Chronic Toxicity of Chloroform to Japanese Medaka Fish

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Japanese medaka (*Oryzias latipes*) were continually exposed in a flow-through diluter system for 9 months to measured chloroform concentrations of 0.017, 0.151, or 1.463 mg/L. Parameters evaluated were hepatocarcinogenicity, hepatocellular proliferation, hematology, and intrahepatic chloroform concentration. Histopathology was evaluated at 6 and 9 months. Chloroform was not hepatocarcinogenic to the medaka at the concentrations tested. Chronic toxicity was evidenced at these time points by statistically significant ($\alpha = 0.05$) levels of gallbladder lesions and bile duct abnormalities in medaka treated with 1.463 mg/L chloroform. We assessed hepatocellular proliferation by exposing test fish to 5-bromo-2´-deoxyuridine in the aquarium water for 72 hr after 4 and 20 days of chloroform exposure; we then quantified area-labeling indices of the livers using computer-assisted image analysis. We observed no treatment-related increases in cellular proliferation. We analyzed cells in circulating blood in medaka after 6 months of chloroform exposure. Hematocrit, leukocrit, cell viability, and cell counts of treated fish were not significantly different from those of control fish. Using gas chromatography (GC), we evaluated intrahepatic concentrations of chloroform in fish after 9 months of exposure. Livers from the 0.151 and 1.463 mg/L chloroform-treated fish had detectable amounts of chloroform, but these levels were always lower than the aquaria concentrations of chloroform. Thus, it appeared that chloroform did not bioaccumulate in the liver. Unidentified presumptive metabolite peaks were found in the GC tracings of these fish livers. *Key words*: aquatic toxicology, 5-bromo-2´-deoxyuridine, carcinogenicity, cell proliferation, chloroform, fish, hematology, histopathology, medaka, *Oryzias latipes*. *Environ Health Perspect* 109:35–40 (2001). [Online 12 December 2000]
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Chloroform is a common drinking water disinfection by-product. Recently, present U.S. Environmental Protection Agency (U.S. EPA) drinking water standards for chloroform have been scrutinized (*1,2*): "Are today's standards based on rodent megadose studies relevant to real world exposures?" Carcinogenic results in these rodent studies were found at concentrations several orders of magnitude higher than the chloroform maximum contaminant level goal (MCLG) of 0 mg/L and the total trihalomethane maximum contaminant level (MCL) of 0.08 mg/L (*3*).

Nontraditional models such as fish can be used to study chloroform-induced toxicity at exposures closer to concentration levels found in drinking water. Fish have been shown to be sensitive to trace levels of contaminants in aquatic media (*4–8*). As water-dwelling organisms, fish receive dermal exposure through whole-body immersion in the exposure solution. Intake of test material also occurs through feeding and respiration.

Japanese medaka have been studied extensively both in the United States and in Japan (*9–13*). Their hardiness, small size, ease of culturing, and relatively short time-to-tumor response make the medaka an attractive test model. Sections of the entire animal will fit on one microscope slide so that examination of every tissue is possible in its anatomical context. The low rate of spontaneous neoplasms in medaka aids in the interpretation of bioassay results (*14*).

Increased cellular proliferation is necessary to transform normal tissue to neoplastic tissue (*15*). 5-Bromo-2´-deoxyuridine (BrdU), a thymidine analog that labels cells in S-phase, has been used extensively to study cellular proliferation in rodents (*16– 18*) and fish (*19,20*). BrdU cell labeling has been accomplished previously via intraperitoneal injection, implantation of an osmotic pump, and aqueous exposure (*19–24*). Previous BrdU range-finding studies with medaka in this laboratory have demonstrated an effective cell-labeling concentration of BrdU in the ambient water to be 75 mg/L for 72 hr (*25,26*).

Our purpose was to determine the effect of chloroform on Japanese medaka after 9 months of continuous exposure. Specific end points evaluated were severity and prevalence of neoplasms, hepatocellular proliferation, hematology, intrahepatic chloroform concentration, fish growth, and fish survival.

Materials and Methods

Test materials. Chloroform (CAS No. 67-66-3) of 99.8% purity was obtained from Aldrich Chemical Company (Milwaukee, WI). Weekly chemical analyses of aquaria water demonstrated that chloroform did not break down to form other compounds. Under the continuous flow-through dosing regimen, sufficient chloroform remained in solution during testing to maintain dosage levels. Approximately 10 L of 100 mg/L chloroform stock were prepared each day. Processed well water was the diluent used. The chloroform stocks were stirred for 24 ± 2 hr in a sealed glass container, and then pumped into a diluter dosing bottle.

BrdU (CAS No. 59-14-3) of 99% purity was obtained from Sigma Chemical Co. (St. Louis, MO). BrdU is water soluble, not volatile, and stable under test conditions. A stock solution of 10 g/L BrdU was prepared in ASTM Type I water on the day of exposure.

Animal care. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals, and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals* (*27*). The facilities are fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International.

Japanese medaka fish (*Oryzias latipes*) were supplied from the U.S. Army Center for Environmental Health Research (USACEHR) in-house cultures and were reared according to USACEHR standing operating procedures. Fish were fed flake food (Aquatox Certified Diet, Ziegler Brothers, Gardners, PA) and live 24-hr brine shrimp daily. Fish were fasted 24 hr before the 6- and 9-month histopathology sacrifices

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and the BrdU exposures. Durotest Optima Choice fluorescent bulbs with a color rendering index (CRI) of 91 (Durotest Lighting, Fairfield, NJ) provided the light for the 16-hr light/8-hr dark cycle of light.

Test design. We chose the amount of chloroform routinely found in dechlorinated tap water as the lowest concentration tested. In 96-hr range-finding studies with juvenile medaka, a length NOEL (no-observed-effect level) of 21–25 mg/L and a weight NOEL of 22–26 mg/L were established for chloroform (*28*). We chose a log scale for the three concentrations of chloroform, which ensured that all concentrations tested were below the NOELs established in acute testing. Four test aquaria were randomly assigned to each nominal concentration of 0, 0.015, 0.15, and 1.5 mg/L chloroform.

The test began when 14-day-old fry (± 1 day) were randomized to each 5-gallon test aquarium, with 80 fry and approximately 15 L test solution per sealed aquarium. The proportional diluter was set to deliver 300 ± 15 mL to each aquarium every 3 min ± 15 sec, yielding 9–10 tank volumes per day.

During the first month of chloroform exposure, five fish per aquarium for each of four time points were used for the hepatocellular proliferation assays. After 6 months of chloroform exposure, we evaluated 20 fish per aquarium by histopathology, and an additional five fish per aquarium by hematology. At 9 months of exposure, five fish per aquarium were used for the chloroform intrahepatic concentration analysis. We euthanized all remaining fish to assess the following tissues by histopathology: bone (vertebra), brain, chromaffin tissue, corpuscle of Stannius, esophagus, eye, gallbladder, gill, heart, hematopoietic tissue, interrenal tissue, intestine, kidney, liver, nares, ovary, pancreas, peripheral nerve, pineal organ, pituitary gland, pseudobranch, skeletal muscle, skin, spinal cord, spleen, stato-acoustic organ, swim bladder, testis, thymus, thyroid tissue, urinary bladder, and gross lesions.

Statistical analyses. We analyzed lengths, weights, and histopathology data statistically for the chronic carcinogenicity test. For these and all other test end points, we applied normalizing transformations where required, and selected the best-fitting model for each test end point. We used analysis of variance (ANOVA) to test for effects, and regression analysis for point estimation. We used SAS PROC GLM computer software (SAS Institute, Inc., Cary, NC) for these analyses (*29*).

Chloroform chemical analyses. All exposure tanks were sampled weekly and analyzed on the day of collection. Daily stock solutions were collected and stored at 5°C until the weekly analysis of the exposure tanks. All samples were collected in 40 mL borosilicate glass U.S. EPA water-sampling vials with Teflon-lined silicone rubber septa in the cap.

To analyze samples we used a Hewlett Packard 6890 gas chromatograph equipped with an electron capture detector and interfaced to Hewlett Packard model 7694 headspace sampler (Agilent Technologies, Palo Alto, CA). We used a Hewlett Packard ChemStation for instrument control and data acquisition. The capillary column used throughout the study was a Hewlett Packard 25-m HP-1 (cross-linked methyl polysiloxane), 0.2 mm i.d. and 0.33 µm film thickness. The oven temperature of the gas chromatograph held isothermally at 40°C, the inlet was set at 250°C, and the electron capture detector was maintained at 300°C.

We chose headspace analysis for this study for its sensitivity, simplicity, and rapid throughput. Headspace analysis has been used elsewhere to analyze rat liver, urine, and blood (*30*,*31*) and was applied here to fish tissue and water samples. For the weekly analysis of exposure tanks, three 5 mL aliquots of each sample were placed in 10 mL headspace vials and sealed with Teflon-lined crimp caps. The vials were then placed in the headspace sampler with the sampler oven set at 60°C, the 1 mL sample loop at 65°C, and the transfer line to the gas chromatograph at 70°C. The sample vial equilibration time was 30 min with agitation set on high. External calibration standards were used for this method and prepared fresh on the day of analysis. The technique required no further sample preparation and there were no interfering contaminants from the sample matrix. The detection limit was 0.003 mg/L and the recovery was 95.2%.

BrdU chemical analyses. We collected samples for chemical analysis at the initiation and termination of each exposure. Spiked samples showed an average recovery of 102%. We used a Hewlett Packard 1050 series high performance liquid chromatograph equipped with a variable wavelength detector, autosampler, and Hewlett Packard Chemstation (Agilent Technologies) to analyze BrdU samples. Samples were filtered through a 0.45 µm membrane before analysis. The solvent delivery system was programmed to deliver 15% methanol/85% water at a flow rate of 1.5 mL/min. The UV detector was set at 277 nm to monitor the eluate. A Supelco TM LC-18 column (25 cm × 0.46 i.d., 5 µm particle size; Supelco, Bellefonte, PA) was used for the separation. The injection volume was 5 µL.

Hepatocellular proliferation assays. Five fish per aquarium were removed from each of the 16 test aquaria on test days 2, 4, 7, and 20, exposed to 75 mg/L BrdU for 72 ± 4 hr, and euthanized with an overdose of tricaine methane sulfonate (MS-222) on test days 5, 7, 10, and 23, respectively. BrdU exposure, fixation, sectioning, staining, and counting methods have been described previously (*26*). We used a Bioquant/True Color Image Analysis System (Bioquant-R&M Biometrics, Inc., Nashville, TN) to evaluate BrdU-labeled slides, with five BrdU-stained sections from each fish. A count labeling index (CLI) was done to validate the area labeling index (ALI) method and to ensure that hepatic cell size did not change due to chloroform treatment. R^2 values comparing the CLI and ALI for the 4-day and 20-day sacrifice points were 0.934 and 0.921, respectively.

Chronic carcinogenicity. At 6 and 9 months of chloroform exposure, fish were euthanized with an overdose of MS-222. Fish necropsy procedures, fixation, sectioning, and staining were done as described previously (*32*). Fish tissues, listed in "Test Design," were evaluated by histopathology.

Fish hematology. During the fish chronic test, five fish per aquarium were removed after 6 months of continuous exposure to chloroform. Fish were euthanized by an overdose of MS-222, and then weighed and measured. A capillary tube (10–20 µL) of blood was removed from each fish, and each fish's anterior kidneys, a major site of hematopoeisis, were excised. Hematocrit and leukocrit were measured for each blood sample. Cell counts were performed using a hemacytometer. Cell viability was assessed (trypan blue exclusion) from kidney cell suspensions.

Fish intrahepatic chloroform concentration. At 9 months of chloroform exposure, five fish per aquarium were removed from the test for analysis of chloroform intrahepatic concentration. Fish were euthanized with an overdose of MS-222, weighed, and measured. The livers were excised, weighed, flash frozen in individual cryovials in liquid nitrogen, and then stored at –70°C. On the day of analysis, livers were thawed and transferred to 10 mL glass head space vials containing 5 mL of 2% (w/v) sodium dodecyl sulfate (SDS). Two percent SDS was added to denature liver enzymes immediately, thus terminating metabolism. The vials were capped and heated at 60°C for 2 hr before headspace analysis by capillary gas chromatography (as described in "Chloroform Chemical Analyses"). The detection limit for chloroform in the SDS solution was 0.5 µg/L, which was converted to milligrams of chloroform per gram of liver tissue. Recovery was 95.2%.

Results

Water quality. Water quality parameters monitored were temperature, pH, dissolved oxygen, conductivity, alkalinity, hardness, and un-ionized ammonia. All water quality parameters were within acceptable limits

based on over 10 years of historical data of our laboratory diluent water.

Chloroform chemical analyses. Over 9 months, we performed 38 weekly chemical analyses on each aquarium. Mean measured concentrations (replicates combined) yielded measured test concentrations of 0, 0.017 ± 0.004, 0.151 ± 0.034, and 1.463 ± 0.242 mg/L.

Hepatocellular proliferation assays. Two of the four hepatocellular proliferation tests had unacceptable mortality (> 10%) in control and treated groups. In the remaining two cell proliferation time points, survival was 100%. Mean measured BrdU concentration for all treatments was 74.3 ± 2.8 mg/L. Results from the two acceptable tests, time point 2 (4 days of chloroform exposure) and time point 4 (20 days of chloroform exposure), suggested chloroform treatment-related effects on hepatocellular proliferation at the concentrations tested (mean values for 20 days of chloroform exposure: 0.014, 0.120, and 1.141 mg/L) at time point 2 but not at time point 4. These results were not statistically significant, however.

Fish growth and survival. Mortality through the 9-month exposure period was < 4% in all control and treated groups. Growth measurements are summarized in Table 1. At 6 months, there was a suggestion of growth reduction, but these results were not statistically significant. Comparison of estimated means and their 95% confidence limits revealed a reduction in fish length (f = 7.66, p = 0.0059) with the highest test-concentration fish smaller than control fish, 23.8 mm (23.43, 24.18) and 24.8 mm (24.23, 25.47), respectively. There was also a reduction in fish weight (f = 5.41, p = 0.025) overall. At 9 months, no reduction in growth was found for length (f = 3.22, p = 0.0732) or weight (f = 1.58, p = 0.2090).

Fish histopathology. At 6 months of chloroform exposure, the only significant (f = 10.74, p = 0.0055) finding for males at the 1.464 mg/L concentration was proliferation, or hyperplasia, of bile ducts of the liver. In contrast, female medaka at the 1.464 mg/L concentration exhibited nine significant findings in the bile ducts of the liver and the gallbladder. As in males, females had an increase (f = 23.46, p = 0.0003) in bile duct hyperplasia. Additionally, females had bile duct epithelium hyperplasia (f = 7.84, p = 0.0142), dilatation of the bile ducts (f = 18.93, p = 0.0007), and concretions in the lumen (f = 42.98, p = 0.0001). Granulomatous pericholangitis—an inflammation around bile ducts characterized mainly by macrophages with variable numbers of lymphocytes—significantly (f = 31.62, p = 0.0001) occurred in females, presumably as a result of bile leakage into the liver parenchyma. Concretions also significantly (f = 35.39, p = 0.0001) occurred in the lumen of the gallbladder. The epithelium of the cystic duct as well as the gallbladder itself exhibited significant hyperplasia (f = 36.17, p = 0.0001 and f = 19.94, p = 0.0005, respectively). Dilatation of the cystic duct was significant (f = 10.60, p = 0.0057) in female medaka.

After 9 months of exposure to various concentrations of chloroform, there were significant differences overall between males and females in their responses to chloroform for gallbladder concretions (f = 5.76; p = 0.0231), cystic duct hyperplasia (f = 4.94; p = 0.0341), and bile duct epithelium hyperplasia (f = 6.43, p = 0.0169). These changes occurred with greater frequency among females than among males.

At 9 months and with increased measured chloroform concentrations, males demonstrated a significantly (f = 9.57; p = 0.0079) higher incidence of dilatation of the cystic duct of the gallbladder and a tendency toward a significantly (f = 4.12; p = 0.0617) higher incidence of hyperplasia of the epithelium of the gallbladder. Females responded to increased measured chloroform concentrations with a higher incidence of hyperplasia of the cystic duct of the gallbladder (f = 25.73, p = 0.0002), hyperplasia of the gallbladder epithelium (f = 5.94, p = 0.0287), concretions in the lumen of the gallbladder (f = 32.51, p = 0.0001), and granulomatous inflammation in the wall of the gallbladder (granulomatous cholecystitis) (f = 8.30, p = 0.0121) characterized by the presence of macrophages and lymphocytes.

Few liver neoplasms were observed for control and treated fish at 6 and 9 months. One hepatocellular adenoma, a benign neoplasm of hepatocytes, was observed in a male exposed to 0.151 mg/L chloroform for 6 months, while no malignant hepatocellular neoplasms occurred in any 6-month-exposed fish. At 9 months, one hepatocellular carcinoma (a malignant neoplasm of hepatocytes) was observed in a control female. Two hepatocellular adenomas occurred in medaka examined at 9 months: one in a male treated with 0.017 mg/L chloroform and one in a female treated with 0.151 mg/L chloroform.

Table 1. Fish chronic chloroform exposure: 9-month survival and growth.

Chloroform treatment (mg/L)	Replicates	Percent survival	Mean wet weight (mg)	Mean standard length (mm)	Treatment mean weight (mg)	Treatment mean length (mm)
Well-water controls	1	100	296	25.3	297	24.9
	2	99	301	24.9		
	3	96	297	24.8		
	4	99	294	24.7		
0.017	1	96	277	25.2	289	24.8
	2	96	282	24.5		
	3	94	311	25.2		
	4	94	288	24.4		
0.151	1	99	293	25.0	291	25.0
	2	99	300	25.3		
	3	99	290	25.0		
	4	96	282	24.7		
1.463	1	90	280	24.8	283	24.4
	2	98	279	24.2		
	3	98	291	24.9		
	4	98	282	23.8		

Table 2. Histopathology of male medaka after chloroform exposure through 6 and 9 months: prevalence of findings.

Tissues	Control (6 m, 9 m)	0.017 mg/L (6 m, 9 m)	0.151 mg/L (6 m, 9 m)	1.463 mg/L (6 m, 9 m)
Gallbladders examined	46, 53	32, 46	44, 47	31, 47
Cholecystitis, granulomatous	0, 0	0, 0	0, 0	0, 0
Concretions	0, 0	0, 0	0, 1	0, 3
Cystic duct concretions	0, 0	0, 0	1, 0	0, 0
Cystic duct dilatation	0, 0	0, 0	1, 0	1, 2
Cystic duct hyperplasia	0, 0	0, 0	0, 0	0, 0
Epithelium hyperplasia	0, 0	0, 0	1, 1	0,2
Livers examined	47, 53	34, 46	46, 47	34, 49
Bile duct concretions	0, 0	0, 1	0, 0	0, 2
Bile duct dilatation	1, 4	1, 5	1, 11	1, 9
Bile duct epithelium hyperplasia	0, 0	0, 0	0, 0	1, 0
Bile duct hyperplasia	0, 0	0, 0	0, 2	2, 1
Pericholangitis, granulomatous	0, 0	0, 0	0, 0	0, 1

m, months.

A tabular summary of non-neoplastic tissue alterations in and around the liver in males is shown in Table 2, females in Table 3. Trends seen in the occurrence of gallbladder lesions and bile duct abnormalities at 6 months were confirmed at 9 months. Representative photomicrographs of tissue alterations occurring at the 1.463 mg/L level of chloroform are shown in Figures 1 and 2.

Table 4 shows relevant findings at 6 and 9 months where responses were observed at both time points. The overall trend of the data was to have findings of no significance or marginal significance at 6 months and to have a significant finding at 9 months, except for cystic duct concretions in 6-month females and bile duct hyperplasia in 9-month males, which showed the opposite trend.

Fish hematology test. The hematology analysis was conducted after 6 months of chloroform exposure. ANOVA performed on data from chloroform-treated fish for hematocrit, leukocrit, cell viability, and cell count demonstrated that none of these parameters were significantly different from those of controls ($p = 0.05$).

Fish intrahepatic concentration. We detected no chloroform in any of the 20 fish livers from the 0 or the 0.017 mg/L aquaria concentration groups. Of the 20 fish analyzed in the 0.151 mg/L aquaria concentration group, 2 fish livers had measured concentrations of chloroform (33 and 133 mg chloroform/g of fish liver). At the 1.463 mg/L aquaria chloroform concentration, 9 fish livers out of the 20 sampled had detectable amounts of chloroform (23, 26, 35, 41, 128, 144, 159, 194, and 219 mg/g).

One or two other unidentifiable peaks were seen frequently on the chloroform chromatographs, as illustrated by a representative chromatograph in Figure 3. One of these peaks was in size equal to or greater than the chloroform peak. Repeated attempts to identify these other peaks through related experiments have not yet been successful.

Discussion

In medaka exposed to chloroform concentrations ranging from 0.017 to 1.463 mg/L, induction of liver neoplasms due to chloroform exposure was not significantly different ($\alpha = 0.05$) in treated fish when compared to control fish, after 6 or 9 months of exposure. Gallbladder abnormalities and bile duct abnormalities were observed in treated fish at significantly increased frequencies at the 1.463 mg/L chloroform level. Chloroform was not hepatocarcinogenic to the medaka after 6 or 9 months of exposure.

Numerous pathology findings were dissimilar between chloroform-exposed rodents and the current fish study. In rats (477 mg/kg chloroform/day, 48-hr duration, single gavage dose) and mice (30 ppm chloroform, 90-day duration, inhalation; and 30 ppm, 14 days of dosing with 2-year duration, inhalation), males had a greater sensitivity to chloroform presumably through testosterone receptor mechanisms in the proximal convoluted tubular cells (*33–36*). In medaka, females demonstrated a greater sensitivity to chloroform at both 6 and 9 months. Chloroform target organs in rodents were kidney, liver, and nasal passages (*33–35*), while in medaka only the gallbladder and bile ducts showed tissue abnormalities. In an inhalation study, rats that were exposed to 300 mg/L chloroform developed intestinal crypt-like ducts with periductular fibrosis from nonbiliary cells in their livers (*37*). No such corresponding abnormality was observed during fish pathology in the current study. Interestingly, the rodent studies revealed an association

Table 3. Histopathology of female medaka after chloroform exposure through 6 and 9 months: prevalence of findings.

Tissues	Control (6 m, 9 m)	0.017 mg/L (6 m, 9 m)	0.151 mg/L (6 m, 9 m)	1.463 mg/L (6 m, 9 m)
Gallbladders examined	31, 62	43, 54	34, 66	48, 55
Cholecystitis, granulomatous	0, 0	0, 0	0, 0	1, 3
Concretions	0, 1	0, 1	0, 5	9, 16
Cystic duct concretions	0, 1	1, 1	0, 1	2, 0
Cystic duct dilatation	0, 0	0, 0	0, 0	3, 1
Cystic duct hyperplasia	0, 0	1, 0	0, 0	1, 6
Epithelium hyperplasia	0, 0	0, 0	0, 2	5, 4
Livers examined	33, 62	47, 58	34, 67	48, 57
Bile duct concretions	0, 0	0, 1	0, 0	5, 6
Bile duct dilatation	1, 4	0, 4	0, 8	10, 12
Bile duct epithelium hyperplasia	0, 0	0, 0	0, 0	3, 4
Bile duct hyperplasia	0, 0	0, 1	0, 0	2, 7
Pericholangitis, granulomatous	0, 0	0, 0	0, 0	3, 4

m, months.

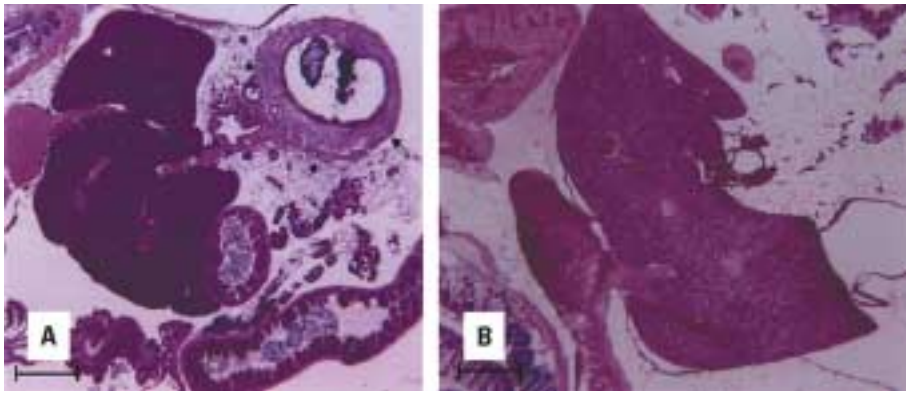

Figure 1. Hematoxylin and eosin-stained slides of medaka tissue after 9 months of continuous dosing. (*A*) Liver and gallbladder (arrows) with concretions in chloroform-treated fish (1.463 mg/L tank concentration); the gallbladder wall was thickened by a granulomatous inflammation. (*B*) Control liver from a fish of the same age. Bar = 300 µm.

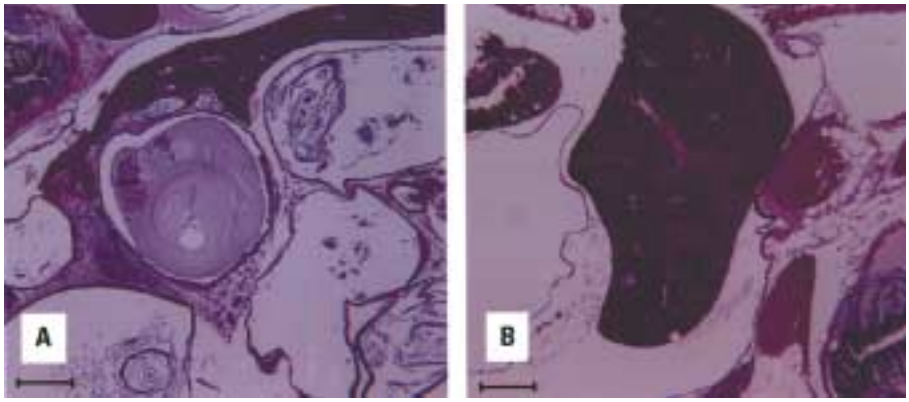

Figure 2. Hematoxylin and eosin-stained slides of medaka liver samples. (*A*) Biliary hyperplasia in liver of a fish treated for 9 months (1.463 mg/L chloroform). (*B*) Liver of a control fish of the same age. Bar = 300 µm.

between hepatocyte labeling indices and prevalence of aberrant ducts (*23*,*33*–*35*). In the fish, we saw no concurrent increase in hepatocyte labeling and tissue abnormalities. Some of these differences can be attributed to route of exposure and applied concentration levels, while others are undoubtedly related to choice of animal model.

The reason for the occurrence of concretions in the gallbladder and the bile ducts of the medaka is unknown. Concretions are rare in animals (*38*,*39*). Previously, biliary concretions have been identified in monkeys, cattle, and pigs (*39*). The principal constituents of these concretions were cholesterol, precipitated bilirubin, and calcium carbonate, respectively. When concretions occur, it is not unusual for them to be composites of these three materials (*39*). The cause of these concretions is usually an infection. In sheep choleliths (concretions), *Pseudomonas aeruginosa* has been identified as the infectious agent (*39*). The mechanism for formation is usually solid particles of dead organisms serving as a nidus for crystallization. Additionally, disturbances in the resorptive activities in the gallbladder may promote the development of concretions (*38*). Further investigations are necessary to determine the origin and makeup of these concretions in chloroform-treated medaka.

Hepatocellular proliferation in chloroform-treated fish livers was not significantly different from proliferation rates observed in control fish livers at 4 and 20 days of chloroform exposure. The exact cause of fish death in the other cell proliferation time points was not determined, but we suspect that the high mortality resulted from fungal and bacterial contamination in the stagnant processed well water delivery line used to make the BrdU exposure solutions.

Rodent studies have shown a positive association between increased cell proliferation and the hepatocarcinogenicity of a compound (*2*,*40*,*41*). In a previous study (*26*), this relationship between carcinogenicity and cell proliferation was also seen in the medaka after a 48-hr exposure to the liver carcinogen, diethylnitrosamine.

Intrahepatic chloroform concentration was less than external aquaria concentrations, with a higher number of fish livers containing chloroform at the 1.463 mg/L concentration. Phosgene is a known mammalian metabolite of chloroform (*42*), but we did not confirm its presence in fish livers exposed to chloroform. Chloroform does not appear to have bioconcentrated in fish liver; the chloroform intrahepatic concentration was always lower than the external aquarium concentration.

Chloroform was not acutely toxic to fish at concentrations two or three orders of magnitude above median drinking water levels. Chronic toxicity effects of chloroform were demonstrated by statistically significant findings in the gallbladder and bile ducts of fish treated with 1.463 mg/L chloroform.

Given the significant biliary findings observed at the high concentrations without a similar early induction of cell proliferation, it is intriguing to hypothesize that in initiated populations of cells (*43*), chronic exposure to the high concentration of chloroform may promote biliary carcinogenesis. It is also significant that although we observed no evidence of early hepatocellular necrosis and compensatory hyperplasia, the highest concentration of chloroform appeared to cause a chronic hyperplasia in animals sacrificed after 9 months of exposure. Further studies are warranted with this nonmammalian vertebrate model to add to the weight of evidence in public health decisions about balancing potential disinfection by-product toxicity and disease risk from microbial contamination.

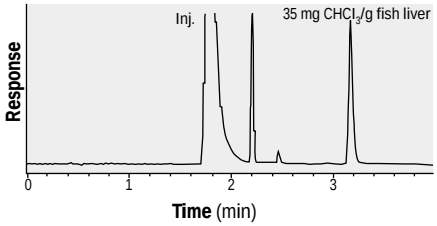

Figure 3. Chromatogram of a fish liver sample used to determine intrahepatic chloroform concentration in medaka fish exposed to 1.463 mg/L chloroform for 9 months. Peaks include the injection peak, two unknown peaks, and the chloroform peak, respectively. Instrumental detection limits of chloroform were 0.001 mg/L.

Table 4. Chloroform concentration effects[a] on fish histopathology end points at 6 and 9 months, by sex.

	Males		Females	
End point	6 months	9 months	6 months	9 months
Gallbladder				
Cystic duct dilatation	No	Marginal	NF	NF
Epithelium hyperplasia	No	Marginal	NF	NF
Granulomatous cholecystitis	NF	NF	Marginal	Yes
Concretions	NF	NF	Yes	Yes
Cystic duct concretions	NF	NF	No	Yes
Liver				
Bile duct concretions	Marginal	No	Yes	Yes
Bile duct dilatation	No	No	Yes	Yes
Bile duct epithelium hyperplasia	NF	NF	Yes	Yes
Bile duct hyperplasia	Yes	No	Yes	Yes
Granulomatous pericholangitis	NF	NF	Yes	Yes

Abbreviations: Marginal, $p > 0.05 \leq 0.10$; NF, none found; No, $p > 0.10$; Yes, $p < 0.05$.
[a]ANOVA significance level = 0.05.

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