poor health. Mice fed the AIN-76 diet recovered when they were intragastrically administered 1 μ g/day of vitamin K₃ from the third day of the diet and all animals in this group survived for 14 days. In the group of mice fed the AIN-93M diet, one of 11 animals died on the sixth day but the rest of the animals survived for 14 days. The survival rate after 14 days of the mice fed only the AIN-76 diet without vitamin K treatment was significantly lower than those of the other groups ($P < 0.05$).

Gross pathologic findings

No apparent lesions, including hemorrhage, were observed grossly in any organs of the surviving animals in the three groups at autopsy after 14 days of feeding. Necropsies were also performed on some of the dead mice that were not decomposed or cannibalized, but no gross lesions, including hemorrhage in the intestinal tract and hemothorax, were observed.

Histopathological findings

Histologically, degeneration of the cardiac muscle cells with hemorrhage, infiltration of inflammatory cells

Fig. 1. Survival rate of germfree mice fed a purified diet of AIN-93M or AIN-76 formula with or without intragastric administration of vitamin K₃. ○: AIN-76 diet, ●: AIN-76 diet and oral vitamin K₃, ■: AIN-93M diet.

and proliferation of collagenous fibers in the interstitium were observed in the ventricular myocardium of mice fed the AIN-76 diet (Fig. 2A). These lesions were mainly distributed in the subepicardial myocardium. No microscopic lesions were observed in the other organs. Microscopic lesions were not found in mice fed the AIN-76 diet and administered vitamin K₃ from the third day of feeding, although the myocardium showed slight edematous loosening of the interstitial tissue (Fig. 2B). No microscopic lesions were observed in mice fed the AIN-93M diet.

Hematological findings

Plasma PT and APTT were extremely prolonged in mice fed the AIN-76 diet without vitamin K treatment, and the plasma did not coagulate within 300 s in both tests. PT and APTT were dramatically shortened to 11.5 ± 2.4 s and 45.9 ± 23.3 s by intragastric vitamin K₃ administration, respectively. However, both PT and APTT in mice fed the AIN-76 diet and administered vitamin K₃ were still significantly longer than in mice fed the AIN-93M diet (8.9 ± 1.7 s and 26.3 ± 3.5 s, respectively, $P < 0.05$).

Analysis of the diets

The concentration of vitamin K₃ in the AIN-76 diet, which was 50 μ g/kg in the formula, decreased to an undetectable level after pelleting and irradiation (Table 3). The concentration of vitamin K₁ in the AIN-93M diet was maintained at 240 μ g/kg even after irradiation; it was added at a concentration of 750 μ g/kg in the formula. Lipid peroxide values were comparable in the

Fig. 2. Histopathological findings of the left ventricle of the heart of germfree mice fed a purified diet of AIN-76 formula with or without intragastric administration of vitamin K₃. H & E stain. Bar = 150 μ m. A: AIN-76 diet, B: AIN-76 diet and intragastric vitamin K₃. Degeneration with hemorrhage, infiltration of inflammatory cells and fibrosis were observed in the subepicardial myocardium of mice fed the AIN-76 diet without vitamin K treatment. Lesions were not found in mice fed the AIN-76 diet and orally administered vitamin K₃, although the myocardium showed slight edematous loosening of the interstitial tissue.

Table 3. Concentrations of vitamin K and lipid peroxide in the purified diet of AIN-76 and AIN-93M formula after 50 kGy γ -irradiation

(/kg)	AIN-76	AIN-93M
Vitamin K (μ g)	ND ^{a, b}	240 ^c
Lipid peroxide (meq)	60.7	62.7

^aNot detected. ^bAs vitamin K₃. ^cAs vitamin K₁.

AIN-76 and AIN-93M diets, and were 60.7 and 62.7 meq/kg, respectively.

Discussion

A disease characterized by spontaneous hemorrhage and death was first described as an epizootic disease of mice in 1943 at the University of Wisconsin [4]. Several other reports followed [5, 9, 11]. A variety of factors other than vitamin K deficiency secondary to altered intestinal microflora of this disease have been suspected of causing or contributing to the outbreaks [5], including infectious agents [4], ethylene glycol contamination of cage bedding [11] and increased dietary lipoperoxides complicated by vitamin K and/or E deficiency [9]; however, the factor(s) responsible for the disease were not fully identified in these reports. Allen *et al.* [1] first described the mortality of mice attributable to hemorrhagic diathesis in NIH Genetic Resource colonies in 1971. They concluded that the cause of hemorrhagic cardiomyopathy and hemothorax was vitamin K deficiency based on controlled studies using germfree mice given a vitamin K-free diet and conventional mice given warfarin in their diet.

In the present study, the mortality rate of germfree mice fed the γ -irradiated diet of AIN-76 formula was 50%, which was significantly higher than that of the mice fed the AIN-93M formula diet. PT and APTT were extremely prolonged in the surviving mice fed the AIN-76 formula diet compared to the mice fed the AIN-93M formula diet. Although the AIN-76 formulation contains 50 μ g/kg of menadione (vitamin K₃), the diet with the AIN-76 formula contained no detectable amount of vitamin K₃ after pelleting and irradiation. Mortality and symptoms were decreased when the mice fed the AIN-76 formula diet were intragastrically administered 1 μ g/day of menadione. The lipid peroxide concentrations of both diets were comparable. These

results suggest that the death of germfree mice fed the γ -irradiated AIN-76 diet was caused by vitamin K deficiency, although it is reported that casein, 200 g/kg in the diet, contains an average of about 800 $\mu\text{g/kg}$ vitamin K_1 and an additional 160 $\mu\text{g/kg}$ of vitamin K_1 was added to the AIN-76 diet [13].

In 1973, an ad hoc committee was formed by the American Institute of Nutrition (AIN) to identify the dietary standards for nutritional studies with laboratory rodents. The committee has established guidelines to help scientists with limited experience in experimental nutrition. The diet following the committee's recommendation was designated the AIN-76 diet [2]. Although the AIN-76 diet has given good results in terms of animal growth, appearance and survival, a few problems, possibly including excessive destruction of menadione or interference with its utilization with a resultant vitamin K deficiency, have been reported [3]. The committee then made the recommendation that the amount of vitamin K should be increased 10-fold to 500 $\mu\text{g/kg}$, and the modified diet has been designated AIN-76A [3]. Based on further discussion on the reported nutritional and technical problems encountered with the AIN-76 and AIN-76A diets at a workshop held in 1989 and their recommendations [12], two new formulations, AIN-93G for growth, pregnancy and lactation, and AIN-93M for adult maintenance, were proposed and adopted [13]. Although no changes in vitamin K were suggested at the 1989 workshop [12], the amount of vitamin K was arbitrarily set at 750 $\mu\text{g/kg}$ diet and phylloquinone (vitamin K_1) was used instead of menadione as the form of vitamin K. Thus, although these revised formulations of standard purified diet for laboratory rodents have been established and contain more vitamin K, the AIN-76 diet remains a well-known standard diet and researchers should take care to use purified diets, especially when they use germfree animals.

Although hypoprothrombinemia due to vitamin K deficiency is occasionally observed in newborn infants and anticoagulant drug- or antibiotic-treated adults, primary vitamin K deficiency is uncommon in both animals and humans because vitamin K is distributed widely in plant and animal tissues, and intestinal flora synthesize menaquinones in amounts that probably supply the nutritional requirement. Therefore, vitamin K deficiency has often been studied in rats or mice fed a

special diet deficient in vitamin K [6, 7, 10, 14]. Komai *et al.* [8] proposed that germfree mice fed a vitamin K-deficient diet were a useful model for studying hemorrhagic diseases without metabolic changes by the administration of antibiotics or vitamin K antagonists. In the present study, we produced vitamin K deficiency merely by feeding a commercially available purified diet with AIN-76 formula, pelleted and irradiated, to germfree mice.

Histologically, mice fed the AIN-76 diet for 14 days in the present study showed myocardial degeneration with hemorrhage, infiltration of inflammatory cells and fibrosis in heart ventricles. In previous studies, multifocal hemorrhage and necrosis with varying degrees of acute inflammation and fibrosis have been reported in vitamin K-deficient mice [1, 5, 9, 11]. Coagulation defects, including prolonged PT, have also been reported [5, 11]. These findings are similar to those found in the present study; however, the key findings in previous studies were hemorrhage in various organs, especially hemothorax, which was not observed in the present study. No gross hemorrhage was observed in any organ. Lesions were found only in the heart muscle both grossly and histologically. The results of the present study indicate that vitamin K deficiency may primarily cause fatal myocardial degeneration with hemorrhage. The mechanisms by which vitamin K deficiency causes myocardial degeneration with hemorrhage and the clinical importance of such lesions due to vitamin K deficiency should be studied further.

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