Pathophysiological Studies on Erythrocytic Inclusion Body Syndrome in Sea-cultured Coho Salmon

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To study pathophysiological changes of sea-cultured coho salmon affected with erythrocytic inclusion body syndrome (EIBS), hemochemical assessments and lipid analyses were carried out using fish from one net-pen during an outbreak of EIBS.

Fish examined were grouped by hematocrit (Ht) values which indicated the disease progression. Few differences in plasma components were observed in fish that were not seriously anemic. In fish with advanced disease (Ht value < 20%), values of plasma total protein, total cholesterol, triglyceride and phospholipid were lower and potassium and GPT activity were higher. A decrease in plasma glucose and increases in plasma sodium, potassium and chlorine were observed in moribund fish. Except for the moribund fish, blood thiobarbituric acid (TBA) values were higher than 20 nmol/ml in almost all of the fish and C22:6 level of liver phospholipids decreased as the disease progressed.

It was considered that the changes in plasma constituent levels in the diseased fish reflected the effects of anemia.

Key words: coho salmon, EIBS, plasma component, fatty acid composition, lipid peroxidation, anemia

Erythrocytic Inclusion Body Syndrome (EIBS) is a viral disease and a major contributor to mortality of coho salmon (Oncorhynchus kisutch) in seawater net-pen culture in Japan (Takahashi et al., 1992a). The epizootiology (Piacentini et al., 1989; Takahashi et al., 1992a), progression of the disease (Takahashi et al., 1992b), susceptibility of salmonid fishes (Okamoto et al., 1992a), resistance to reinfection (Okamoto et al., 1992b) and the relationship between fish growth and the disease progression (Takahashi et al., 1994) have been studied. However, there have been few studies of the physiological effects of EIBS.

In this study, hemochemical assessment and chemical analysis of lipids of the liver were carried out to understand the physiological condition of coho salmon affected with EIBS.

Materials and Methods

Sampling and grouping of the fish and preparation of the materials

Sampling of coho salmon affected with EIBS reared in a seawater net-pen was carried out in Shizugawa Bay, Miyagi prefecture, Japan in April 23, 1987. An outbreak of EIBS in this net-pen was confirmed by the symptoms and detection of erythrocytic inclusion bodies, according to Takahashi et al. (1992a). Water temperature was 8°C at the time of sampling. Five moribund fish with abnormal swimming and 37 fish that came up to feed were caught with a spoon net. Blood samples were obtained from caudal blood vessels with heparinized syringes fitted with 20G needles. Immediately after taking blood, hematocrit (Ht) values were measured by the method of Ikeda et al. (1992). A part of the blood
was frozen on dry ice and kept at $-80^\circ$C for analysis of blood thiobarbituric acid (TBA) values. The remainder of the blood was centrifuged (1,500 $\times$ g, 10 min) to separate plasma. The plasma was kept at $4^\circ$C and used for analyses within 24 h. A portion of the liver was frozen on dry ice and kept at $-80^\circ$C for analysis of hepatic lipids. Takahashi et al. (1992b) reported that Ht values are a good indicator of the progression of EIBS. According to their standards, specimens that appeared healthy were divided into three groups on the basis of Ht values: group I (Ht: greater than 28%), group II (20–27%) and group III (less than 20%). Moribund fish were grouped separately. Results were analyzed by Student’s or Welch’s t-test from the data obtained from individual or pooled samples in each group.

**Analysis of plasma constituent levels and enzyme activities**

Plasma constituent levels, enzyme activity and blood TBA values were determined by the following methods with commercial clinical investigation kits (Wako Pure Chemical Co., Ltd.): total protein, Biuret method; glucose, enzymatic method; total cholesterol, enzymatic method; triglyceride, enzymatic method; phospholipid, enzymatic method; chlorine, Shales-Shales method; alanine aminotransferase (GPT), UV-Kinetic method. Sodium and potassium were determined with a clinical anion meter (CIM 101 type, electrode method; Shimazdu Co., Ltd.).

**Analysis of lipids**

Livers from three fish in each group were pooled and lipids were extracted by the Bligh-Dyer method (Bligh and Dyer, 1959). Phospholipids and neutral lipids were separated by column chromatography (66 cm $\times$ 3 cm, internal diameter) using Biobeads SX-2 and benzol.

Fatty acid methyl esters prepared with boron trifluoride methanol complex (Wako Pure Chemical Co., Ltd.; $100^\circ$C for 5 min) and fatty acid compositions of each lipid were analyzed with a gas-liquid chromatograph (GC-7A, Shimadzu Co., Ltd.) equipped with a flame ionization detector and a glass column (3 m $\times$ 3 mm, internal diameter) packed with Unisole 3000 on Unisep C (80–100 mesh, Gasukuro Kogyo Inc.). Column temperature was kept at $220^\circ$C.

**Results**

Although the fish that came up to feed looked healthy, their Ht values varied from 5.7% to 36.5%. Some fish in group III (Ht < 20%) showed severe anemia and yellowish liver, which are the typical symptoms of EIBS. In addition to these symptoms, all the moribund fish had stomachs filled with mucous liquid with a salinity of 30–31‰.

Few differences in the plasma components were observed in group II, compared with group I. Total protein, total cholesterol, triglyceride and phospholipid were significantly lower and potassium and GPT were significantly higher in group III than in group I ($p < 0.01$). In moribund fish, the changes were more pronounced than in group III. In addition to these changes, plasma glucose decreased to less than 20 mg/dl in moribund fish, and plasma sodium, potassium and chlorine increased, compared with all other groups (Table 1).

Polyunsaturated fatty acid composition of liver phospholipids decreased in the order of group I, group II, group III and moribund fish.

### Table 1. Plasma components of coho salmon affected with EIBS

<table>
<thead>
<tr>
<th>Group</th>
<th>Hematocrit (Ht) value (%)</th>
<th>Total protein (g/dl)</th>
<th>Glucose (mg/dl)</th>
<th>Total cholesterol (mg/dl)</th>
<th>Triglyceride (mg/dl)</th>
<th>Phospholipid (mg/dl)</th>
<th>Sodium (mEq/l)</th>
<th>Potassium (mEq/l)</th>
<th>Chlorine (mEq/l)</th>
<th>GPT (Karmen Unit)</th>
</tr>
</thead>
</table>
| I (12) | 31.4 ± 2.4
| II (15) | 24.6 ± 1.5
| III (10) | 12.3 ± 4.7
| Moribund (5) | 7.5 ± 2.7

* Groups were made by difference of Ht values, according to Takahashi et al. (1992b).

* Numbers of fish examined.

* Data are shown as mean ± standard deviation.

* Student’s or Welch’s t-test; significantly different ($p < 0.01$) against the results in group I.
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Table 2. Main fatty acids composition of phospholipids from the liver of coho salmon affected with EIBS

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Moribund</th>
</tr>
</thead>
<tbody>
<tr>
<td>C16:0</td>
<td>17.2a</td>
<td>17.9</td>
<td>18.6</td>
<td>12.9</td>
</tr>
<tr>
<td>C18:0</td>
<td>7.1</td>
<td>8.6</td>
<td>10.3</td>
<td>8.5</td>
</tr>
<tr>
<td>C18:1</td>
<td>13.8</td>
<td>15.2</td>
<td>17.9</td>
<td>12.8</td>
</tr>
<tr>
<td>C20:4</td>
<td>4.6</td>
<td>4.6</td>
<td>3.9</td>
<td>5.5</td>
</tr>
<tr>
<td>C20:5</td>
<td>7.7</td>
<td>7.9</td>
<td>7.9</td>
<td>9.3</td>
</tr>
<tr>
<td>C22:6</td>
<td>34.1</td>
<td>29.2</td>
<td>23.2</td>
<td>32.4</td>
</tr>
<tr>
<td>Total saturates</td>
<td>28.3</td>
<td>30.7</td>
<td>32.9</td>
<td>25.2</td>
</tr>
<tr>
<td>Polyunsaturates</td>
<td>49.2</td>
<td>44.3</td>
<td>37.9</td>
<td>49.7</td>
</tr>
</tbody>
</table>

a  Groups were made by difference of Ht values, according to Takahashi et al. (1992b). See Table 1.

b  Fatty acid composition data are expressed as a percentage of total area.

Discussion

Hematocrit values of fish that looked healthy varied from 5.7% to 36.5%. This indicates that the population examined in this study included fish that were at different stages of the disease. Piacentini et al. (1989) suggested that horizontal transmission contributes to the occurrence of fish being at different stages of the disease during an epizootic. We were unable to examine fish that were not affected with EIBS as a control, because outbreaks of EIBS were observed throughout the bay at the time of this study. However, it seems reasonable to group fish in different stages of the disease by Ht values because it is well known that Ht values indicate the progression of this disease (Piacentini et al., 1989; Takahashi et al., 1992b).

Severe anemia is a main symptom of this disease. Anemia reduces oxygen transport and causes various alterations in fish. As few differences in plasma constituent levels between groups I and II were observed, it was assumed that fish that were not so seriously anemic were not pathophysiologically affected by the disease. It appeared that the decreases in plasma total protein, total cholesterol, triglyceride and phospholipid observed in group III were due to the progression of the disease. Decreases in these plasma components are often observed in starved fish. As the fish examined in this study came up to feed, it is unlikely that the decrease in the plasma components were caused by starvation. Decreases in the Ht value, plasma total protein and cholesterol were observed in rainbow trout and Atlantic salmon infected with *Renibacterium salmoninarum* (Bruno and Munro, 1986; Bruno, 1986). Bruno (1986) suggested that a decline in serum...
protein and cholesterol could occur through a loss of their production and therefore could be attributed to liver disfunction. Plasma cholesterol and phospholipid of rainbow trout were decreased under the condition of low dissolved oxygen (Maita et al., unpublished data). The decreases in plasma total protein, total cholesterol, triglyceride and phospholipid may be caused by derangement of oxygen transport induced by anemia and/or liver disfunction. The observed increase in GPT activity may be due to the partial necrosis of hepatocytes (Takahashi et al., 1992a), and the increase in plasma potassium seems to reflect the progression of hemolysis. The changes observed in group III are not necessarily the cause of death in diseased fish, because many fish classified into group III in challenge tests recovered (Takahashi et al., 1992b). Homeostasis would be kept even in group III because changes in plasma glucose, sodium and chloride were not observed. In moribund fish, significant increases in plasma sodium and chloride were observed, indicating the occurrence of osmoregulatory failure. These results show that maintaining homeostasis is necessary to recovery from the disease. In viral erythrocytic necrosis (VEN), which is characterized by a severe anemia that is similar to that encountered in EIBS, it was pointed out that a decrease in the ability to regulate serum sodium and potassium was a secondary consequence of the disease (MacMillan et al., 1980; Haney et al., 1992).

Hayakawa et al. (1989) suggested that lipid peroxidation might occur in sea-cultured coho salmon affected with EIBS, and Sakai et al. (1994) showed that erythrocytic superoxide dismutase (SOD) activity, which is induced in tissues that are oxidatively stressed, was induced in infected fish. Lipid peroxidation affects mainly polyunsaturated fatty acids, which can result in profound membrane disorganization and cell injury (Slater, 1984). Fatty acid composition of fish is known to be affected by diet (Greene and Selivonchick, 1987). However, as the results in this study were obtained from fish that were reared in one net-pen, and thus from fish that presumably had the same diet, no differences in fatty acid composition due to diet would be expected. Therefore, it was considered that the decrease in C22:6 of liver phospholipids might be caused by lipid peroxidation. The TBA value, which estimates the malondialdehyde that is produced by decomposition of peroxidized fatty acid, is one of the indicators of lipid peroxidation. The normal range of TBA values in coho salmon has not been reported. Therefore, it is not clear whether the blood TBA values obtained in this study, more than 20 nmol/ml, are higher than normal. Sakai et al. (1994) observed hyperbilirubinemia in coho salmon affected with EIBS. It is known that bilirubin has a beneficial role as a physiological antioxidant of lipid peroxidation (Stocker et al., 1987). The lower blood TBA values and the higher levels of liver C22:6 in moribund fish than in group III may be interpreted as antioxidative effects caused by advanced hyperbilirubinemia in the moribund fish.

It remains to be determined, through artificial infection of fish with EIBS, whether lipid peroxidation accelerates the progression of this disease.

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