Bull. Environ. Contam. Toxicol. (2002) 69:593–600

DOI: 10.1007/s00128-002-0102-x

Bulletin of Environmental Contamination and Toxicology

Effects of Dietary Exposure to Environmentally Relevant Concentrations of Weathered Prudhoe Bay Crude Oil in Ranch-Raised Mink (*Mustela vison*)

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Received: 28 October 2001/Accepted: 26 April 2002

Oil spills, such as the *Exxon Valdez* oil spill (EVOS) in Prince William Sound (PWS), Alaska, have the potential to be very destructive to the environment and to have long-term impacts on wildlife species (Taylor et al. 2000; Duffy et al. 1999; EVOS 1994). Biological effects associated with crude oil exposure include reduced growth rates, reproductive dysfunction, developmental abnormalities, hepatotoxicity, as well as immune dysfunction, carcinogenesis, and central nervous system damage (Ben-David et al. 2000; Neff et al. 2000; Williams et al. 1995; Leighton 1990). However, no studies have attempted to identify specific neuropathological changes of chronic exposure to crude oil in mammals.

The purpose of the present study was to determine the effects of prolonged ingestion of weathered Prudhoe Bay crude oil (WPBCO) using concentrations of oil that could be encountered in the environment by wild mink. A ranch-raised strain of American mink (*Mustela vison*) was used as an animal model to represent a wildlife species that occupies a high trophic level and consumes prey affected by environmental contaminants, such as crude oil. Mink, as an indicator species of environmental contaminant exposure and ecosystem health, are approved by the U.S. Environmental Protection Agency and recognized by the National Academy of Science as a preferred toxicological model, and can be studied in a controlled laboratory situation (Aulerich et al. 1999; Calabrese et al. 1992). Mink have also been suggested as a model for sea otters (*Enhydra lutra*) (Mazet et al. 2000; Schwartz et al. 1997). By exposing post-natal mink in the late stages of development to non-lethal doses of weathered crude oil, we investigated the impacts on various parameters including hematology, serum clinical chemistries, and tissue pathology, including neuropathology.

MATERIALS AND METHODS

Thirty 8-week-old natural dark male mink kits were individually housed in wire-mesh cages (61 cm L x 31 cm W x 38 cm H) that were suspended above the ground in an open-sided building at the Michigan State University (MSU) Experimental Fur Farm. The mink were assigned to three groups with 10 animals per group, and fed diets containing 0, 100, or 1000 ppm weathered crude oil. Siblings were not placed in the same group to minimize any sensitivity to treatment attributable to genetic pre-disposition.

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Environmentally relevant concentrations of WPBCO used in this study were based on reported concentrations still found in the marine environment through 1996 (EVOS 2000). Prudhoe Bay crude oil was weathered as described by Ormseth and Ben-David (2000). The WPBCO was blended into standard mink feed formulated to meet the nutrient requirements of mink (NRC 1982) at concentrations of 100 and 1000 ppm. Control animals received the untreated basal diet. A sample of each diet was submitted to Litchfield Analytical Services (Litchfield, MI) for proximate analysis. Feed and drinking water were provided *ad libitum*. The diets were fed to the mink during a four-month period (July – November, 2000). Feed consumption was measured on two consecutive days per month to determine palatability of the diets and consistency of consumption across treatment groups. Ceramic crocks were used for the feed to be placed in individual cages. Feed was weighed in the morning and placed in the cage for each study animal. The crocks were then taken out the next morning and the uneaten portion was weighed. These steps were then repeated for the second day. The difference between the weight of the feed placed in the cage and the weight of the feed remaining after 24 hours was considered the amount of feed consumed.

The mink were observed daily for clinical signs of toxicity. Body weights were recorded prior to the start of the study and bi-weekly thereafter for the duration of the trial. At the end of the trial, animals (n = 29) were anesthetized with ketamine hydrochloride (Ketaset®; 25 mg/kg body weight; intramuscular injection; Fort Dodge Animal Health, Fort Dodge, IA), and approximately 5 ml of blood was collected from each mink via cardiac puncture for assessment of hematological parameters and serum clinical chemistries. Approximately half of the collected blood was added to a Vacutainer® tube (Becton Dickinson, Franklin Lakes, NJ) containing EDTA for hematology, and the remaining blood was added to a Vacutainer® tube containing no additive for serum chemistries. The blood samples were submitted to the MSU Clinical Pathology Laboratory for analysis. Hematologic parameters included red blood cell (RBC) count, white blood cell (WBC) count, hemoglobin (HGB) concentration, hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), spun packed cell volume (PCV), platelet volume, and leukocyte differential cell counts. Serum clinical chemistry parameters included calcium (Ca), chloride (Cl), iron (Fe), phosphorus (P), potassium (K), magnesium (Mg), sodium (Na), total carbon dioxide (TCO_2), anion gap, total protein (PROT), albumin (ALB), total globulin (GLOB), cholesterol (CHOL), glucose (GLUC), triglyceride (TRIG), blood urea nitrogen (BUN), and creatinine (CREAT) concentrations, albumin-globulin ratio (A/G ratio), alkaline phosphatase (ALK PHOS), alanine amino transferase (ALT), aspartate amino transferase (AST), amylase, creatinine kinase (CK) and sorbitol dehydrogenase (SDH) activities, and osmolality.

Three mink per treatment were randomly selected and deeply anesthetized with sodium pentobarbital (Nembutal® Sodium Solution, 125 mg/kg body weight; intraperitoneal injection; Abbott Laboratories, North Chicago, IL). Animals were

perfused transcardially with 4% paraformaldehyde for 10 minutes. Adrenal glands, kidneys, liver, pancreas, and spleen were removed, trimmed, weighed, and placed in 10% buffered formalin (pH 7.4) for subsequent routine histopathologic assessment. Brains were also preserved in 10% buffered formalin and processed for histologic examination as follows: longitudinal sections were taken to represent all major areas of the brain, including the cerebrum, hippocampus, brainstem, and cerebellum. Three hematoxylin-and-eosin-stained sections, two Kluver-Barrera-stained (for myelin) sections, and two Bodian-stained (for axons) sections were examined from each animal.

Data were analyzed using Statistical Analysis System (SAS) program version 8.0 (SAS Inst. Inc., Cary, NC). A series of univariate tests was used to determine normality of data. Analysis of variance III for Random Complete Block Design was used to analyze the differences between treatment diets (control, 100 ppm, and 1000 ppm) for feed consumption, body and organ weights, and blood parameters. The model included fixed effects of diet and random effects of litter. Analyses were followed by Tukey's multiple comparisons to establish where significant differences in the effects of treatment diets on variables occurred. The level of significance was based on a Type I error rate of $\alpha = 0.05$ ($P \leq 0.05$).

RESULTS AND DISCUSSION

The results of the proximate analysis indicated that the feed consisted of 48% moisture, 18.3% fat, 31% protein, 7.15% crude fiber, 10.16% ash, 1.94% calcium, 1.70% phosphorus, 0.82% potassium, 0.20% magnesium, and 0.73% sodium, with total digestible nutrients at 85.33%.

A significant decrease was recorded in feed consumption between the control and both oil-treated diets ($P \leq 0.0001$). Mean daily consumption values for the groups were 251.1 g (control), 217.8 g (100 ppm), and 196.6 g (1000 ppm). However, White et al (1991) reported no differences in consumption of diets containing up to 500 ppm weathered crude oil by mink.

Consumption of WPBCO did not adversely affect body weight gain in the present study. Mink in all groups gained weight during the 123-day feeding trial. Average weight gains were 1012 g, 1035 g, and 977 g for mink in the control group, and the 100, and 1000 ppm crude oil groups, respectively. Contrary to these findings, river otters (*Lutra canadensis*) that were live-captured in oiled areas in PWS, AK in 1991, two years after the EVOS, had significantly lower body weights than otters captured in non-oiled areas (Duffy et al. 1994). However, these wild river otters experienced multiple natural stressors besides oil exposure.

No clinical signs of toxicity (e.g. include lethargy, weight loss, general poor health) were noted on daily observation of the mink. One animal in the 100 ppm group died prior to the end of the study. The cause of death was determined to be urolithiasis in the left kidney and the urinary bladder, as well as an unidentified systemic infection that was unrelated to treatment.

Changes in hematology and serum clinical chemistry parameters were noted in mink consuming feed containing 100 ppm and1000 ppm WPBCO. Blood samples from one 100 ppm and two samples from the 1000 ppm groups clotted, so hematologic analysis was not completed on those samples. Observed hematologic changes included a significant decrease in HGB, and associated RBC, HCT, and concentrations and an increase in MPV (Table 1). Serum chemistry parameters that significantly decreased in mink consuming 100 and/or 1000 ppm WPBCO diets included Na ($P \leq 0.03$), PROT ($P \leq 0.0001$), ALB ($P \leq 0.0002$), GLOB ($P \leq 0.02$), Ca ($P \leq 0.0001$), Fe ($P \leq 0.02$), Mg ($P \leq 0.05$) and CHOL ($P \leq 0.003$) concentrations, and ALK PHOS ($P \leq 0.002$) activity (Table 2). Serum CREAT ($P \leq 0.0001$) concentrations were significantly elevated in mink fed the 1000 ppm crude oil diet. Results from this study indicated that trend increases in WBC were not significant between the treatment and the control groups ($P < 0.09$). However, they were comparable to the trend reported by Mazet et al (2000) in mink exposed to various oil conditions. That study reported an increase in WBC count in mink that had been externally exposed to Bunker C fuel oil.

In the present study, data suggest that the mink exposed to WPBCO suffered from anemia. Ben-David et al (2000) suggested that captive river otters exposed to crude oil also suffered from mild anemia in addition to a reduction in WBC. Anemia, as well as hypoproteinemia and hypoalbuminemia, are conditions reported in this study that have been reported as being indicative of oil toxicity (Spraker et al. 1994; Williams et al. 1995).

Several of the serum chemistry values reported in this study fell just outside the reported reference ranges for healthy mink (data not shown). Two primary pathological considerations are suggested by the changes in serum chemistry parameters. First, the decreases reported in ALB, ALK PHOS, CHOL, GLOB, and PROT support the above diagnosis of non-specific anemia (Tip 1993). Second, the changes are suggestive of possible protein malnutrition as indicated by changes in ALB, ALK PHOS, Ca, CHOL, CREAT, GLOB, and the hematologic parameters of HGB and HCT (Tip 1993). White et al (1991) reported that ingestion of oil by mink decreased gut passage time. Ormseth and Ben-David (2000) reported a similar effect of decreased gut passage time in river otters. The fact that feed consumption decreased with increasing WPBCO concentrations, and the potential of decreased absorption of necessary nutrients through the gastrointestinal tract because decreased gut passage time would contribute to the condition of protein malnutrition. Speculatively, several of the parameter changes may be indicative of gastrointestinal or digestive disturbances, suggested by decreases in Na, PROT, ALB, GLOB, Ca, HCT, and HGB (Tip 1993). These data are also suggestive of altered renal and/or hepatic function, although if this was the case, renal and hepatic damage had not progressed to the point of being evident histologically.

No significant differences were reported for relative organ weights (expressed as a percent of body weight). However, there was an upward trend in liver and spleen weights with increasing concentrations of WPBCO. Average liver weight

Table 1. The effects of weathered crude oil on hematologic values for 7- month-old natural dark male mink.

Parameter	Unit	Control n = 10	100 ppm n = 8[1]	1000 ppm n = 8[2]
Red blood cell count**	$x10^6$/uL	9.35 ± 0.07[a]	9.16 ± 0.28[a]	8.50 ± 0.06[b]
Hemoglobin**	g/dL	17.4 ± 0.2[a]	17.2 ± 0.3[a]	15.9 ± 0.2[b]
Hematocrit **	%	57.7 ± 0.7[a]	56.6 ± 1.1[a]	52.6 ± 0.6[b]
MPV*	fL	61.7 ± 0.4[a]	62.0 ± 0.8[ab]	61.8 ± 0.5[b]
White blood cell count	$x10^3$/uL	5.33 ± 0.58[a]	6.52 ± 0.89[a]	6.93 ± 0.68[a]

Data expressed as least squares mean ± standard error of the mean. Means in the same row with different superscripts are significantly different at P* < 0.01; P** < 0.0001. [1] One animal died mid- trial from bladder stones; one blood sample clotted and could not be run. [2] Blood samples from two animals clotted and could not be run.

Table 2. The effects of weathered crude oil on serum chemistry values for 7- month-old natural dark male mink.

Parameter	Unit	Control n = 10	100 ppm n = 9[1]	1000 ppm n = 10
Albumin	g/dL	3.55 ± 0.05[a]	3.38 ± 0.04[b]	3.12 ± 0.07[c]
Alkaline Phosphatase	IU/L	55.80 ± 3.85[a]	47.11 ± 3.13[ab]	41.60 ± 2.13[b]
Calcium	mg/dL	9.36 ± 0.09[a]	9.08 ± 0.07[b]	8.68 ± 0.09[c]
Cholesterol	mg/dL	288.1 ± 14.3[a]	243.3 ± 8.6[b]	223.3 ± 9.8[b]
Creatinine	mg/dL	0.54 ± 0.04[a]	0.60 ± 0.03[a]	0.85 ± 0.04[b]
Total Globulin	g/dL	2.67 ± 0.08[a]	2.61 ± 0.11[a]	2.35 ± 0.08[b]
Iron	µg/dL	202.80 ± 13.99[a]	152.38 ± 11.50[b]	166.20 ± 12.53[ab]
Magnesium	mg/dL	2.71 ± 0.07[ab]	2.77 ± 0.09[a]	2.58 ± 0.06[b]
Total Protein	g/dL	6.22 ± 0.12[a]	5.98 ± 0.08[a]	5.44 ± 0.07[b]
Sodium	mmol/L	153.48 ± 0.53[a]	152.88 ± 0.41[ab]	151.33 ± 0.70[b]

Data expressed as least squares mean ± standard error of the mean. Means in the same row with different superscripts are significantly different at P < 0.05. [1] One animal died mid- trial from bladder stones.

was 52.66 g for the control, 59.78 g for 100 ppm, and 71.61 g for 1000 ppm groups. Average spleen weights were 3.29 g, 5.25 g, and 5.61 g, respectively. Histopathological examination of tissues, including brain sections, indicated no significant alterations.

Crude oil exposure has been reported to induce severe, generalized cerebral morphological changes, associated with suppression of neuroblastic proliferation, during postnatal developmental stages. Petroleum components, such as benzene, are known to cause neuronal degradation in the thalamus, hypothalamus, hippocampus, and amygdala (de-Gandarias et al. 1992; Kandel et al. 2000). Histopathologic findings in Pacific herring (*Clupea pallasi*) after EVOS indicated degeneration or necrosis of developing brain cells (Marty et al. 1997). In a follow up study after EVOS, harbor seals (*Phoca vitulina*) that died from oil exposure were investigated by Spraker et al. (1994). Neurological lesions were reported in the midbrain region of those seals, which included intramyelinic edema, axonal degeneration, and neuronal swelling and necrosis (Spraker et al. 1994). The neurological effects reported by Spraker et al. (1994) were not observed in our study. This may be in part to the fact that we used weathered oil as a condition of chronic exposure, and low molecular weight hydrocarbons that are suspect in neurotoxicity are decreased through volatilization via the weathering process. Exposure during EVOS was internal and external in very high doses of fresh spilled crude.

Although these results were obtained under experimental conditions, wild mustelids can encounter similarly contaminated food, especially after a widespread event such as the *Exxon Valdez* oil spill (Duffy et al. 1999). Wild carnivores will most likely consume contaminated prey items that contain detectable amounts of weathered crude oil. Based on the results from this study, 1000 ppm WPBCO will not cause extensive clinical chemistry changes in the short-term. Anemia and immune effects should be monitored under laboratory conditions because multiple stressors that are present in the natural environment might elicit compounded long-term impacts on an animal's physiological state, and eventually lead to population effects. More neurological and gestational exposure studies should be conducted to investigate offspring of female mink fed oil-contaminated diets. In light of the studies by Ben-David et al (2000) that showed behavioral effects, these multi-generational neurological studies will help explain population trends.

Acknowledgments. We thank the Laboratory for Histological and Molecular Services, Clinical Center, MSU for their help with the neurological tissue processing and slides; Dr. Lee Lipsitz for her assistance with the perfusion of the animals; and the staff of the MSU Experimental Fur Farm.

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