Blood Properties of Rainbow Trout in Acute Hepatotoxicity by Carbon Tetrachloride

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The liver injury caused by carbon tetrachloride (CCI₄) injection to dorsal aorta of rainbow trout, Salmo gairdneri irideus, was evaluated by the changes of some blood parameters. Whereas significant changes of plasma constituents by CCI₄ injection were accompanied by the appearance of necrotic area in liver, the hematological values of affected specimens were nearly equal to those of normal ones. The electrophoretic examination showed depression of albumin fraction and lipoproteins in the CCI₄-injected specimens. A compensational increase of certain globulin components in them was noticed. The plasma lipid level was observed to decrease with increasing dose in those specimens. The decrease of lipid was for the most part accounted for by the decrease of triglyceride fraction. No significant change was observed in the plasma cholesterol level.

Little is known about relationships between physical conditions and blood parameters in fish. Liver is specifically affected by carbon tetrachloride (CCI₄), resulting in an acute injury. The studies on hepatic response to the chemical administration were carried out in the yellow tail Seriola quinqueradiata1), rainbow trout Salmo gairdneri2-4), Pacific salmon Oncorhynchus sp.5), and eel Anguilla japonica6-7). These reports describe that the damage of liver cells is accompanied by increasing blood enzyme activities.

It would, therefore, be necessary to know how far the hematological and plasmological changes occur in a liver-injured fish. The physiological parameters would be useful for diagnosing or controlling the health of cultured fish. For the above purpose, the present study was conducted with the rainbow trout.

Materials and Methods

Fish
Two year-old rainbow trout, Salmo gairdneri irideus, had been cultured with fish pellets in an outdoor breeding tank of Kumano Freshwater Biological Station of Hiroshima University.

All fish were transferred in indoor aquaria for a day prior to the administration of a chemical. Feed was withheld prior to sacrifice. After blood sampling, body weight and body length were recorded.

Administration of Chemical
CCI₄ was injected to dorsal aorta of the fish anesthetized lightly with tricaine methansulfonate (MS222). The injection volumes were 0.25 and 0.50 µg/100 g body weight. The second administration was carried out 3 days after the first injection. The effect of the CCI₄-injection was examined 3 days after the second injection.

Blood Collecting
Blood was directly withdrawn by cardiac puncture.

An anticoagulant, Angrot (Nippon Shoji Co., Ltd.), was used. A small amount of the blood was used for hematological measurements. The remainder was centrifuged at 3,000 rpm for 10 min. and the plasma obtained was subjected to the physico-chemical and chemical determinations.

Histological Studies
The paraffin sections of 8 µ in thickness of liver, spleen, and kidney were prepared for microscopic observation, according to Hematoxylin and Eosin staining using Bouin’s solution for fixation.

Frozen sections 10 µ thickness of liver were stained with Sudan black B for the observation of lipid deposition after fixation with formalin-calcium.

Glycogen deposition was observed in liver which was sectioned in 8 µ paraffin fixed with Gendre’s solution and stained with periodic acid-Schiff
Hematological Determination

The heparinized blood was submitted to the determination of hematocrit value and hemoglobin content. For the determination of hematocrit value, the blood drawn into a capillary tube was centrifuged for 10 min. at 11,000 rpm in a micro-hematocrit centrifuge.

Hemoglobin content was measured spectrophotometrically by cyanmethemoglobin method. The mean corpuscle hemoglobin concentration (MCHC) was calculated from hematocrit value (%) and hemoglobin content (g/100 ml) according to the following equation:

\[ \text{MCHC} \% = \frac{\text{hemoglobin}}{\text{hematocrit}} \times 100 \]

Plasmological Determination

Osmotic pressure was measured by a Halbmicro osmometer.

Total plasma protein and lipid were determined by Biuret and sulfo-phospho-vanilline methods, respectively. Albumin and globulin ratio (A/G ratio) was estimated by cellulose acetate electrophoresis and by ammonium sulfate salting-out method (Al-Glo ratio test kit, Kyokuto Seiyaku Kogyo Co., Ltd.). The contents of Na, K, and Ca in plasma were measured by flame-photometrically using a Hitachi 208 atomic absorption photometer.

Separation of Albumin and Globulin Fractions

To the pooled plasma sample was added an equal volume of saturated ammonium sulfate solution, and the mixture allowed to stand overnight in a cold room and centrifuged. The resulting precipitate was designated globulin fraction, and the soluble protein albumin fraction.

Electrophoresis

The plasma sample (3 µl) was spotted on a film (Sartorius-Membranfilter) of 7 cm length and 1.5 cm width. Migration was carried out in a field strength of 0.5 mA per cm width, in veronal buffer of pH 8.6 and ion strength of 0.06. Protein bands were detected by Ponceau 3R staining. Disc electrophoresis was done by 7.5% acrylamide gel.

Protein and lipoprotein were visualized by Coomassie blue and Sudan black B stainings, respectively.

Lipid Analysis

Plasma lipid composition was analyzed by thin-layer chromatography according to the following method. The pooled plasma of each group was directly spotted on a silica gel plate (E. Merck, Darmstadt) by the procedure of Marinetti and Srotts. The development was followed by the method of Sohdi and Gould. Namely, the plate was firstly developed by the solvent system for phospholipid separation (chloroform-methanol-water, 65:25:4, v/v/v) until the solvent front reached to the center of the plate. Then the plate was air-dried and developed further for the separation into lipid classes with a mixture of petroleum ether-ether-acetic acid (80:20:1, v/v/v) until the solvent front reached to the top. The lipid fractions were detected by spraying potassium dichromate saturated 70% sulfuric acid, and charring at 120°C.

Quantitative Measurement

Quantitative analysis of electrophoretic pattern and thin-layer chromatogram were conducted by an Ozumor densitometer 82 (Asuka Kogyo Co., Ltd.).

Results

Immediately after the CCl₄ injection, a part of the fish surface was temporarily darkened. This phenomenon was suggestive of an incoordination of pigment control, as observed in the case of yellow tail. With the increasing dose, mortality of the fish was increased. The accumulated mortalities of two experimental groups were 21 and 62%, respectively. Experimental results were obtained using the survival fish, of which nature was tabulated in Table 1.

Table 1. Nature of rainbow trout analyzed

<table>
<thead>
<tr>
<th>Chemical (µl/100g B.W.)</th>
<th>Control</th>
<th>0.25 µl</th>
<th>0.50 µl</th>
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<tbody>
<tr>
<td>Mortality (%)</td>
<td>21</td>
<td>62</td>
<td></td>
</tr>
<tr>
<td>Number analyzed</td>
<td>9</td>
<td>11</td>
<td>5</td>
</tr>
<tr>
<td>Mean body weight (g)</td>
<td>395</td>
<td>370</td>
<td>348</td>
</tr>
<tr>
<td>Mean body length (cm)</td>
<td>29.2</td>
<td>29.4</td>
<td>27.0</td>
</tr>
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</table>

In a preliminary study, it was confirmed that the injection of sterilized physiological saline solution to dorsal aorta of rainbow trout hardly influenced on blood properties. Necrotizing changes were seen in the liver cells of the fish in the experimental groups by staining with Hematoxylin and Eosin. Fig. 1 shows the liver aspect of the fish injected with CCl₄.
significant deposition of fat and glycogen was observed in the liver cells stained with Sudan black B or PAS, respectively. Liver was an only organ visibly affected by the injection of dose. Two different levels of the dose caused the practically similar extent of necrosis. Neither necrosis nor inflammation was seen in other viscera.

Table 2 shows some blood properties in the control and two experimental groups. The administration of CCl₄ resulted in the depression of plasma protein and lipid contents, and elevation of mineral content. The increase of the dose induced a tendency to cause the decrease of A/G ratio measured by the salting-out method. The plasma mineral level of the experimental groups seemed to fluctuate. There appeared minor changes in the hematocrit value, osmotic pressure, hemoglobin content, and MCHC.

Schematic electrophoretic diagrams of human serum and rainbow trout plasma are shown in Fig. 2. The pooled plasma protein of rainbow trout was separated into albumin and globulin by salting-out, and both fractions were subsequently subjected to electrophoresis. Cellulose acetate electrophoresis of the rainbow trout plasma produced mainly 8 protein components. Polymorphic 2 components designated Fr. II behaved as albumin fraction in the solubility test. Lipoproteins were localized in Fr. I, II, and III. In addition, Fr. II, III, IV, and VI were positive to carbohydrate staining.

Table 3 shows the plasma protein composition calculated from the cellulose acetate electrophoretic pattern. From the electrophoretic results of Fig.

**Table 2. Blood properties** of rainbow trout injected with carbon tetrachloride

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Chemical (µl/100 g B.W.)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>0.25 µl</td>
<td>0.50 µl</td>
</tr>
<tr>
<td>Hematocrit value (%)</td>
<td>41.8 ± 2.7</td>
<td>42.7 ± 4.8</td>
<td>41.1 ± 6.8</td>
</tr>
<tr>
<td>Hemoglobin (g/100 ml)</td>
<td>9.9 ± 1.2</td>
<td>9.2 ± 1.4</td>
<td>9.4 ± 1.9</td>
</tr>
<tr>
<td>MCHM*2 (%)</td>
<td>23.6 ± 2.3</td>
<td>21.7 ± 1.8</td>
<td>22.8 ± 1.9</td>
</tr>
<tr>
<td>Plasma osmotic pressure</td>
<td>256 ± 1</td>
<td>254 ± 25</td>
<td></td>
</tr>
<tr>
<td>(m osM/l)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma total protein (g/100 ml)</td>
<td>4.16 ± 0.70</td>
<td>3.66 ± 0.71</td>
<td>3.33 ± 0.69</td>
</tr>
<tr>
<td>Plasma total lipid (mg/100 ml)</td>
<td>789 ± 191</td>
<td>547 ± 172</td>
<td>505 ± 169</td>
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<tr>
<td>A/G ratio*3</td>
<td>0.34</td>
<td>0.27</td>
<td>0.25</td>
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<tr>
<td>Na (mEq/l)</td>
<td>100.0 ± 7.4</td>
<td>152.0 ± 20.0</td>
<td>140.8 ± 5.8</td>
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<tr>
<td>K (mEq/l)</td>
<td>4.3 ± 1.9</td>
<td>10.8 ± 4.1</td>
<td>10.7 ± 6.1</td>
</tr>
<tr>
<td>Ca (mEq/l)</td>
<td>8.1 ± 1.6</td>
<td>27.0 ± 4.7</td>
<td>26.9 ± 9.2</td>
</tr>
</tbody>
</table>

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*1 Mean ± S.D.
*2 Mean corpuscle hemoglobin concentration.
*3 Determined by ammonium sulfate salting-out method.
A/G ratio was also estimated by identifying Fr. I, II, and IV as albumin fraction. In response to CCl₄, changes in the protein composition occurred, especially in Fr. II, III, V, and VI. The proportions of Fr. III and VI were likely to increase as compensation for the decreases of Fr. II and V. When expressed as concentration in the plasma, Fr. III and VI markedly increased. Consequently, there occurred a marked depression of A/G ratio.

Table 3. Plasma protein composition of carbon tetrachloride-injected rainbow trout

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Control</th>
<th>Chemical (μg/100g B.W.)</th>
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<tbody>
<tr>
<td></td>
<td>0.25 μl</td>
<td>0.50 μl</td>
</tr>
<tr>
<td>I</td>
<td>1.5±1.9%</td>
<td>2.2±2.3% 4.0±1.2%</td>
</tr>
<tr>
<td>II</td>
<td>40.6±3.9</td>
<td>30.9±7.0 21.5±4.2</td>
</tr>
<tr>
<td>III</td>
<td>5.5±1.5</td>
<td>9.9±2.6 14.5±2.2</td>
</tr>
<tr>
<td>IV</td>
<td>22.5±3.5</td>
<td>22.5±2.4 24.3±3.1</td>
</tr>
<tr>
<td>V</td>
<td>16.9±3.3</td>
<td>4.5±4.6 1.2±2.7</td>
</tr>
<tr>
<td>VI</td>
<td>12.2±2.9</td>
<td>27.4±5.7 34.1±5.2</td>
</tr>
<tr>
<td>VII</td>
<td>0.4±0.6</td>
<td>2.6±2.5 1.3±0.5</td>
</tr>
<tr>
<td>A/G ratio*</td>
<td>1.82</td>
<td>1.25 0.96</td>
</tr>
</tbody>
</table>

* Calculated from electrophoretic pattern.

Fig. 3 shows disc electrophoretic diagrams of the plasma protein. Albumin fraction was comprised of the components with relatively higher mobility. Approximately 7 lipoproteins were observable by Sudan black B staining. The most dominant lipoprotein was ascertained to behave as albumin, corresponding to Fr. II on the cellulose acetate film.

The change of blood constituents by the injection occurred significantly in lipoprotein fraction. Three lipoprotein bands on a cellulose acetate film gradually diminished as the dose of administration increased. Apart from Fr. II, the other two lipoprotein bands disappeared. On the other hand, both Fr. III and VI having globulin-like character proportionally were increased by the CCl₄ injection. When an aliquot of the pooled plasma was subjected to disc electrophoresis, a fair amount of the lipoprotein components decreased as the dose increased, as shown in Fig. 4.

The plasma lipid composition is given in Table 4.
There was a marked decrease in plasma triglycerides. While the proportions of cholesterol seemed to increase by the CCl₄ injection, when expressed as the concentration in plasma, it remained constant. In addition, there was a slight increase in partial glycerides and phosphatidyl ethanolamine concentrations.

Discussion

Gingerich et al. demonstrated that CCl₄ serves as a suitable hepatotoxic agent in rainbow trout. CCl₄ injection to fish is accompanied with the histological change of liver and the changes of blood properties. Since the synthesis of albumin and most globulins in mammal takes place in liver, liver cell damage directly influences upon serum protein composition. In the experimental groups, no significant changes occurred in hematological values, but some changes in both plasma protein and lipid. The CCl₄ injection to rainbow trout influenced on the amount of albumin fraction which was simultaneously lipid-stainable. The presence of lipid-containing albumin, as seen in electrophoretic analysis of other fish species, was probably the evidence of insufficient differentiation in protein function. Significant depression caused in the lipid-containing albumin seemed likely to be the result of the interruption of albumin and/or lipid in the fish liver.

The degradation of liver function by the injection seemed to follow the fluctuation of plasma osmotic pressure. Main function of mammalian serum albumin is transportation of low-molecular substances required by the tissue and maintenance of an osmotic equilibrium. The lack of albumin in many fish species means the low level of the colloid osmotic pressure in the capillaries. In elasmobranch serum, Drilhon and Fine discussed a relationship between the absence of albumin and the presence of urea which regulates the osmotic pressure. The relationship between the amount of serum albumin and the osmotic regulation in animal has been discussed with respect to evolutional significance. As-
suming that the albumin components of the fish plasma correspond to a mammalian serum albumin, the depression of the albumin in the plasma may affect on plasma osmotic regulation.

The marked effects on plasma lipid composition had some meaning in elucidating lipid metabolism in the fish liver. It is quite reasonable to assume that the degradation of plasma lipoprotein and lipid components was caused by the retardation of lipid metabolism in liver. However, the CCI4 injection gave no effect on cholesterol level in the plasma. It is established in mammal that both liver and intestinal mucosa are capable of serum lipoprotein synthesis. The studies on fish serum lipoprotein demonstrated some marked differences in lipid circulation between fish and mammals18-22). The main lipid transport form in carp and yellow tail plasma was a lipid-albumin complex23,24), which was incompletely adapted to the definition of human serum albumin in several chemical properties. MILLS and TAYLAR20) suggested that lipid transform mechanism develops during the evolution of phylum. Despite of insufficiency of applying mammalian metabolic feature to fish, as to plasmological observation, the toxicity to fish was similar to mammal. In fish, though the lipid metabolism is not so specialized as in higher vertebrates, the liver plays an important part in forming the lipid-albumin complex. It can therefore be presumed that the depression of lipoprotein in the plasma of rainbow trout injected with CCI4 might be related to the decrease of triglycerides and/or the small molecular proteins. A disease of cultured yellow tail caused by inadequate feed stuff was accompanied by atrophy of liver and alternation of plasma lipid composition. Moreover, the confusion of electrophoretic mobility of lipoprotein components resulted in the diseased fish plasma25). Therefore plasma constituents relating to lipid metabolism could be influenced by certain hepatic damages.

References