The effect of dietary vitamin K (phylloquinone and menadione) levels on the vertebral formation in mummichog Fundulus heteroclitus

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SUMMARY: The effect of different dosages of vitamin K, either as phylloquinone or menadione sodium bisulfite (MSB), on vertebral formation during growth of mummichog Fundulus heteroclitus was investigated. Diets without vitamin K caused a significantly higher incidence of bone deformity in fish than diets supplemented with MSB or phylloquinone (P<0.01). These results indicate that vitamin K is essential for mummichog. A massive dose of MSB caused a high incidence of bone deformity compared with a lower dose, while an increased dose of phylloquinone brought about no significant difference in deformity incidence. The vertebral deformity was significantly higher (P<0.01) in fish fed a vitamin K-free diet (Control Group) and MSB-supplemented diet (Group MSB25) compared to fish fed a phylloquinone-supplemented diet (Group PK1). These results indicate that phylloquinone is more suitable than MSB as a vitamin K source in fish feed.

KEY WORDS: larvae, menadione sodium bisulfite, phylloquinone, vertebral formation.

INTRODUCTION

The biological function of vitamin K was only known as a cofactor of blood coagulation. Recently however, an important role of vitamin K in healthy bone development has been revealed in mammals. A residue-specific carboxylation of proteins is a known function of vitamin K and calcium-binding proteins such as osteocalcin and matrix-Gla protein also require vitamin K for their carboxylation. Thus, the importance of vitamin K in mammalian bone development has drawn the interest of many researchers, while no reports have dealt with the effect of vitamin K deficiency in fish bone. There are some reports describing vitamin K nutrition in fish. Poston reported on brook trout that the blood coagulation time was delayed and the microhematocrit value was lowered by vitamin K deficiency, but no apparent influence on the growth rate was observed. Kitamura et al. also reported that, in rainbow trout Oncorhynchus mykiss, no perceivable syndrome was caused by vitamin K deficiency, and only a tendency toward anemia was caused by this deficiency. As for catfish Ictalurus punctatus, no symptom of vitamin K deficiency was observed. On the other hand, Taveekijakarn et al. reported that mortality in vitamin K-deficient amago Oncorhynchus rhodurus reached 50%. There is another report that the fish was affected by a deficiency in vitamin K during spawning season. However, as the participation of vitamin K in fish bone development had not been investigated, one of the purposes of this study was to elucidate the effect of vitamin K deficiency on bone development in fish, using mummichog Fundulus heteroclitus as an experimental animal.

A variety of naturally occurring vitamin K compounds are known, including phylloquinone (PK), which is of plant origin, and menaquinones (MK), which are of bacterial origin. Furthermore, synthetic vitamin K exists and there are many different derivatives. They have the same naphthoquinone structure but different aliphatic side chains. Menadione is generally used for fish feeds as a vitamin K supplementary agent in the form of a water-soluble sodium bisulfite derivative (MSB). Menadione, a synthetic product of vitamin K, is not itself biologically active. The majority of menadione taken in is easily excreted in rat, and only a part becomes effective after being converted into...
an active form, MK-4, in the liver in rat\textsuperscript{10} and in cod.\textsuperscript{11} Hence, in practice, MSB is added to feed in excess. However, it has been reported that menadione and its analogs are toxic to certain animals (mice, rats, horses and humans) and cause abnormalities in the liver, kidneys and lungs, as well as hemorrhage, hemolytic anemia and other physiological abnormalities.\textsuperscript{12-15} In nature, fish ingest PK and/or MK-4 as the vitamin K source and it is also known that PK and MK-4, particularly PK, are accumulated by certain fish species in the liver at considerably high concentrations.\textsuperscript{16-18} However, no study has investigated the toxicity of PK and MK to fish. Therefore, another purpose of the present study was to estimate suitable doses of PK and MSB for mummichog.

**MATERIALS AND METHODS**

**Experimental diets and fish**

Approximately 100 mature mummichog were fed a commercial diet supplemented with MSB in a laboratory aquarium (60 L) in order to produce larvae for the experiment. Spawned and fertilized eggs were collected and kept in a fine-net cage which was set in flowing natural seawater in an aquarium until hatching. Some 2000 larvae with an average bodyweight of 2.2 mg were divided into five groups of 400 each. Each group was kept in an 8 L indoor aquarium. Flow rate was about 0.4 L/min. The water temperature was kept at 22\degree C throughout the experiment. Larvae in each group were fed for 4 weeks on experimental diets differing in vitamin K source and content. Vitamin K was not supplemented to the Control Group diet. Diet MSB25 and MSB2500 are supplemented with MSB at concentrations of 25 and 2500 mg/kg, respectively. Diet PK1 and PK100 are supplemented with PK at concentrations of 1 and 100 mg/kg, respectively (Table 1). These diets were given every hour between 07:00 and 18:00. Illumination of the laboratory was controlled with fluorescent lamps for a 13 h light photoperiod from 06:30. The experiment was run in two replicates.

After 4 weeks of rearing, all fish were starved for 20 h, and 50 fish (4-week-old fish) from each group were taken for vitamin K content analysis. Forty of the 2-week-old fish and 20 of the 4-week-old fish were removed from each group, and the remaining fish were checked for vertebral abnormalities.

**Observation and analysis of vertebral abnormalities**

The skeletal structure was observed by double staining with alizarin red and Alcian blue according to the method of Kawamura and Hosoya,\textsuperscript{19} and

<table>
<thead>
<tr>
<th>Component</th>
<th>Control</th>
<th>MSB25</th>
<th>MSB2500</th>
<th>PK1</th>
<th>PK100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin-free casein\textsuperscript{1}</td>
<td>300</td>
<td>300</td>
<td>300</td>
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<td>300</td>
</tr>
<tr>
<td>Gelatin\textsuperscript{2}</td>
<td>50</td>
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<tr>
<td>Dextrin</td>
<td>300</td>
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<tr>
<td>Feed oil\textsuperscript{3}</td>
<td>80</td>
<td>80</td>
<td>80</td>
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</tr>
<tr>
<td>Mineral mix\textsuperscript{4}</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Vitamin mix\textsuperscript{5}</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>40</td>
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</tr>
<tr>
<td>Calcium lactate</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>Sodium phosphate, monobasic</td>
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<tr>
<td>CMC\textsuperscript{6}</td>
<td>50</td>
<td>50</td>
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<tr>
<td>Cellulose</td>
<td>134.4</td>
<td>134.4</td>
<td>134.4</td>
<td>134.4</td>
<td>134.4</td>
</tr>
</tbody>
</table>

Vitamin K (mg/kg)

| MSB\textsuperscript{7}              | 0       | 25    | 2500    | 0    | 0     |
| Phylloquinone                       | 0       | 0     | 0       | 1    | 100   |

\textsuperscript{1} Lot no. ECF7046; Wako Pure Chemical Industries, Osaka, Japan.
\textsuperscript{2} DIFCO Laboratories, Detroit, MI, USA.
\textsuperscript{3} Lot no. SNo.J-450; Riken Vitamin Company, Ltd., Tokyo, Japan.
\textsuperscript{4} Mc.Collum Salt 820517; Iwai Chemical Co., Ltd., Tokyo, Japan.
\textsuperscript{5} The premix reported by National Research Council\textsuperscript{23} was partly modified as follows (mg/4 g premix): thiamin HCl 5, riboflavin 20, pyridoxine HCl 5, choline chloride 500, nicotinic acid 75, ca-pantothenate 50, inositol 200, biotin 0.5, folic acid 1.5, ascorbic acid 100, alpha-tocopherol 40, vitamin B\textsubscript{12} 0.01, activated 7-dehydro-cholesterol 200IU, retinol 3200IU.
\textsuperscript{6} Carboxymethylcellulose-Na.
\textsuperscript{7} Menadione sodium bisulfite.
examined in detail under a microscope at low magnification. All abnormal cases found were counted so as to calculate the occurrence incidence, and abnormality type was simultaneously classified. The data of abnormality were statistically analyzed by the pairwise chi-squared tests. The abnormality type was statistically analyzed by the t-test or Cochran–Cox test.

**Determination of the vitamin K content in fish bodies**

The PK and MK were determined by high-performance liquid chromatography (HPLC) using a Pt column by the same method previously reported. Fifty test fish from each group and 100 larvae immediately after hatching were pooled separately and homogenized with 5 mL of a mixture of ethanol and water (4:1). Six milliliters of n-hexane was added to each homogenate. After mixing, the mixture was passed through a Sep-Pak silica cartridge (Waters, Milford, MA, USA). The filtrate was evaporated to dryness under reduced pressure at 30°C. The residue was subjected to vitamin K determination using HPLC, after being dissolved in 200 µL of ethanol. The HPLC was equipped with a Cosmosil 5C18-Ar column (4.6×100 mm; Nacalai Tesque, Inc., Kyoto, Japan) and postcolumn reduction by Pt column (4×20 mm; EICOM Co., Kyoto, Japan). According to the method of Shino, vitamin K detection was detected fluorometrically (excitation, 254 nm; emission, 430 nm). The peaks were identified by comparison of the retention times with those of the standards and the disappearance of peaks with reduction.

Authentic PK was purchased from Sigma Chemical Co. (St Louis, MO, USA). Authentic MK-4 was kindly provided by Eisai Co. (Tokyo, Japan). The HPLC grade ethanol, methanol and n-hexane were obtained from Wako Pure Chemicals Industries (Osaka, Japan).

**RESULTS**

**The incidence of abnormalities in vertebrae**

The incidence of abnormalities in the vertebrae and caudal skeleton of mummichog fed diets with differing vitamin K contents in the duplicated experiments is shown in Table 2. The incidence was significantly higher in the Control Group fish, while the incidence in Group PK1 fish (fed a diet supplemented with PK at 1 mg/kg) is considered to be at a low level under these study conditions. The incidence in Group PK100 fish was essentially the
same as that in Group PK1, suggesting that the higher dosage of PK does not affect the incidence of vertebral abnormalities. Furthermore, the incidence in Group MSB25 fish (MSB at 25 mg/kg) was also much the same as that in Group PK1. This finding suggests that the content of MSB of 25 mg/kg in the feed might be appropriate. This view is also supported by vitamin K activity of MSB and its effect on bone formation. In contrast to fish fed a higher dose of PK (100 mg/kg), the fish fed a higher dose of MSB showed a significantly higher incidence of abnormalities. The mortality of fish during this experiment ranged from 18.1% to 40.6%, and did not differ significantly between the groups.

Types of abnormality

Vertebral abnormalities were classified into three types based on appearance (Fig. 1). Although it was not exact morphologically, these three types were termed types A, B and C for convenience. Type A includes abnormalities such as vertebral fusion, vertebral deformity and vertebral row irregularity. Type B comprises neural and hemal spine abnormalities such as fusion, extra-ossification and a combination of the two. Cases which could not be categorized into either type A or B were lumped together under type C. The incidence of the three different types of abnormality is shown in Fig. 2. The incidence of type A abnormalities is apparently significantly high in the Control Group ($P < 0.001$) and Group MSB25 ($P < 0.05$) compared to that in Group PK1, with Group PK1 having the lowest incidence among the five groups. The incidence of type C abnormalities is relatively high in the Control and MSB25 Groups. However, the incidence of type B abnormalities is similar across the five groups.

PK and MK fish body content

Both content and form (PK or MSB) of vitamin K in experimental fish bodies were analyzed to determine differences between fish fed differing experimental diets. The results of this analysis are given in Fig. 3. The vitamin K content and form in the fish
DISCUSSION

The incidence of bone abnormalities is apparently high in fish fed with a vitamin K-free diet, compared with those fed diets supplemented with PK or MSB ($P<0.01$). This result clearly indicates that vitamin K is necessary for normal bone development in mummichog as is the case for mammals. Although it is reported that many factors cause bone abnormalities, it is supposed that vitamin K is one of them.

Wild fish ingest PK as a vitamin K source, while cultured fish are generally given MSB as a vitamin K source. The effects of PK- and MSB-supplemented feed on bone development of mummichog larvae differ in two ways. First, when the incidence of bone abnormalities is compared between fish given either a low dose of PK (1 mg/kg of diet: Group PK1) or MSB (25 mg/kg of diet: Group MSB25), there is no significant difference between the two groups. In contrast, when fish given either a high dose of PK (100 mg/kg of diet: Group PK100) or MSB (2500 mg/kg of diet: Group MSB2500) are compared, the incidence is significantly higher in fish given MSB than in fish given PK ($P<0.05$). Furthermore, the incidence rate between higher and lower doses of PK was not significantly different. These findings suggest that a massive dosage of MSB is harmful to bone development.

Second, the incidence of the different types of abnormalities differed between fish given a lower dose of either PK or MSB (Fig. 2). As noted in terms of results, MSB mainly causes significant ($P<0.01$) type A abnormalities, namely, fusion, deformity and row irregularity of the vertebrae from PK. Although it has been reported that the growth rate of cultured Atlantic salmon *Salmo salar* fed MSB-supplemented feed was lower than that of PK-supplemented feed, the effect of MSB on bone development was not determined in that study. Therefore, more extensive studies on the relationship between dietary MSB supplement and bone health are necessary.

The PK and MK contents in the bodies of larvae differed depending on the diet fed. Figure 3 shows that a PK dose 100 times higher than that of a low dose caused a tissue PK concentration about 50 times higher in fish, although an equivalently large dose of MSB caused only a 2.5 times increase in MK-4 concentration. In wild fish such as sardine *Sardinops melanostictus*, mackerel *Scomber japonicus* and ayu *Plecoglossus altivelis*, considerably large quantities of PK have been observed in the liver, while in cultured fish, fed a diet containing MSB, not as much MK-4 was observed. Although the reason why a large amount of PK, not MK, becomes accumulated in fish livers has not yet been elucidated, it is speculated that PK, rather than MK, plays a significant role in preventing vitamin K deficiency diseases of fish in nature.

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