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Acute and Subchronic Mammalian Toxicity of Naphthenic Acids from Oil Sands Tailings

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Naphthenic acids are the most significant environmental contaminants resulting from petroleum extraction from oil sands deposits. In this study, a mixture of naphthenic acids isolated from Athabasca oil sands (AOS) tailings pond water was used in acute and subchronic toxicity tests with rodents, in order to assess potential risks posed to terrestrial wildlife. Dosages were chosen to bracket worst-case environmental exposure scenarios. In acute tests, adult female Wistar rats were given single po dosages of naphthenic acids at either 3, 30, or 300 mg per kg body weight (mg/kg), while adult male rats received 300 mg/kg. Food consumption was temporarily suppressed in the high-dose groups of both sexes. Following euthanasia 14 days later, histopathology revealed a significant incidence of pericholangitis in the high-dose group of both sexes, suggesting hepatotoxicity as an acute effect. Other histological lesions included brain hemorrhage in high-dose males, and cardiac periarteriolar necrosis and fibrosis in female rats. In subchronic tests, naphthenic acids were po administered to female Wistar rats at 0.6, 6, or 60 mg/kg, 5 days per week for 90 days. Results again suggested the liver as a potential target organ. The relative liver weight in the high-dose group was 35% higher than in controls. Biochemical analysis revealed elevated blood amylase (30% above controls) and hypocholesterolemia (43% below controls) in high-dose rats. Excessive hepatic glycogen accumulation was observed in 42% of animals in this group. These results indicate that, under worst-case exposure conditions, acute toxicity is unlikely in wild mammals exposed to naphthenic acids in AOS tailings pond water, but repeated exposure may have adverse health effects.

Key Words: **naphthenic acids; oil sands; mammalian; acute and subchronic toxicity.**

Naphthenic acids are natural constituents of petroleum, where they evolve through the oxidation of naphthenes (cycloalkanes). This diverse group of saturated mono- and polycyclic carboxylic acids can account for as much as 4% of raw petroleum by weight (Brient *et al.,* 1995), and represents an important component of the waste generated during petroleum processing in some situations. For example, in the Athabasca oil sands industry near Fort McMurray, Alberta, Canada, naphthenic acids become dissolved and concentrated in tailings water as a result of the hot-water process used to extract bitumen from mined oil sands. These tailings are then amassed in large holding ponds in the immediate area, and must eventually be incorporated into aquatic and terrestrial landscape reclamation schemes. Since the contaminated water represents a long-term source of naphthenic acid exposure to indigenous wildlife, it is of interest to better characterize the toxicity of these compounds in order to determine potential risks to wildlife health.

The Athabasca oil sands (AOS) deposit covers about 42,000 km^2 surrounding Fort McMurray (57° 3.07′ N, 111° 36.02′ W), approximately 440 km northeast of Edmonton, Alberta. It is the largest of 4 major oil sands deposits in the province and contains over 200 billion cubic meters of petroleum, making it the world's largest single oil deposit (AOSTRA, 1990). Oil sands are a mixture of bitumen (crude petroleum), sand, and water. Separation of bitumen from other components is accomplished by the Clarke Hot Water Extraction process, where mined oil sands are mixed with hot (79–93°C) water and caustic soda (sodium hydroxide). After the bitumen is removed, residual sand, clay, and water, along with other inorganic and organic contaminants, are diverted to settling ponds. By 2025, an estimated 1 billion m^3 of tailings pond water (TPW) will have accumulated as a result of mining the AOS (Herman *et al.,* 1994).

A consequence of the hot water extraction process is that the alkalinity (pH = 8) promotes solubilization of naphthenic acids (pKa ~5), thereby concentrating them as mixtures of sodium salts in aqueous tailings. The actual amounts of naphthenic acids in the holding ponds of the two major companies operating in the AOS, Syncrude Canada Ltd. (Syncrude) and Suncor Energy Inc. (Suncor), are typically between 80 and 110 mg/l (FTFC, 1995). The bioavailability and persistence of naphthenic acids in contaminated waters are believed to be high. These compounds are highly soluble and have an extremely low volatility (Henry's constant = 8.56×10^{-6} atm × m^3/mol). Sorption to dissolved and particulate organic matter is limited by the polarity of dissolved naphthenates. The settling

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of fines in tailings ponds could decrease naphthenic acid bioavailability to some extent since naphthenic acids are amphipathic compounds that have surfactant-like properties and will accumulate at aqueous/nonaqueous interfaces, such as between water and fines. Physical biodegradation mechanisms for naphthenic acids have not been well investigated, but microbial biodegradation does occur, although primarily for naphthenic acids with carbon numbers ≤21 (Holowenko *et al.,* in press).

Although there are numerous contaminants in TPW, evidence suggests that naphthenic acids are the component primarily responsible for toxicity to aquatic organisms. For example, acute lethality of TPW from Mildred Lake Settling Basin (the primary holding pond of Syncrude) to rainbow trout (*Onchorhynchus mykiss*) and water fleas (*Daphnia magna*) was significantly reduced when tailings were treated to remove naphthenic acids. The treatment did not significantly influence the concentrations of other potentially important contaminants in the TPW (MacKinnon and Boerger, 1986). Another aquatic toxicity study with freshwater fish used commercial sodium salts of naphthenic acids and produced 96-h LC_{50} values of 50 mg/l for adult kutum (*Rutulis frisii* kutum) and sturgeon (*Acipenser gueldenstaedi*), and 75 mg/l for roach (*Rutulis rutulis* caspicus). Sixty-day LC_{50} values for these species were 2, 11, and 14 mg/l, respectively (Dokholyan and Magomedov, 1983). Significant nonlethal effects observed included decreased blood glucose levels, reduced leukocyte count, and increased muscle glycogen. It should be emphasized that the chemical composition of naphthenic acid mixtures is source-specific, so extrapolation of toxicity test results to different sources should be done cautiously.

Compared to aquatic organisms, there is limited information on the mammalian toxicity of naphthenic acids. The po LD_{50} of male mice was found to be 3.55 g/kg body weight for commercial sodium salts of naphthenic acids. Symptoms observed included central nervous system depression, convulsions, and respiratory arrest, leading to death (CEATAG, 1998). For rats, the po LD_{50} was 3.0 g/kg for commercial naphthenic acids (U.S. EPA, 1987), while a mixture of isomers of dimethylcyclohexane (a specific naphthenic acid) had a po LD_{50} of 1.75 g/kg (Uzhdavini and Glukharev, 1984). Intramuscular administration of cyclopentane naphthenic acid to rats at 0.15 g/kg/day for 10 days resulted in increased vascular permeability of capillaries (Maizelis *et al.,* 1980). To the authors' knowledge, results of mammalian toxicity testing involving repeated po exposure to naphthenic acids have not been reported previously.

The objective of the current study was to determine whether AOS naphthenic acids posed a health risk to indigenous mammals by means of exposure through their drinking water. Naphthenic acids were isolated from TPW collected from the upper (0–3 m), clarified zone of Mildred Lake settling basin. Wistar rats, serving as surrogate mammals, were used to test the acute po toxicity as well as the po subchronic toxicity of the naphthenic acids. Animals were gavaged with either single (acute study) or repeated (subchronic study) doses of the purified naphthenic acids, reconstituted in distilled water. Dosage levels in each study were chosen to bracket worst-case environmental exposures that small mammals could achieve if their drinking water was contaminated with naphthenic acids at concentrations comparable to TPW (i.e., 100 mg/l). Effects on body weight, water and food consumption, and other clinical signs of toxicosis were monitored, followed by histopathological examination of major organs. In subchronic testing, plasma biochemical and hematological effects were also evaluated.

MATERIALS AND METHODS

Isolation and purification of naphthenic acids. The procedure for isolating naphthenic acids from TPW has been described previously (Rogers *et al.,* in press). Briefly, the neutral, organic fraction of acidified TPW (including unionized naphthenic acids) was extracted with dichloromethane. Naphthenic acids were subsequently purified by reconstitution of the water soluble component of this fraction in basic solution, followed by ultrafiltration with a 1000 MW cutoff membrane. The filtrate was primarily low MW naphthenic acids. Identification and quantification of the naphthenic acids was performed using Fourier Transform Infrared (FTIR) analysis. The concentration of naphthenic acids in the stock solution was determined to be 8549 mg/l. A small portion of stock was dehydrated to provide a concentrated naphthenic acid solution for high-dose acute toxicity testing (see below). Since polycyclic aromatic hydrocarbons (PAHs) are known to be present in TPW, there was a concern about possible contamination of the purified naphthenic acid stock solution with these compounds. Upon analysis, however, the total concentration of PAHs in the stock solution was found to be low (75 ng/ml), and well below toxicological significance, based on the volumes administered in this study. The isolated naphthenic acid mixture was partially characterized using mass spectrometry, revealing the stock solution to be a highly heterogeneous mixture of acyclic and 1-, 2-, 3-, and 4-ringed compounds (Rogers *et al.,* in press).

Animals and animal husbandry. All animals used in this study were outbred Wistar rats, purchased from Charles River Canada (St. Constant, Ontario, Canada) and were 9 weeks old at the onset of dosing. Rats were housed individually in polycarbonate cages lined with heat-treated hardwood bedding, with *ad libitum* access to Prolab Lab Diet™ 5P00 rat chow (PMI® Nutrition International Inc., St. Louis, MO) and tap water. Animals were maintained under a light:dark schedule of 12h:12h at a temperature of 20°C. In the acute study, 40 female rats were randomly assigned to 4 groups of 10 animals each, designated control, low-dose, medium-dose, and high-dose. Twenty male rats were randomly assigned to 2 groups of 10 animals each, designated control and high-dose. In the subchronic study, 48 female rats were randomly assigned to 4 groups of 12 animals each (control, low, medium, and high dose). Animals were allowed a 2-week acclimatization period prior to toxicity testing during which body weight and food and water consumption were monitored. All aspects of this study were conducted with approval from the University of Saskatchewan Committee on Animal Care and Supply, in compliance with the Principles of Animal Care of the Canadian Federation of Biological Societies.

Acute toxicity test. Animals were fasted for 12 h prior to dosing on day 0. Treatment females were dosed by po gavage, using a stainless steel feeding needle, with 5.45 ml/kg body weight of aqueous solutions containing either 55,080, 5508, or 550.8 mg/l naphthenic acids. The dosages delivered were therefore 300 (high dose), 30 (medium dose), or 3 (low dose) mg/kg body weight naphthenic acids. These dosages reflected 50, 5, and 0.5 times, respectively, a worst-case, single day exposure for wild mammals. This is based on a naphthenic acid concentration in environmental drinking water of 100 mg/l, typical of relatively fresh TPW (FTFC, 1995), and a water consumption rate of 60 ml/kg/day. Treatment males received the high dose in the same volume (per

unit body weight) as females. Female and male controls were given 5.45 ml/kg tap water by gavage. Animals were returned to an *ad libitum* diet immediately after dosing.

All rats were monitored continuously for 12 h after dosing for signs of toxicosis. For the remainder of the 14-day study period, animals were monitored daily for changes in body weight, food and water consumption, and any additional behavioral or clinical signs of toxicosis. Rats were euthanized on day 14 by CO_2 inhalation, and selected organs removed, weighed, and fixed in 10% neutral buffered formalin. Fixed tissues were sectioned, stained with hematoxylin and eosin, and examined microscopically for toxicant-induced changes.

Subchronic toxicity test. For the repeated dose study, animals were exposed to naphthenic acids by po gavage 5 days per week (Monday–Friday), for a 90-day period (68 total doses). Treatment animals (all females) were dosed by po gavage, using a stainless steel feeding needle, with 7.0 ml/kg of aqueous solutions containing either 8549, 854.9, or 85.5 mg/l naphthenic acids. The dosages delivered were therefore 60 (high dose), 6 (medium dose), or 0.6 (low dose) mg/kg body weight naphthenic acids per day. Control females were gavaged daily with 7.0 ml/kg tap water. These dosages reflected 10, 1, and 0.1 times, respectively, a worst-case, daily exposure for wild mammals, based on daily water consumption rates and environmental naphthenic acid concentrations discussed above. Animals were maintained on an *ad libitum* diet of rat chow and tap water throughout the test, with body weight, food and water consumption, and behavioral and clinical signs monitored daily.

Blood samples were collected from the ventral tail vein of each rat on day 45 of dosing. Samples were submitted to Prairie Diagnostic Services, Western College of Veterinary Medicine, Saskatoon, SK, Canada, for comprehensive plasma biochemical and hematological analyses. At the completion of the repeated dose study on day 91, rats were anesthetized with isofluorane, and blood samples collected by cardiac puncture were immediately submitted for plasma biochemical and hematological analyses as above. All plasma biochemical analyses were performed using an automated chemistry analyzer (Abbott Spectrum II™, Chicago, IL). Sorbitol dehydrogenase activity was measured in place of alanine aminotransferase and aspartate aminotransferase in the standard clinical chemistry panel, since the former plasma enzyme is considered more hepatospecific and sensitive in the detection of hepatocellular injury in rats (Loeb, 1997). Following cardiac puncture, rats were euthanized by exsanguination. Selected organs were removed, fixed, sectioned, and stained for histopathological examination as previously described. To evaluate the presence of glycogen in hepatocytes, duplicate sections of liver were stained with Periodic Acid-Schiff, with and without prior treatment with diastase. Positive staining that was lost after diastase treatment was considered to be due to the presence of glycogen (modified from Luna, 1968).

Statistical analyses. Statistical analysis was performed using SigmaStat™ statistical software, version 2.0. In the acute study, a one-way ANOVA was used to compare means of female dose and control groups (4) with respect to consumption and body weight at regular timepoints, as well as the postmortem organ weights. A pairwise multiple comparison test was then used in cases where statistical significance was reached. For the male dose and control groups (2), a Student's *t*-test was used to compare group means. In the subchronic study, one-way ANOVA was again used to compare group means for consumption, plasma biochemical/hematological parameters, and organ weights, while a one-way repeated measures ANOVA was used to compare body weight trends. An ANOVA on ranks was performed on histological scoring of tissue lesions for both studies. Probability values of $p \leq 0.05$ were considered statistically significant.

RESULTS

Acute Toxicity Test

Behavioral observations. Immediately following dosing, 2 of 10 females and 3 of 10 males in the high-dose groups were lethargic and mildly ataxic. These symptoms disappeared within 1–2 h. With this exception, no outward behavioral abnormalities were noted during the 2-week posttreatment period.

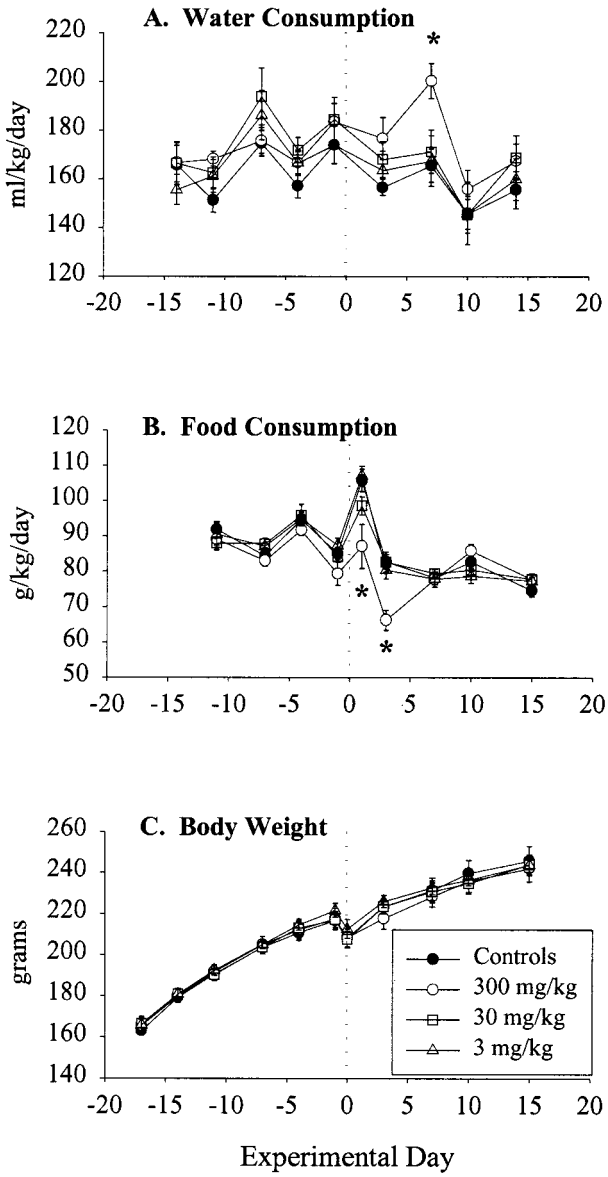

FIG. 1. Water consumption (A), food consumption (B), and body weight (C) trends for 9-week-old female Wistar rats dosed once with naphthenic acids at 3, 30, or 300 mg/kg body weight. Each data point represents the mean ± SEM ($n = 10$). Values for consumption are based on total intake and average body weight of the preceding time interval. *Significantly different from other groups ($p < 0.05$).

Consumption and body weight trends. Mean water and food consumption and body weight trends are illustrated in Figures 1 and 2 for female and male rats, respectively. Water consumption of dosed and control groups was similar for each sex. High-dose females and males exhibited a significant decrease in food consumption for 4 days following dosing, but all

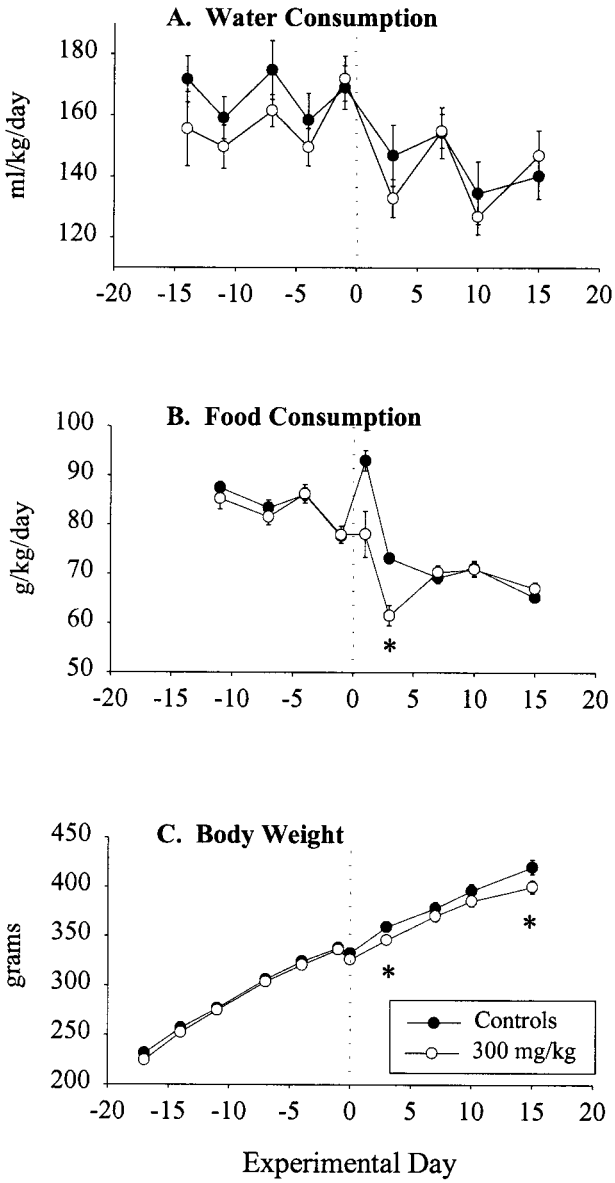

FIG. 2. Water consumption (A), food consumption (B), and body weight (C) trends for 9-week-old male Wistar rats dosed once with naphthenic acids at 300 mg/kg body weight. Each data point represents the mean ± SEM (n = 10). Values for consumption are based on total intake and average body weight of the preceding time interval. *Significantly different from other groups ($p < 0.05$).

groups were similar thereafter. A small but significant post-treatment drop in body weight was observed in males, but not females. The sudden drop in body weight from day –1 to day 0 for all groups resulted from the predosing fast. Gorging on day 0 (after food was reintroduced) was evidenced by the spike in food consumption, but was less marked in dosed animals, particularly the high-dose groups of both sexes.

Organ weights. Minor but statistically significant increases were observed in the relative organ weights (as % of total body weight) of high-dose rats compared with other dose groups for ovaries and spleen in female rats, and for testes and heart in male rats (Tables 1 and 2).

TABLE 1
Organ Weights (% Total Body Weight) of Female Rats following Single Oral Exposure to Naphthenic Acids

Organ	Controls	High dose	Medium dose	Low dose
Liver	4.05 ± 0.139	4.40 ± 0.133	3.91 ± 0.064	3.96 ± 0.070
Kidneys	0.77 ± 0.017	0.79 ± 0.023	0.76 ± 0.015	0.75 ± 0.014
Heart	0.34 ± 0.012	0.35 ± 0.006	0.35 ± 0.008	0.34 ± 0.006
Spleen	0.26 ± 0.011	0.29 ± 0.012*	0.26 ± 0.008	0.27 ± 0.013
Ovaries	0.06 ± 0.002	0.07 ± 0.004*	0.06 ± 0.002	0.06 ± 0.002
Lungs	0.59 ± 0.030	0.62 ± 0.019	0.61 ± 0.017	0.56 ± 0.012

Note. Values represent the mean ± SEM (n = 10). High dose, 300 mg/kg; medium dose, 30 mg/kg; low dose, 3 mg/kg.
*Significantly different from other groups ($p < 0.05$).

Histology. Median scores for lesions observed in specific organs were determined in a blind pathological analysis. A significant dose-related effect was most evident in livers, with 6 of 10 high-dose males exhibiting mild, and 7 of 10 high-dose females exhibiting moderate, eosinophilic pericholangitis (Fig. 3A). Mild myocardial necrosis with periarteriolar fibrosis was significantly more prevalent in medium-dosed females, with 7 of 10 animals affected (Fig. 3B). Heart lesions were observed in only 4 of the 10 dosed males. Brain hemorrhage was present in 6 of 10 high-dose males (Fig. 3C). The hemorrhage predated euthanasia and included macrophages containing hemosiderin. By comparison, only 2 of 10 high-dose females exhibited cerebral hemorrhage.

Subchronic Toxicity Test

Behavioral observations. A small portion of rats in each group experienced seizures, which were only observed at the time of gavaging. Some rats had only one such episode, while others had repeated seizures. These events were not observed

TABLE 2
Organ Weights (% Total Body Weight) of Male Rats following Single Oral Exposure to Naphthenic Acids

Organ	Controls	High dose
Liver	4.16 ± 0.067	4.20 ± 0.125
Kidneys	0.72 ± 0.016	0.76 ± 0.018
Heart	0.29 ± 0.006	0.32 ± 0.005*
Spleen	0.24 ± 0.010	0.24 ± 0.011
Testes	0.73 ± 0.023	0.84 ± 0.020*
Lungs	0.47 ± 0.029	0.49 ± 0.011

Note. Values represent the mean ± SEM (n = 10). High dose, 300 mg/kg.
*Significantly different from controls ($p < 0.05$).

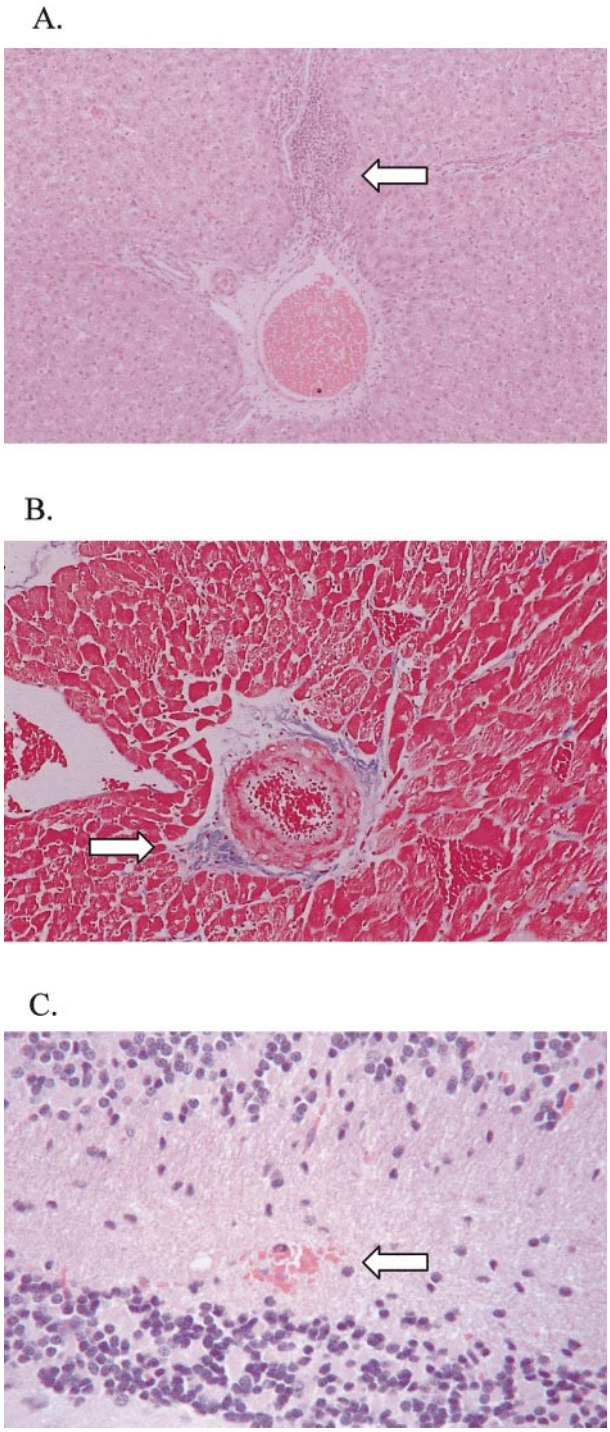

FIG. 3. Lesions in rat tissues 2 weeks after administration of a single po dose of naphthenic acids to Wistar rats. (A) Pericholangitis, evidenced by dark-stained lymphocytes, plasma cells and eosinophils (white arrow) along biliary duct (hematoxylin and eosin [H/E] stain). (B) Myocardial periarteriolar fibrosis. Blue-stained amorphous collagen (white arrow) is seen surrounding a blood vessel in the heart (Masson's Trichrome stain). (C) Cerebral hemorrhage, illustrated by a focus of extra-vascular erythrocytes (white arrow) in brain section (H/E stain).

until dosing day 40, with the earliest and most severe seizures occurring in the high (25% of rats) and medium (17% of rats) dose groups. Severe, clonic seizures typically lasted about 20 s, followed by a brief tetanic contraction and a 2–3 min period of immobility. Animals resumed normal behavior and activity immediately after each of these episodes, except one rat from the high-dose group that died following a seizure. By comparison, rats in the low-dose (8%) and control (17%) groups demonstrated milder episodes, characterized primarily by muscle twitching.

Consumption and body weight trends. Mean water and food consumption and body weight trends are illustrated in Figure 4. Compared with controls, water consumption by high-dose rats was significantly elevated throughout most of the experiment, but was depressed for the low-dose group. Food consumption was significantly less in high-dose rats for the first 11 days of dosing. However, from day 11 to the end of the study, food consumption of all groups was similar. Mean body weight of animals in the high-dose group was significantly lower than the weight of other groups throughout the exposure period.

Organ weights. Significant differences existed between the relative liver, kidney, and brain weights of high-dose rats and those of other groups (Table 3). Livers were 36% heavier than those of controls, while kidney and brain weights of high-dose rats were 12 and 9.1% heavier than controls, respectively.

Plasma biochemistry. Plasma biochemistry results are provided in Table 4. For brevity, results are restricted to those parameters for which significant differences existed amongst groups, although a more comprehensive analysis of plasma solutes and enzymes was performed. The most apparent treatment-related effects were changes in total cholesterol concentration and amylase activity. On dosing days 45 and 91, total plasma cholesterol concentration in the high-dose rats was reduced by 41 and 43% compared with control values, respectively. Amylase activity was 33 and 30% higher than in control rats on days 45 and 91, respectively. Differentiation of amylase isozymes was not provided by the automated analytical technique used. Less pronounced but significant differences were observed between the high-dose group and other groups with respect to total protein concentration (increased in high-dose rats) and albumin/globulin ratio (decreased in high-dose rats).

Hematology. Hematological parameters for which significant differences existed between groups are shown in Table 5. Minor reductions in hemoglobin concentration, mean corpuscular volume, and hematocrit were apparent in the high-dose group, but differences were not large, and values were within or close to normal ranges for this species.

Histology. Dose-related, histological changes were only observed in liver tissue in the subchronic test. The prevalence of pericholangitis was low in contrast to the acute study, but there was a positive correlation between dose and liver glycogen accumulation. Five of 12 high-dose rats exhibited above-

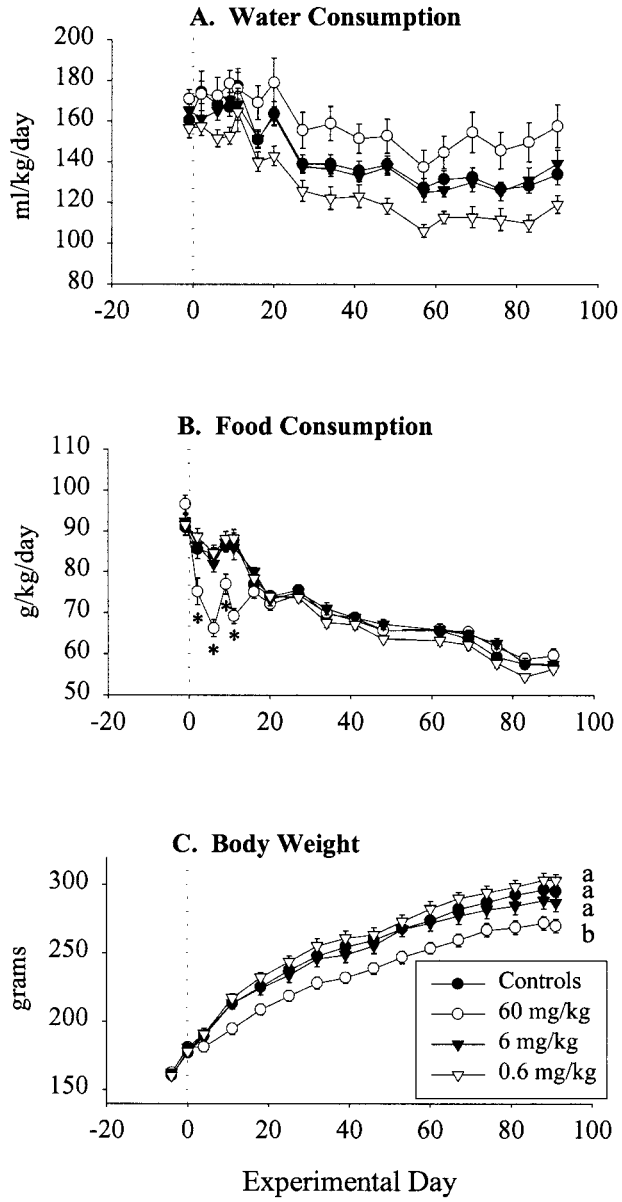

FIG. 4. Water consumption (A), food consumption (B), and body weight (C) trends for 9-week-old female Wistar rats during subchronic (90-day) dosing with naphthenic acids at 0.6, 6, or 60 mg/kg/day. Each data point represents the mean ± SEM ($n = 12$). Values for consumption are based on total intake and average body weight of the preceding time interval. *Significantly different from other groups ($p < 0.05$).

normal (moderate to intense) hepatic glycogen stores (Fig. 5), compared with 3 medium- and 2 low-dose rats. One control had mild glycogen accumulation. There were no treatment-related cardiovascular lesions.

DISCUSSION

Exposure of Wistar rats to single or repeated po doses of AOS naphthenic acids produced a number of treatment-related effects, particularly in the highest dose groups. Marked reduction in food consumption was observed immediately following dosing in the high-dose group of the acute toxicity study. A similar decrease in food consumption was observed in the subchronic study, but in both cases the effect was short-lived. Appetite suppression was probably not due to direct irritation of the gastrointestinal lining, since repeated exposure did not

TABLE 3
Organ Weights (% Total Body Weight) of Female Rats following Repeated Oral Exposure to Naphthenic Acids

Organ	Controls	High dose	Medium dose	Low dose
Liver	3.07 ± 0.044	4.15 ± 0.147*	3.34 ± 0.093	3.17 ± 0.066
Kidneys	0.57 ± 0.011	0.63 ± 0.016*	0.59 ± 0.014	0.58 ± 0.011
Heart	0.32 ± 0.007	0.33 ± 0.011	0.33 ± 0.007	0.32 ± 0.008
Spleen	0.24 ± 0.008	0.26 ± 0.010	0.25 ± 0.007	0.23 ± 0.010
Ovaries	0.05 ± 0.004	0.05 ± 0.003	0.05 ± 0.003	0.05 ± 0.002
Brain	0.61 ± 0.010	0.67 ± 0.016*	0.64 ± 0.011	0.61 ± 0.011

Note. Values represent the mean ± SEM ($n = 12$). High dose, 60 mg/kg/day; medium dose, 6 mg/kg/day; low dose, 0.6 mg/kg/day.
*Significantly different from other groups ($p < 0.05$).

TABLE 4
Selected Plasma Biochemistry Parameters of Female Rats following Repeated Oral Exposure to Naphthenic Acids

Parameter	Day 45	Control range	Day 90	Control range
Cholesterol (mmol/l)		2.25–3.25		1.57–2.56
Controls	2.81 ± 0.11[a]		2.11 ± 0.08[a]	
High dose	1.67 ± 0.09[b]		1.20 ± 0.05[b]	
Medium dose	2.84 ± 0.15[a]		1.97 ± 0.11[a]	
Low dose	2.50 ± 0.07[a]		1.99 ± 0.08[a]	
Amylase (U/l)		1256–1804		1228–1592
Controls	1656 ± 76[a]		1417 ± 34[a]	
High dose	2198 ± 91[b]		1838 ± 80[b]	
Medium dose	1856 ± 72[a]		1565 ± 57[a]	
Low dose	1786 ± 109[a]		1635 ± 91[a,b]	
Total protein (g/l)		76.0–92.0		62.0–73.0
Controls	82.1 ± 1.5[a]		66.4 ± 1.0[a]	
High dose	82.9 ± 1.3[a]		72.3 ± 2.0[b]	
Medium dose	80.7 ± 1.6[a]		65.3 ± 1.3[a]	
Low dose	82.1 ± 0.8[a]		68.3 ± 1.3[a,b]	
Albumin/globulin ratio		1.22–1.99		1.39–2.09
Controls	1.63 ± 0.12[a]		1.71 ± 0.07[a]	
High dose	1.44 ± 0.10[a]		1.40 ± 0.05[b]	
Medium dose	1.64 ± 0.10[a]		1.63 ± 0.05[a]	
Low dose	1.57 ± 0.08[a]		1.65 ± 0.04[a]	

Note. High dose, 60 mg/kg/day; medium dose, 6 mg/kg/day; low dose, 0.6 mg/kg/day naphthenic acids. Values represent the mean ± SEM ($n = 12$). NOEL (no observed effect level) and LOEL (lowest observed effect level) = 6 and 60 mg/kg/day, respectively.
[a,b]Similar letters indicate no significant difference between groups ($p < 0.05$).

TABLE 5
Selected Hematological Parameters of Female Rats following Repeated Oral Exposure to Naphthenic Acids

Parameter	Day 45	Control range	Day 90	Control range
Hemoglobin (g/l)		148–171		135–153
Controls	161 ± 3.6[a]		145 ± 1.9[a]	
High dose	145 ± 1.7[b]		136 ± 2.2[b]	
Medium dose	157 ± 3.3[a]		143 ± 2.2[a,b]	
Low dose	156 ± 2.3[a]		142 ± 2.3[a,b]	
Mean corpuscular hemoglobin (pg/l)		18.4–19.3		17.9–19.7
Controls	19.0 ± 0.14[a,b]		18.9 ± 0.18[a]	
High dose	18.2 ± 0.14[a]		17.9 ± 0.13[b]	
Medium dose	19.2 ± 0.17[b]		18.8 ± 0.19[a]	
Low dose	18.3 ± 0.30[a]		18.5 ± 0.29[a,b]	
Mean corpuscular volume (fl)		57.8–61.1		55.9–60.7
Controls	59.7 ± 0.5[a]		58.7 ± 0.4[a]	
High dose	56.8 ± 0.4[b]		56.8 ± 0.3[b]	
Medium dose	60.1 ± 0.7[a]		58.9 ± 0.4[a]	
Low dose	57.8 ± 0.9[a,b]		57.8 ± 0.7[a,b]	
Hematocrit (l/l)		0.47–0.54		0.42–0.48
Controls	0.51 ± 0.01[a]		0.45 ± 0.01[a]	
High dose	0.45 ± 0.01[b]		0.43 ± 0.01[a]	
Medium dose	0.49 ± 0.01[a]		0.45 ± 0.01[a]	
Low dose	0.49 ± 0.01[a]		0.45 ± 0.01[a]	

Note. High dose, 60 mg/kg/day; medium dose, 6 mg/kg/day; low dose, 0.6 mg/kg/day naphthenic acids. Values represent the mean ± SEM ($n = 12$). NOEL (no observed effect level) and LOEL (lowest observed effect level) = 6 and 60 mg/kg/day, respectively.

[a,b]Similar letters indicate no significant difference between groups ($p < 0.05$).

sustain the effect in the subchronic study. In addition, there was no histopathological evidence of gastrointestinal irritation in either study. The mechanism of toxicant-induced anorexia has yet to be determined.

The results of the acute toxicity test suggested exposure to AOS naphthenic acids at levels of 300 mg/kg in rats had both cardiovascular and hepatic effects. A single po dose of 300 mg/kg produced significant cerebral hemorrhage in male rats. Vasoactive effects of naphthenic acids were also noted in a previous study by Maizelis *et al.* (1980). In that study, rats injected intramuscularly with 150 mg/kg cyclopentane naphthenic acid for 10 days demonstrated increased vascular permeability of cerebral capillaries. A similar effect on vascular permeability was not directly investigated in our study, but such an effect could be linked to the cerebral hemorrhaging or periarteriolar necrosis/fibrosis in the heart that was apparent here following acute exposure to naphthenic acids. It is unclear why the cerebral hemorrhage observed in the present study was more prevalent in male than in female rats.

The effects of acute naphthenic acid dosing on cardiac tissue have not previously been documented. Whether the observed periarteriolar necrosis/fibrosis was attributable to parent naphthenic acids or their metabolites is unknown at this time.

The clearest demonstration of a target organ in the acute toxicity test was the liver, where the occurrence of pericholangitis was consistent between sexes and highly dose-dependent. However, the pathogenesis of the observed pericholangitis remains to be determined.

Although sex differences may exist with respect to naphthenic acid toxicity, the subchronic toxicity test was restricted to female Wistar rats for logistical reasons (e.g., there was a limited supply of purified naphthenic acids). In addition, for these initial investigations of naphthenic acid toxicity, female

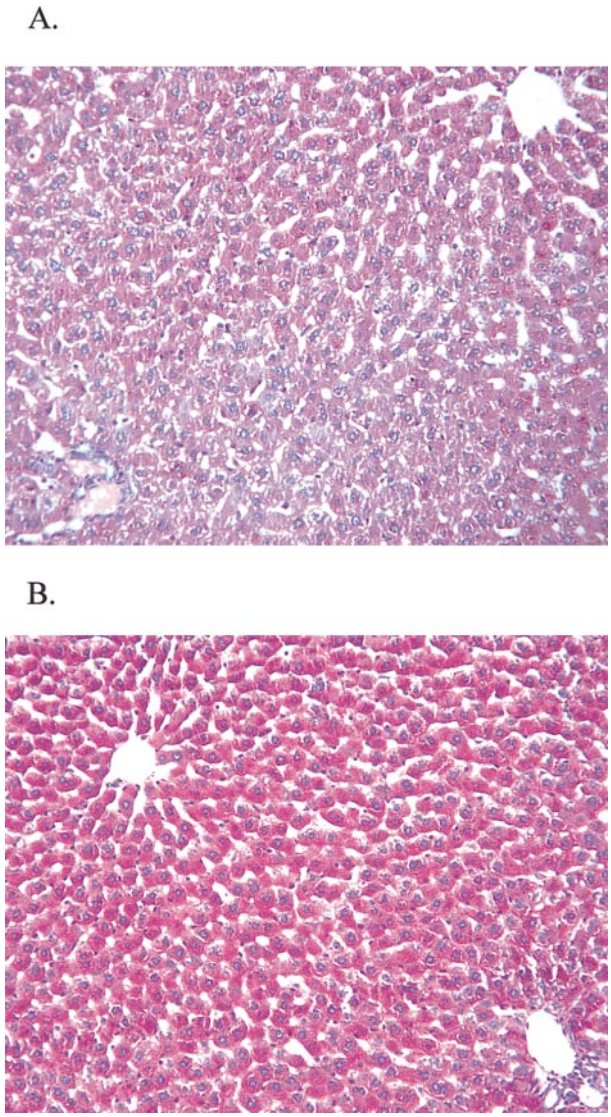

FIG. 5. Glycogen accumulation in female rat liver following subchronic (90-day) dosing with naphthenic acids at 60 mg/kg/day. Periodic Acid Schiff-stained liver sections with diastase digestion from control (A) and high-dose (B) rats show more intense coloration, and hence glycogen concentration, in treated animals.

responses to subchronic exposure were of greater interest since the data were required for future reproductive toxicity tests.

Subchronic exposure of female rats to naphthenic acids affected both food and water consumption. Although the suppression in food consumption was short-term, the elevation in water consumption in high-dose rats persisted throughout most of the 90-day exposure period. It is possible that the higher water consumption was simply due to elevated sodium intakes in the high-dose animals. Naphthenic acids were administered as sodium salt solutions, with the high-dose solution having a Na^+ concentration of 0.1 molar. Since the gavage volume was 7 ml/kg body weight, naphthenic acid administration resulted in a daily sodium dose of 16.1 mg/kg body weight. This was over and above the 50 mg/kg body weight per day normally derived from the diet (Koolhaas, 1999), such that treatment increased the sodium intake of high-dose rats by 32%. Medium- and low-dose rats received dilutions of the high-dose solution, and therefore incremental sodium intake was negligible by comparison.

Although food consumption of all dose groups and controls were similar beyond day 11 in the subchronic study, the average body weight of the high-dose rats was significantly lower than that of other groups. The difference was consistent until the end of dosing, with high-dose animals being 8–9% lighter than controls. The effect on body weight was not extreme, but suggested that the 60 mg/kg naphthenic acid treatment had a sustained, suppressive influence on growth. It was not determined whether the loss in body mass was predominantly in the form of muscle or adipose tissue.

Subchronic dosing produced a marked increase in relative liver weight, which was 35% above control values for the high-dose group. This effect did not appear to be attributable to glycogen accumulation, since the relative liver weight of rats exhibiting high hepatic glycogen stores were similar to liver weights of rats not having high glycogen. A possible explanation for the increased liver weights of high-dose rats could be the induction of hepatic detoxification enzymes such as cytochrome P450 (Woodman, 1996), a mechanism currently being investigated. The relative weights of brain and kidneys in the high-dose group were also statistically greater than in control rats (11.5 and 9.1% higher, respectively). However, these differences are modest, and may simply reflect the slightly lower body weights of animals in the high-dose group. The elevations in terminal organ weights were not likely due to sodium and water retention. No pathological lesions were observed in the kidneys, rats had free access to drinking water, and plasma levels of sodium were normal both at the midpoint and end of the subchronic study.

Plasma biochemical analysis at the midpoint (day 45) and end (day 91) of the subchronic study also suggested liver as a target organ for naphthenic acid toxicity, particularly the suppression in plasma cholesterol concentration. Hypocholesterolemia in the high-dose group was not likely due to a difference in diet, since food consumption patterns of all groups were similar beyond day 11, and because severe dietary restrictions are required to induce this effect in rats (Levin *et al.*, 1993). Baseline plasma cholesterol levels are largely regulated via synthesis in the liver, and suppression with naphthenic acid exposure suggests interference with synthetic, feedback, or mobilization pathways associated with that organ. Some of these mechanisms are currently being explored. Changes in cholesterol or other plasma lipids are often preceded by more obvious changes in other diagnostic tests (Woodman, 1999), so the presence of hypocholesterolemia without other evidence of major hepatic dysfunction is surprising. Another possible mechanism for hypocholesterolemia that is being investigated is cytochrome P450 induction, since cholesterol is a substrate for the CYP 7 subfamily of this enzyme group (Okuda *et al.*, 1993). Changes in plasma amylase are usually associated with pancreatic disease in most species. However, in rats it may also be indicative of a hepatic effect, since all isozyme forms of amylase are also present in rat hepatocytes (Loeb, 1999). In addition, there was no evidence of pathological lesions in the pancreas of treated rats. However, since organ-specific amylase isozymes were not quantified in this study, it is uncertain at this time whether dose-related changes in amylase reflect a liver and/or pancreatic effect.

The major histopathological observation associated with repeated naphthenic acid dosing was increased glycogen storage in the liver. Hepatic glycogen storage is a normal metabolic function, but abnormally high intracellular accumulation can be a signal of hepatotoxicity or perturbations of carbohydrate metabolism. Interestingly, increased muscle glycogen was reported by Dokholyan and Magomedov (1983) in fish exposed to sodium naphthenates. The possible pathogenic mechanism for these observations are unknown. Other histopathological lesions that were seen in the acute study were not observed to a significant degree in the subchronic study. For example, the prevalence of pericholangitis in high-dose females in the subchronic study was low. This may suggest that 60 mg/kg, even on a repeated-exposure basis, is below the threshold required to induce this type of change.

In conclusion, a number of significant physical, clinical, and pathological changes were associated with the po administration of AOS naphthenic acids to Wistar rats, either acutely (300 mg/kg) or subchronically (60 mg/kg/day). Several of these observations support the conclusion that the liver is a plausible target organ in both cases and for both sexes, although the mechanism of toxic injury appears distinct between short- and long-term exposures to naphthenic acids.

Based on the Wistar rat as a surrogate mammal, the results of this study suggest the risk of acute po toxicity to small, wild mammals exposed to AOS naphthenic acids in contaminated drinking water is low, since 300 mg/kg is approximately 50 times a likely worst case single-day exposure. This exposure estimate is based upon a typical tailings pond water concentration of 100 mg/l and an average water consumption rate of 60 ml/kg/day (60 ml/kg/day $\times$ 100 $\times$ 10^{-3} mg/ml = 6 mg/kg/

day). However, adverse health effects from repeated low-dose exposure may be more likely, since 60 mg/kg is only 10 times the estimated worst-case daily exposure for small mammals. Naturally, the extrapolation of data from the laboratory to the field always involves uncertainty, and there exists the possibility that indigenous mammals could be more sensitive to naphthenic acids than Wistar rats. Age, season, diet, general health and contemporary interacting stressors, contaminant interaction, and interspecic differences in metabolism or excretion are just a few factors that could influence the response to naphthenic acid consumption. It should also be noted that the naphthenic acids used in the present toxicity studies were relatively "fresh" since they were isolated from recently produced tailings. Knowing that naphthenic acids will undergo some microbial biodegradation over time, mixture composition will change, and the overall concentration will decrease in the future. However, this study was a logical starting point for assessing the mammalian toxicity of these compounds, and will be a valuable reference for others researching the toxicity of naphthenic acids from the AOS or other sources.

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