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Effects of Toluene-2,4-Diamine on Red Sea Bream, *Pagrus major*: Biochemical and Histological Evaluation

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The subacute toxicity of toluene-2,4-diamine (2,4-TDA) on marine fish was investigated in laboratory toxicity tests using red sea bream, *Pagrus major*. The fish were exposed to subacute concentrations of 2,4-TDA (measured concentrations: 0.0628, 0.234 and 0.878 mg/L) during the 14-day toxicity test and the effects on growth as well as on some biochemical parameters in the plasma, gills, liver and kidney structures were studied. The body weight and body length of the red sea bream exposed to the highest concentration were lower than those of the control. This study also showed a tendency towards decreases in three hematological parameters, i.e., the number of red blood cells, hemoglobin level and hematocrit, as well as blood chemical parameters in the plasma, i.e., the levels of total cholesterol, triglyceride, total protein and albumin, of fish exposed to the high concentrations. These findings were attributable to the reduced feeding of fish exposed to this concentration. In addition, cytoplasmic changes were observed in the cells of fish exposed to the highest concentration. It is suggested that the changes were caused by the decrease in feed efficiency derived from the exposure to 2,4-TDA .

1. Introduction

Toluene-2,4-diamine (2,4-TDA) is an amine compound used as an intermediate by the Japan chemical industry for the production of polyurethanes, dies, pigments and other rigid forms. Significant releases of 2,4-TDA into the environment are not expected because this process is performed in closed systems. However, 2,4-TDA can be formed by the hydrolysis of tolylene diisocynate (TDI) under certain conditions. This reaction is dependent on the ratio of TDI/water mixing. If the pure isocyanate is spilled into water, polyurea is formed as the main product; on the other hand, if small amounts of TDI are mixed with much water, 2,4-TDA will be formed.[1]

It is clear that 2,4-TDA is not highly toxic to freshwater fish. Several studies that have been carried out on fish have shown that it is not toxic. The 96-h LC_{50} (median lethal concentration) values range between >100 and 1,420 mg/L for medaka, *Oryzias latipes*[2,3] and the fathead minnow, *Pimephales promelas*;[4] however, the toxicity of 2,4-TDA to marine fish, such as red sea bream, *Pagrus major* is particularly high and the 96-h LC_{50} measured is in the range of 0.733 to 0.826 mg/L.[5] The toxicity of 2,4-TDA to red sea bream is 2,000-fold higher than that to fathead minnow.

Generally, the sensitivity of marine fish species is relatively higher than that of freshwater fish. However, the difference between the LC_{50} of red sea bream and that

of freshwater fish seems larger than that expected from the known toxicities of some chemicals with nonspecific toxicity to these fish. Red sea bream are endemic to coastal waters surrounding Japan and are some of the most important fish in Japan, where their importance lies in fish farming for human consumption. These are sufficient reasons to call for further studies on the toxicities to other marine fish and to determine the cause of the species-specific toxicity of 2,4-TDA on both freshwater and marine fish. Data on the effects of 2,4-TDA on red sea bream could also be useful in its risk assessment.

In this study, the possible adverse biochemical and histological effects of 2,4-TDA on red sea bream were investigated.

2. Materials and Methods

2.1 *Fish*

The red sea bream (*Pagrus major*) used in this study were obtained from the Nagasaki-gyogyokosha fish farm (Kozasa, Nagasaki, Japan) and maintained at our laboratory. The fish (mean body weight, 39.6–39.8 g) were kept for at least two weeks prior to tests in a flow-through system at 20±1°C under a 16-h light: 8-h dark photoperiod. The fish were fed twice a day with commercial pellets suitable for red sea bream.

2.2 *Chemical*

2,4-TDA of 98% purity was purchased from Tokyo Kasei Kogyo Co., Ltd.

2.3 *Test conditions and dosing regimen*

The fish were exposed for 14 days to 2,4-TDA nominal concentrations of at 0, 0.100, 0.316 and 1.00 mg/L. The nominal concentrations tested were chosen on the basis of the result of a preliminary 96-h acute toxicity test. Natural seawater filtered through an activated carbon filter at sufficient aeration and a controlled temperature was used as the dilution water. Before starting the experiments, the fish were weighed and their lengths were measured, and then allocated to each test group.

A glass tank (length, 60.0 cm; width, 29.5 cm; depth, 36.0 cm) containing 50 L of test solution was used for each test concentration with seven fish in each tank. A flow-through diluter system was used in this study. The stock solution was injected using a glass plunger pump purchased from EYELA GMW-A (Tokyo Rikakikai, Tokyo, Japan). Appropriate amounts of the stock solution and dilution water were placed in a mixing vessel before introducing them into the test tanks. The flow rates of the stock solution and dilution water were checked daily. The renewal rate of the test solution for each tank was six times per day. A 16-h photoperiod was used for this study. The fish were fed with commercial pellets twice daily at a rate of 2% of body wet-weight during the exposure. Mortality, abnormal behavior and appearance were observed daily. The temperature, dissolved oxygen (DO) concentration and pH of each treatment and control aquaria were measured five times (0, 3, 7, 10 and 14 days) throughout the exposure.

2.4 *Analytical chemistry*

The TDA concentrations in the test solutions were measured at the beginning, after seven days and at the end of the exposure. The TDA solution was collected and used as the analytical samples directly or with diluted water. The 2,4-TDA samples were analyzed using a Shimadzu high-performance liquid chromatography (HPLC) system (Tokyo, Japan) equipped with an L-column ODS (length, 250 mm; inner diameter, 4.6 mm; Chemicals Evaluation and Research Institute, Tokyo, Japan). The samples were injected and eluted at a flow rate of 1 ml/min in a mobile phase of acetonitrile/0.01% phosphate buffer containing 5 mmol/L of 1-octanesulfonic acid sodium salt, 25/75 (V/V). The elution profile was monitored with a Shimadzu SPD-10AV at 240 nm.

2.5 *Biochemical investigation*

After the 14-day exposure period, fish from each treatment were anesthetized with FA100 (Tanabe Seiyaku Co., Ltd., Osaka, Japan), and blood from the fish was collected from the caudal peduncle using heparinized tuberculin syringes. Subsamples for determining the number of red blood cells, hemoglobin level and hematocrit were taken, and the rest of the blood was centrifuged (3,000 rpm, 10 min) for chemical examinations of aspartate aminotransferase (AST, UV method), alanine aminotransferase (ALT, UV method), alkaline phosphatase (ALP, p-nitrophenyl phosphate method), total cholesterol (T-Cho, COD·DAOS method), triglyceride (TG, GPD·DAOS method), blood urea nitrogen (BUN, urease indophenol method), creatinine (Jaffé's method), total protein (T-Protein, Biuret method), albumin (Bromocresol green method), total bilirubin (T-Bil, Azo bilirubin method), inorganic phosphorus (IP, Fiske-Subbarow method) and calcium (Ca, OCPC method).

2.6 *Histological investigation*

For histological investigation, portions of the brain, gills, liver, kidney, spleen, gall bladder and intestines were sampled from the fish, fixed with Bouin's fluid, and processed for histological examination using standard techniques with hematoxylin and eosin staining.

2.7 *Statistical analysis*

The results of growth, and hematological and biochemical examinations were analyzed using Bartlett's test for homogeneity of variance. If the variances were homogeneous, the one-way layout test was used. If a significant difference was found, Dunnett' s test was carried out between the control group and each of the treatment groups. If the variances were not homogeneous, the Kruskal-Wallis test was used. If a significant difference was found, the nonparametric Dunnett's test was carried out between the control group and each of the treatment groups. Differences were considered to be significant at $p \leq 0.05$.

3. Results

3.1 *Concentrations of chemicals in test solutions*

The means (percentage of nominal) of the measured concentrations in the test solutions during the exposure period were 0.0628 (62.8%), 0.231 (73.1%) and 0.878 (87.8%) mg/L.

3.2 *Water quality measurements*

The water temperature was maintained at 20±1°C. The DO concentration was from 6.4 to 6.8 mg/L (not less than 60% of the air saturation value) and pH was from 7.7 to 8.0 (did not vary by more than 1.5 units in any one of the tests).

3.3 *Mortality*

One fish died after five days of exposure at the highest concentration. Both 96-h LC_{50} and 14-day LC_{50} were >0.878 mg/L. No mortality was observed at other concentrations or in the control.

3.4 *Abnormal responses*

The following results were based on a comparison with the control. After two days of exposure at 0.878 mg/L, the fish began to show reduced swimming activity and reduced feeding behavior. In addition, the affected fish showed dark pigmentation with time and swam at the water surface. At 0.231 mg/L, all fish began to show reduced feeding behavior after six days of exposure; afterwards, one fish showed reduced swimming activity and dark pigmentation. At 0.0628 mg/L, one fish showed dark pigmentation after 14 days of exposure.

3.5 *Body weight and body length*

Body weight and body length measured at every exposure level are shown in Tables 1 and 2. Both the body weight and body length of the fish exposed to the highest concentration were lower than those of the control, although these differences were not significant.

3.6 *Hematological and biochemical examinations*

3.6.1 *Hematological examinations*

Table 3 shows the numbers of red blood cells, hemoglobin levels and hematocrit values of fish exposed for 14 days. A tendency towards anemia was observed at each concentration in comparison with the control. However, this trend was not significant.

Table 1
Effects on increase in body weight after exposure to 2,4-TDA.

	Treatment			
	Control	0.0628 mg/L	0.231 mg/L	0.878 mg/L
Number of samples	7	7	7	6
Initial weight (g)	39.5±6.7	39.8±7.0	39.6±6.5	39.5±5.2
	(31.4–44.4)	(33.2–53.3)	(31.8–50.5)	(32.2–45.0)
Final weight (g)	46.8±7.9	47.0±8.3	42.9±6.3	37.6±7.0
	(38.6–60.1)	(38.7–60.7)	(35.2–52.0)	(28.3–44.4)
% Increase	18.5	18.1	8.3	–4.8

Values are means±SD, with ranges in parentheses.

Table 2
Effects on increase in body length after exposure to 2,4-TDA.

	Treatment			
	Control	0.0628 mg/L	0.231 mg/L	0.878 mg/L
Number of samples	7	7	7	6
Initial length (cm)	10.9±0.6	10.9±0.5	10.8±0.4	11.1±0.6
	(10.4–11.6)	(10.4–11.9)	(10.6–11.8)	(10.3–12.0)
Final length (cm)	11.2±0.6	11.1±0.5	11.1±0.4	10.3±0.6
	(10.2−12.0)	(10.2–11.5)	(10.3–11.3)	(9.6–10.9)
% Increase	2.8	1.8	2.8	–7.2

Values are means±SD, with ranges in parentheses.

Table 3
Hematological values for red sea bream after 14 days of exposure to 2,4-TDA.

	Treatment			
	Control	0.0628 mg/L	0.231 mg/L	0.878 mg/L
Number of samples	5	5	6	6
Number of RBCs ($\times 10^4/\mu l$)	278±42	255±28	245±52	215±52
Hb (g/dl)	6.7±1.0	6.3±0.5	6.4±1.2	6.2±2.0
Ht (%)	32.0±2.3	30.2±5.1	32.5±4.5	26.3±6.0

Values are means±SD.

3.6.2 *Biochemical examinations*

The results show no significant differences among the fish exposed to 0.0628, 0.231 and 0.878 mg/L, except for the decrease in TG level at 0.878 mg/L. Table 4 shows this in terms of each plasma component. A tendency towards decreases in the levels of T-Cho, TG, BUN, T-Protein, albumin, IP and Ca was observed. However, these decreases were not statistically significant; only the difference in TG at 0.878 mg/L was significant ($p<0.05$). The T-Bil levels of fish exposed to 0.231 and 0.878 mg/L increased, and creatinine levels were unchanged.

There was no clear effect of 2,4-TDA on the activities of enzymes such as AST, ALT and ALP, because there were wide variations and considerably high values for these parameters in the control.

3.7 *Histology*

The histological structures of the gills, brain, kidney, spleen, gall bladder, pancreas and intestines of the fish treated with 2,4-TDA were not substantially different from those of the control group.

Some changes were observed in the livers of the treated groups in comparison with those of the control group. The liver from the control showed a network structure under low magnification. The liver cells showed a clear border and poorly stained cytoplasm. Nuclei were roundish and sparingly surrounded by basophilic components. The cytoplasm contained a small number of fine acidophilic structures, which were possibly visible glycogen, against a poorly stained background (Fig. 1). The number of acidophilic components ranged from a few to almost none in the control. However, the number of acidophilic components in the liver of the fish exposed to the test substance increased with exposure concentration; the basophilic area near the nuclei also increased. All the livers from the fish exposed to the highest concentration (0.878 mg/L) and three livers from those exposed to the second-highest concentration (0.231 mg/L) showed a deeply stained cytoplasm, an unclear cell border and a slightly decreased cell size (Fig. 2). In some sections, the cell border appeared as unstained gaps between deeply stained cytoplasm. The appearance of the cytoplasm ranged from acidophilic granular to basophilic. At the highest concentration, fatty degeneration, which was considered to be a disruption of the balance between the production of lipid and its use, was partly observed in three out of six specimens (Fig. 3). The cytoplasmic changes in the liver cells of the fish exposed to the highest and second-highest concentrations were never observed in the fish exposed to the lowest concentration or in the control fish.

Table 4
Blood chemical values for red sea bream after 14 days of exposure to 2,4-TDA.

Parameter	Measured concentration (mg/L)			
	Control (n=7)	0.0628 (n=7)	0.231 (n=7)	0.878 (n=7)
AST (IU/L)	97±53	29±31	64±66	60±88
ALT (IU/L)	24±25	5±3	13±12	42±82
ALP (IU/L)	100±73	95±74	60±10	57±32
T-Cholesterol (mg/dl)	230±93	225±44	153±20	145±72
Triglyceride (mg/dl)	191±123	155±59	103±57	70±40*
BUN (mg/dl)	9.4±2.4	6.9±2.0	6.4±2.3	7.7±3.1
Creatinine (mg/dl)	0.37±0.06	0.38±0.05	0.37±0.07	0.36±0.08
T-Protein (g/dl)	3.4±0.6	3.9±0.4	3.4±0.3	2.8±0.8
Albumin (g/dl)	1.5±0.3	1.7±0.2	1.6±0.2	1.2±0.4
T-Bilirubin (mg/dl)	0.11±0.04	0.12±0.02	0.15±0.04	0.17±0.08
I-Phosphorous (mg/dl)	12.0±6.4	10.4±1.2	9.2±1.0	10.3±4.2
Calcium (mg/dl)	12.2±1.3	13.6±1.0	12.5±1.1	11.5±2.3

Values are means±SD.
*Significantly different from control at $p<0.05$.

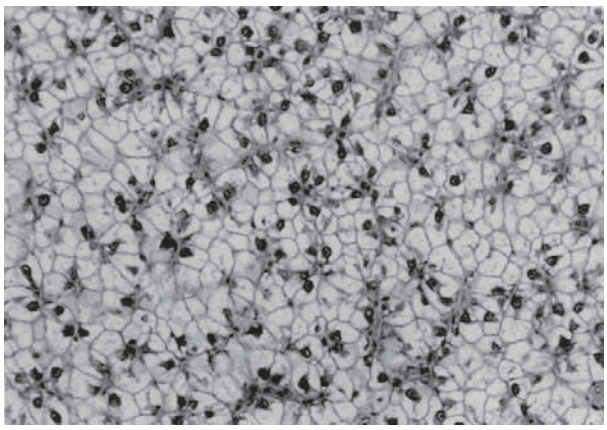

Fig. 1. Liver tissue of red sea bream in control. HE, ×400.

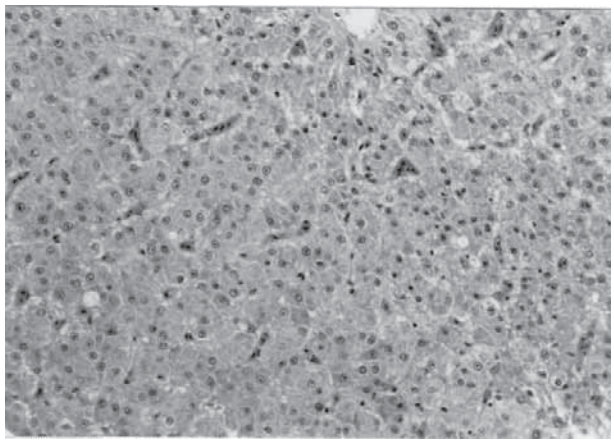

Fig. 2. Liver tissue of red sea bream exposed to 0.878 mg/L 2,4-TDA for 14 days. HE, ×400. Deeply stained cytoplasm.

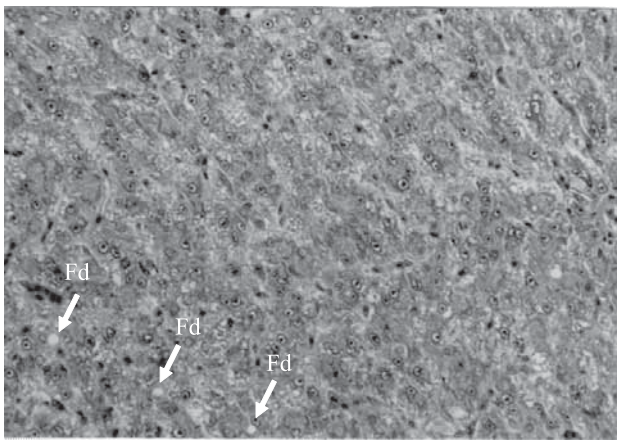

Fig. 3. Liver tissue of red sea bream exposed to 0.878 mg/L 2,4-TDA for 14 days. HE, ×400. Deeply stained cytoplasm and fatty degeneration (Fd).

4. Discussion

In this study, the mortalities at 96 h and 14 days were 0 and 14.3%, respectively, and both LC_{50} values were >0.878 mg/L. The reason why the toxicity of 2,4-TDA to *Pagrus major* was slightly low compared with the existing values is considered to be due to the different growth stages of the fish exposed to 2,4-TDA, i.e., about 40 g of body weight in this study and 10 to 20 g in the reference cited,[5] and the presence or lack of feeding during exposure.

The majority of the fish exposed to the 2,4-TDA concentration of 0.878 mg/L showed reduced feeding behavior and were found at or near the water surface. Some fish exposed to 0.23l mg/L also showed reduced feeding behavior. Holcombe *et al.* tested the long-term toxicity of 2,4-TDA in a larval test using medaka, *Oryzias latipes*.[2] Larvae between zero and three days of age were exposed in a flow-through system to 2,4-TDA concentrations of 40.3, 68.8, 131, 263 and 551 mg/L for 28 days. No abnormal responses were observed in the fish during the exposure; however, in the same larval tests using other amine substances, such as aniline and 4-chloroaniline, in all exposure tanks, swimming at or near the water surface and loss of appetite were observed during the last two weeks of the test. From histological observations, the authors concluded that gas bladder development might have affected the ability of the exposed fish to regulate buoyancy, and might explain the observed behavior.[2] However, no histological changes in the gas bladders of the sea bream exposed to 2,4-TDA were found in this study, although similar abnormal behaviors were observed.

At the end of the test, the body weight and body length of the fish exposed to the highest concentration were lower than those of the control, perhaps owing to the reduced feeding observed at that concentration. Our results also showed a tendency towards decreases in three hematological parameters, i.e., the number of red blood cells, hemoglobin level and hematocrit, as well as blood chemical parameters in the plasma, i.e., the levels of total cholesterol, triglyceride, total protein and albumin, of fish exposed to the high concentrations. Because decreases in the number of red blood cells, hemoglobin level, hematocrit and serum protein level are considered as effects of starvation in mammals and fish,[6–8] the results obtained in the current study could also explain the effect of starvation.

The histological alterations observed were deeply stained cytoplasm, an unclear cell border and decreased cell size in the liver of fish exposed to the high 2,4-TDA concentrations. Similar histological appearances in the eel liver were observed during one month of starvation.[9] This suggests that the decrease in heamatological parameters, total protein level and other indicators probably led to the appearance of the structural alterations in the liver. The cytoplasmic changes observed in the cells of fish exposed to concentrations of 0.231 and 0.878 mg/L were never observed in the cells of those exposed to the lowest concentration or in the control. It is suggested that the changes were caused by the decrease in feed efficiency derived from exposure to 2,4-TDA.

After medaka fry (one to three days old) were exposed to 2,4-TDA for 28 days and then reared in clean water for an additional five months, the 2,4-TDA produced pathological effects such as pyknosis, tubular degeneration, hepatocyte atypia, and necrosis in erythrocytes, the kidney and liver.[10,11] These histological effects were not observed in the present study. This might be due to the difference in the species used or the shorter test period. These findings demonstrate the necessity of carrying out long-term toxicity studies and explaining the cause of the species-specific toxicity of 2,4-TDA in red sea bream.

In summary, after exposure to 2,4-TDA for 14 days, the body weight and body length of red sea bream exposed to the highest concentration were lower than those of the control fish. This study also showed a tendency towards decreases in three he-

matological parameters, i.e., the number of red blood cells, hemoglobin level and hematocrit, as well as blood chemical parameters in the plasma, i.e., the levels of total cholesterol, triglyceride, total protein and albumin, of fish exposed to the higher concentration. These findings were attributable to the reduced feeding of fish exposed to these concentrations. In addition, cytoplasmic changes were observed in the cells of those exposed to the highest concentration. It is suggested that these changes were caused by the decrease in feed efficiency derived from exposure to 2,4-TDA.

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