Content of Phyloquinone and Menaquinone in the Tissues of Mummichog Fundulus heteroclitus Fed Diets Containing Different Forms of Vitamin K

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Summary The contents of vitamin K in the plasma and tissues (kidney, liver and gonad) of mummichog Fundulus heteroclitus fed diets supplemented with different vitamin K groups were determined. The vitamin K mainly detected in the gastrointestinal tract of each experimental group was the one supplemented in the respective diet, and all other forms of vitamin K were observed at low concentrations. This implies that the main vitamin K source for mummichog is their food. Further evidence that the main vitamin K source is the food is that the elevation of vitamin K concentrations in the plasma and other tissues in this experiment was brought about by vitamin K added to the feed. The phyloquinone-rich diet raised the phyloquinone concentration in the plasma and the tissues much higher than the diets supplemented with short and/or long chain menaquinones. This indicates that phyloquinone is more easily accumulated into the body of fish than the menaquinone homologues. There were apparent differences in absorption and deposition of vitamin K between females and males. This may be a factor in the high mortality in male mummichog during the spawning season but further clarification of the causes of the mortality is required.

Key Words phyloquinone, menaquinone, tissue distribution, diet, fish

The dietary necessity and physiological roles of vitamin K are fairly well established for mammals (1) and its importance in bone health has recently been a focus for research (2). However, it is still not clear whether dietary supplementation of this vitamin is indispensable for the growth of fish because contradictory results have been reported between fish species (3–6). Lately, we have found that like in the amago salmon, its deficiency leads to high mortalities in mummichog, but only in males during the spawning season (7). Thus, determination of any difference in the mechanism of absorption and deposition of the various vitamin K derivatives into the body between females and males might be a first step to elucidate the roles of vitamin K in fish.

Vitamin K is a general term for several derivatives such as phyloquinone (PK), menaquinone (MK) and menadione (MD). PK, called vitamin K1, is produced by plants. MK, called vitamin K2, is of bacterial origin, and has a homologous series differing in the length of the side chain made up by repeated isoprene units expressed as the MK-n series. MD is vitamin K having no side chain, and is called vitamin K3. The water soluble salt of MD, menadione sodium bisulfite (MSP), is commonly added to commercial feeds for domestic animals including fish. PK, MK and MD are known to be converted partly into MK-4 in the tissues of calves (8). Similarly, PK has been shown to be converted into MK-4 in mice and chickens (9), and MD into MK-4 in cod (10). The distribution pattern of vitamin K in the tissues of fish has been reported to differ with the feeding habits among fish species and the PK content is usually much higher than MK in wild fish (11–13). When the PK content in the gastrointestinal tract is high, then a high PK level is always detected in the liver, while even though relatively large amounts of MKs are found in the gastrointestinal level, not all forms of MKs are detected in fish. However, the mechanism and rate of absorption of the various vitamin K groups have not been clarified yet in fishes.

This paper describes the concentrations of the vitamin K derivatives in the tissues of male and female mummichog after being fed diets supplemented with different forms of vitamin K as a first step to elucidate the physiological role of vitamin K in fish.

MATERIAL AND METHODS

Fish and diets. Mummichog Fundulus heteroclitus were fed a commercial diet for common carp in an outdoor tank of 4.5 m3 (sea water, depth: 1.5 m). All the experimental fish were acclimated to the experimental conditions described below by being fed a vitamin K deficient diet for three weeks prior to the start of the experiment. The experimental fish (average body weight of 18.1 g) were divided into seven groups of 3 pairs each. Each group was transferred to respective 20 L glass aquariums placed in the laboratory and supplied with running natural seawater. The experimental fish were reared in a 3 mm mesh screen caged placed in the aquar-
Table 1. Composition of the experimental diet.

| Ingredient (g/100 g) | No. 1 Group 1 | No. 2 Group 2 | No. 3 Group 3 | No. 4 Group 4 | No. 5 Group 5 | No. 6 Group 6 | No. 7 Group 7 |
|---------------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|
| Vitamin-free casein*1 | 30           | 30           | 30           | 30           | 30           | 30           | 30           |
| Gelatin*2           | 5            | 5            | 5            | 5            | 5            | 5            | 5            |
| Dextrin             | 30           | 30           | 30           | 30           | 30           | 30           | 30           |
| Feed oil*3          | 8            | 8            | 8            | 8            | 8            | 8            | 8            |
| Mineral mix*4       | 4            | 4            | 4            | 4            | 4            | 4            | 4            |
| Vitamin mix*5       | 4            | 4            | 4            | 4            | 4            | 4            | 4            |
| Calcium lactate     | 0.08         | 0.08         | 0.08         | 0.08         | 0.08         | 0.08         | 0.08         |
| Sodium phosphate, monobasic | 0.48    | 0.48         | 0.48         | 0.48         | 0.48         | 0.48         | 0.48         |
| CMC*6               | 5            | 5            | 5            | 5            | 5            | 5            | 5            |
| Cellulose           | 13.44        | 13.44        | 13.44        | 13.44        | 13.44        | 13.44        | 13.44        |
| Vitamin K (mg/kg)   |              |              |              |              |              |              |              |
| Phylloquinone       | —            | 1            | —            | —            | —            | —            | —            |
| Menaquinone-4       | —            | —            | 1            | —            | —            | —            | —            |
| Menaquinone-6       | —            | —            | —            | 1            | —            | —            | —            |
| Menaquinone-7       | —            | —            | —            | —            | 1            | —            | —            |
| Menaquinone-8       | —            | —            | —            | —            | —            | 1            | —            |
| Menaquinone-9       | —            | —            | —            | —            | —            | —            | 1            |

*1: (Lot no. ECF7046) Wako Pure Chemical Industries (Osaka, Japan).
*2: DIFCO Laboratories (Detroit, USA).
*3: (Lot no. SN-3850) Riken Vitamin Co., Ltd. (Tokyo, Japan).
*4: Ca-pantothenate.
*5: The premix reported by the National Research Council was partly modified as follows (mg/4 g premix): thiamin HCl 5, riboflavin 20, pyridoxine HCl 5, choline chloride 500, niacinamide 75, calcium pantothenate 50, inositol 15, folic acid 1.5, ascorbic acid 100, alpha-tocopherol 40, vitamin B12 0.01, activated 7-dehydrocholesterol 200 IU, retinol 3,200 IU.
*6: Carboxymethylcellulose- Na.

ium so that they were unable to eat their own feces and eggs. The water temperature was kept at 20°C and the laboratory was lit with fluorescent lights for 13 h from 7:00 throughout the experimental period.

The basal diet was designed according to Murai et al. (14). Fish in each group were fed an experimental diet respectively supplemented with different vitamin K derivatives (Table 1). No. 1: a vitamin K deficient diet; Nos. 2 to 7: supplemented with PK, MK-4, -6, -7, -8 and -9 at a ratio of 1 mg/kg, respectively. Experimental diets were given at 2% of the body weight per day for 12 d.

Approximately 3 h after the final feeding, samples of blood, kidney, liver, gonad and gastrointestinal tract were collected from individual fish to analyze the content of vitamin K derivatives in each specimen.

Chemicals and analysis of vitamin K. Authentic PK was purchased from Sigma Chemical Co. (St Louis, MO). Authentic MK-4 was kindly provided by Eisai Co. (Tokyo, Japan), and MK-6, 7, 8 and 9 by Nippon Roche Tokyo (Tokyo, Japan). Ethanol, methanol and n-hexane, HPLC grade, were purchased from Wako Pure Chemical Industries (Osaka, Japan).

PK and MK were analyzed by HPLC using a Pt column by the same method as previously described (12). Samples of both plasma and kidney were separately pooled prior to measurement. The plasma and tissue samples were homogenized with a mixture of n-hexane, H2O and ethanol (6 : 1 : 4). After the homogenate had been shaken vigorously and allowed to stand, the separated solvent layer was pre-treated by passing it through a Sep-Pak silica cartridge (Waters, Milford, MA, USA) using a n-hexane-ether mixture (93 : 7). The eluate was evaporated and the residue dissolved in 200 μL ethanol. An aliquot of this solution was subjected to HPLC.

HPLC methods. The HPLC system used was composed of a pump (Hitachi L-6000, Japan), a chromatograph-integrator (Hitachi D-2500), a fluorescence spectrophotometer (Hitachi F-1050), a Cosmosil 5C18-Ar column (4.6×100 mm, Nacalai Tesque Inc., Kyoto, Japan) and a platinum oxide column (4×20 mm, Eicom Co., Kyoto, Japan) as a reduction column. Methanol containing 2% sodium perchlorate was used as the mobile phase, after being deaerated by bubbling with argon gas. The flow rate was adjusted to 0.5 mL/min, and vitamin K detection was performed fluorometrically at an excitation wavelength of 254 nm and at an emission wavelength of 430 nm (15). The peaks were identified by comparison of the retention times with those of the standards and disappearance of peaks with reduction.

RESULTS

The contents of PK and MKs in the gastrointestinal
tract which were collected at about 3 h after the final feeding of the respective diets are shown in Fig. 1. The contents of the various vitamin K derivatives reflected almost exclusively the dietary supplements even though there were some differences in their levels. These levels were somewhat higher in females than in males, especially for Group 5. Also, a small but significant quantity of MK-7 was detected in the females only of Group 1 although both females and males of this group were fed the vitamin K-free diet for 33 d including the acclimatization period.

The concentrations of PK and MKs in the plasma, kidney, liver and gonad are shown in Fig. 2 for females and Fig. 3 for males. In both females and males, eating the vitamin K-free diet for an additional 12 d (control group) made no change in the concentrations of vitamin K in the plasma and tissues which consisted only of PK and MK-4. The plasma levels of the various vitamin K derivatives, which are a certain indication of absorption from the intestine, differed according to the vitamin K consumed, and between females and males. The plasma level of PK in Group 2 fed the PK supplemented diet was 30 ng/mL in females but only about 1/3 of that value in males. Values of the MK derivatives were much lower than the PK value not only in males but also in females. On the whole, the values for females were much higher than the corresponding values for males. MK-9, having the longest side chain, was not detected at all in the plasma.

Fig. 1. Phyloquinone and menaquinone contents in the gastrointestinal tract contents of each group of fish. Values are means±SE of 3 fish.

Fig. 2. Phyloquinone and menaquinone contents in four different tissues of female mummichog of each group of fish. Control fish (no vitamin K added) and each vitamin K added to the experimental diet. Values are means±SE of 3 fish.
PK was accumulated in the kidney, liver and gonad of fish fed the PK-supplemented diet in both females and males. Reflecting the plasma level, the PK level in the kidney of females was much higher than that of males, but this was not true for the liver and gonad. In the case of males, the contents of MK-4 in the liver were highest even in the groups fed the other derivatives, while, MK-6, MK-7, and MK-8 were more clearly detected in the tissues of females than in males. However, MK-9 was not detected at all in any tissues examined.

PK and MKs which were not supplemented in the diet were detected in the tissues of almost every dietary group at similar levels as prior to the experiment and of the control group (Group 1).

DISCUSSION

As shown in Fig. 1, the major vitamin K in the gastrointestinal tracts of each experimental group was that administered in the feed to the respective group and only minute amounts of the other homologues were recognized in each group. This clearly indicates that the major vitamin K source is almost entirely of dietary origin.

The fish were found to contain very small amounts of PK and MK-4 in the plasma at the beginning of the experiment. Because the experimental fish were fed a commercial diet with MSB until the start of feeding the experimental diets, the small amount of MK-4 detected in all groups should be taken into account. The plasma levels of vitamin K derivatives shown in Figs. 2 and 3 imply that PK is the most efficiently absorbed into the blood in both females and males because its level was much higher than those of the MK derivatives. This result agrees well with Birgit and Suttie who reported that the PK level in the plasma of rats fed a PK-supplemented feed was about three times higher than rats fed a MK-9 supplemented feed (16). Ichihashi et al. (17) described that the absorption rates of MKs decrease markedly with an increase in the number of isoprenoid units in rats. Akiyama et al. pointed out that the number of isoprene units of MK is an important factor in its absorption and incorporation into the liver in rats (18). No clear relationship between the number of isoprene units and absorption was detected in this study. However, the side chain of MK-9 may be too long for mummichog to absorb it into the blood, because MK-9 was not detected at all in the plasma of either females or males.

As to the difference in the effectiveness in vitamin K activity, Groenen-van Dooren et al. observed that MK-9 was definitely more active than PK and MK-4 in rats (19). Thijssen et al. reported that MK-4 rather than PK may be the functional vitamin in rats (20). From the viewpoint of the effective accumulation of vitamin K in the tissues, PK can be considered to be a more available vitamin K in fish than any of the MK homologues and
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MD, differing from the case of rats. This view may be supported by the fact that the major vitamin K detected in the body of fish is generally PK (11–13). There is a report that the PK and MK contents in the bodies of larvae differ depending on their feeds, and PK is more suitable than MSB for fish bone health (21). Therefore, further studies on the differences in the mechanism of absorption of PK and MKs are needed with respect to the supplementation of commercial feed for fish culture with vitamin K.

Judging from the vitamin K derivatives in the tissues, PK and MK-4 may play a major role in males as has been reported in wild fishes (12). However, not only PK but also other MKs except MK-9 may play a certain important role in females. There were also apparent differences in the absorption of PK and MK-4, and in the accumulation of MKs in the tissues, especially MK-4 in the liver, between females and males. The high mortality in mummichog occurred only in males during the spawning season, unlike amago salmon (7). However, these differences in absorption and deposition of vitamin K between males and females alone cannot explain the high mortality in males. Thus, we need further studies to elucidate the relationship between the high mortality in males and the role of vitamin K in mummichog.

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