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Effects of Dietary Administration of CL-20 on Japanese Quail *Coturnix coturnix japonica*

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Abstract. Hexanitrohexaazaisowurtzitane, or CL-20, is an emerging highly energetic compound currently under consideration for military applications. With the anticipated wide use of CL-20, there is the potential for soil and groundwater contamination resulting in adverse toxicologic effects on environmental receptors. Presently, there is a lack of data describing the toxic effects of CL-20 on avian species. The present study describes the effect of CL-20 on Japanese quail (*Coturnix coturnix japonica*) modified from standard toxicity test guidelines. First, a 14-day subacute assay was adopted using repeated gavage doses (0, 307, 964, 2439, 3475, or 5304 mg CL-20/kg body weight (BW)/d for 5 days followed by no CL-20 exposure (vehicle only) for 10 days. Second, a subchronic feeding assay (0, 11, 114, or 1085 mg CL-20/kg feed) was done for 42 days. During both studies, no overt toxicity was observed in the CL-20-treated birds. During the first 5 days of the subacute study, CL-20-exposed birds showed a dose-dependent decrease in BW gain, whereas increased liver weight, plasma sodium, and creatinine levels were observed in birds receiving the highest dose tested. For the subchronic study, embryo weights were significantly decreased in a dose-dependent manner. Embryos from CL-20-exposed birds were observed to have multiple cranial and facial deformities, beak curvatures, possible mid-brain enlargement, and classic one-sided development with microphthalmia (nonstatistical comparisons with control embryos). A trend toward decreased number of eggs laid per female bird was also observed. We conclude that CL-20 (or its degradation products) elicits few effects in adults but may affect avian development, although these preliminary findings should be confirmed.

Hexanitrohexaazaisowurtzitane, or CL-20 (Fig. 1), is a high-density polynitro compound currently under consideration for military application. CL-20 is envisaged to deliver 10% to 20% higher performance than octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) and better performance than hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) (Nielsen *et al.* 1998; Geetha *et al.* 2003). RDX and HMX are common contaminants found in soil at military-related sites, and the toxicity of these nitramines to terrestrial vertebrate species such as mammals, amphibians, and birds is becoming better known (Schneider *et al.* 1976; Levine *et al.* 1981; Talmage *et al.* 1999; Gogal *et al.* 2003; Johnson *et al.* 2004). Gogal *et al.* (2003) found the approximate lethal dose of acute oral RDX exposure in northern bobwhite (*Colinus virginianus*) to be 280 mg/kg body weight (BW) for male and 187 mg/kg BW for female birds. The dose-dependent effects of RDX also included a decrease in food intake, BW, and spleen and liver mass for 14 days but not for 90 days of exposure. Although the effects of CL-20 in plant and soil invertebrate species have been recently reported (Gong *et al.* 2004; Robidoux *et al.* 2004), no data are available on the toxicity of CL-20 in birds.

CL-20 is a polycyclic nitramine characterized by having N-NO₂ bonds, similar to the two monocyclic nitramines RDX and HMX. Based on these structural similarities, we hypothesized that the effects of CL-20 to birds will be similar to those of RDX. In the present study, we investigated the toxic effects of CL-20 on the gallinaceous test species Japanese quail (*Coturnix coturnix japonica*) exposed to repeated daily doses of CL-20. We selected this species because it is amenable to laboratory toxicity testing, and species of this order can have significant dietary exposure to soil based on their foraging and behavioral habits. This species has been used in many standard toxicity testing regimes for standard avian toxicity testing (American Society for Testing and Materials [ASTM] 1997; United States Environmental Protection Agency [USEPA] 1996a, 1996b; Organization for Economic Cooperation and Development [OECD] 1984a, 1984b). Data generated using these standardized toxicity test methods will be useful for the ecotoxicologic risk assessment of birds exposed to CL-20.

Fig. 1. Molecular structures of the cyclic nitramine explosives CL-20, HMX, and RDX. CL-20 = hexanitrohexaazaisowurtzitane; HMX = octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine; RDX = hexahydro-1,3,5-trinitro-1,3,5-triazine

Materials and Methods

Chemicals and Reagents

CL-20 (Chemical Abstracts Service [CAS] no. 135285-90-4) in the ϵ form (purity >95%) was obtained from ATK Thiokol Propulsion (Brigham City, UT). Acetonitrile and acetone (high-performance liquid chromatography [HPLC]-grade) were obtained from EM Science (Darmstadt, Germany). All other chemicals and reagents were of the highest grades of purity available and were obtained from Anachemia (Milwaukee, WI). Deionized water was obtained using a Zenopure Mega-90 water purification system (Zenon Environmental Inc, Burlington, Ontario). All glassware was washed with phosphate-free detergent, rinsed with acetone, and acid washed before a final rinse with deionized water.

Raising of Test Species

Fertilized Japanese quail (*C. coturnix japonica*) eggs ($n = 70$ for subacute study and $n = 120$ for subchronic study) obtained from a local breeder (Couvoir Simetin, Mirabel, PQ, Canada) were placed in an environment-controlled incubator at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ (SD), with $65\% \pm 5\%$ relative humidity, and turned 180° every fourth hour. Three days before hatching, rotation of eggs was stopped and the

relative humidity increased to 70%. Eggs were grouped at the center of the incubator on a 5-mm mesh until they hatched. When dry, quail chicks were transferred to a heated brooder (35°C achieved using an electric resistance element) under constant illumination with red incandescent light to prevent eye damage. Birds were fed a diet of powdered Turkey Starter (Purina Mills, St Louis, MO) and tap water *ad libitum* until 14 days of age. Quail were then maintained under controlled conditions of temperature ($22.0^{\circ}\text{C} \pm 3.0^{\circ}\text{C}$), relative humidity ($50.0\% \pm 6.0\%$), and lighting (14 hours light to 10 hours dark).

Experimental Design

The subacute study (Fig. 2A) described in this article was modified from the USEPA (1996a) and OECD (1984a) guidelines. Forty-four 2-week-old quail were weighed, banded, and observed for any abnormalities. A blood sample (1 ml) was taken from the jugular vein (described later), and each bird was then randomly assigned to one of six groups ($n = 7$ to 8/group for $n = 44$ total). Animals were housed together in stainless steel cages (61 cm wide x 76 cm long x 41 cm high; 10-mm mesh floor) (2 to 3 individuals/cage) and were uniquely identified by a leg band. Two days later, birds were gavaged daily for 5 days with pulverized commercial feed (Ralston Purina Turkey Grower) containing CL-20. Briefly, the feed was pulverized using a hand-held blender until a granular consistency was formed, then CL-20 was added. The CL-20-contaminated feed was prepared daily, and the doses were adjusted to the BW that was taken daily during the dosing period (1% of BW). To deliver the contaminated feed, the desired quantity was transferred into a 5-ml syringe, and 1 ml water was added to the syringe to form a slurry. A 10-gauge stainless steel curved feeding tube equipped with a Luer Lock fitting (Becton Dickinson, Canada) onto the syringe was inserted down the esophagus to the crop. The slurry was administered gently to ensure minimal regurgitation. The calculated doses delivered (mg CL-20/kg BW) were 307 ± 1 , 964 ± 8 , 2439 ± 12 , 3475 ± 5 , and 5304 ± 53 . A second blood sample (from 1 to 1.5 ml) was collected 5 days after completion of the dosing. At the end of the experiment (day 14 of study), birds were anesthetized and a third blood sample (5 ml) was procured. While under deep anaesthesia, birds were killed (as desired later), and the organs (heart, brain, liver, and spleen) were weighed and frozen at -80°C for residual CL-20 analysis. The somatic index (%) was calculated as the wet organ weight (g) per 100 g BW.

The subchronic study described in this article was modified from the USEPA (1996b) and OECD (1984b) guidelines. The lighting period was changed (16 hours light to 8 hours dark) to provide optimal reproductive conditions when the quail were 2 weeks old. The birds ($n = 48$) were then randomly assigned to one of each of the four dose groups (described below) in triplicate. Each exposure was conducted in triplicate, each group (three female and one male bird) was housed in stainless steel cages (described above). Quail were uniquely identified by a leg band. During this period, close surveillance was required to monitor any signs of increased aggression. Individuals injured or demonstrating incompatible behavior relative to the group were replaced. Eggs were collected and incubated during the pre-exposure period to verify fertility and thus ensure that only proven breeders were included in this study. Two days before the start of feeding CL-20 diets when quail were 54 days old, a blood sample (2 ml) was taken from each bird. The diets were prepared by mixing CL-20 (previously dissolved in acetone) with commercial feed (Ralston Purina Turkey Grower) to achieve nominal concentrations described later. Acetone was allowed to evaporate, and the CL-20 feed mixture was then placed in glass jars and stored at $4.0^{\circ}\text{C} \pm 2.0^{\circ}\text{C}$ in the absence of light. Measured concentrations of

Fig. 2. Schedule for the two CL-20 exposure studies. (A) Subacute study. (B) Subchronic study

CL-20 in the feed were analyzed using HPLC (11 ± 1 , 114 ± 26 , and 1085 ± 52 mg CL-20/kg feed corresponding to the nominal exposure groups of 10, 100, and 1000 mg/kg BW, respectively), and were found to be consistent throughout the study. All feed was provided daily *ad libitum*. Daily feed consumption was estimated as the difference between initial and final feed weight plus the quantity of unconsumed pellets recovered from the litter pans, which was determined daily for the first 2 weeks of the study and then on every alternate day.

A subsample of eggs (based on total eggs collected, $n = 15$ to 20/d) were collected and frozen at -80°C ; the remaining eggs ($n = 10$ to 12 collected/d) were stored in a refrigerator ($16^{\circ}\text{C} \pm 1^{\circ}\text{C}$ and 60% to 70% humidity) for 5 days. Eggs of the latter group were then incubated at 37.8°C and 60% relative humidity. After 8 days of incubation, the eggs were cooled to 4°C , and embryos were removed and weighed. Embryos were then preserved in capped vials containing 10% formalin, and they were stored at room temperature in the dark until further evaluation of developmental effects. Embryos were evaluated according to the Hamburger-Hamilton system (1951) for normal stages of chick embryo development. At the end of the 6-week exposure period, the adult birds were killed and examined for gross lesions. Liver, spleen, heart, and brain were excised, weighed, frozen in liquid nitrogen, and stored at -80°C before residual CL-20 analyses.

For both studies, birds were monitored daily for changes in health or disposition (*i.e.*, alertness, appearance). For the subacute study, BW gain was measured daily for the duration of the gavage phase and then on study days 10 and 14. Body weight and feed consumption for the subchronic study were measured daily for a 2-week period and then on every alternate day. At the end of the experiments, birds were anesthetized (2% to 5% isoflurane), and killed (90% CO_2 and 10% O_2). Moribund animals were treated in the same fashion. Manipulation and handling were in accordance with the Canadian Council on Animal Care guidelines (1984).

Blood Collection and Analysis

For clinical chemistry and hematological analyses, female quail blood was collected from the jugular vein with a 27-gauge needle and 3-ml syringe containing 100 μl 1% ethylenediaminetetraacetic acid solution in saline. Male birds were not considered for this study because of the low number of male birds per exposure group (1 male bird/3 female

birds/replicate, 3 replicates/exposure group). Blood was thoroughly mixed, and 250 μl was removed for whole-blood analysis. Plasma was prepared in 1.5-ml Eppendorf tubes centrifuged at $13,000 \times g$ for 5 minutes and was used either fresh for clinical chemistry analysis to screen for biochemical and pathologic abnormalities or was stored at -80°C for plasma residual CL-20 analysis. Quail plasma was analyzed to determine its clinical chemistry profile (Beckman LX20 Pro, Rochester, NY); the tests measured Na, K, Cl, glucose, creatinine, total protein, PO_4 , total bilirubin, direct bilirubin, uric acid, Mg, cholesterol, alanine aminotransferase, aspartate aminotransferase (AST), alkaline phosphatase, amylase, lactate dehydrogenase, and triglycerides. For hematologic analysis, one drop of anticoagulated blood was placed on a microscope slide, and a smear was made. The blood smear was fixed in methanol, stained with modified Wright-Giemsa, and the leukocyte differential performed when 100 leukocytes (lymphocytes, heterophils, monocytes, basophils, and eosinophils) were enumerated. Heterophil-to-lymphocyte ratios were calculated to investigate stress. For hematocrit (HCT) determination, a nonheparinized hematocrit capillary tube was filled with anticoagulated blood. The bottom of the capillary tube was sealed with a clay plug and centrifuged for 5 minutes at 3000 rpm, and the HCT was determined as the ratio between total capillary volume and the packed cell volume (PCV).

CL-20 Extraction from Quail Tissue

CL-20 extractions were performed according to USEPA Method No. 8330A (USEPA 1998) with the following modifications. For CL-20 exposure studies, whole organs taken from test animals were immediately homogenized on ice in 7 ml cold acetonitrile using a Polytron tissue homogenizer (30 seconds for brain and spleen or 1 minute for heart and liver) in Teflon tubes. After overnight sonication in the dark at 8°C (60 Hz; Branson 3200, Danbury, CT), samples were centrifuged at 4°C for 10 minutes at $10,000 \times g$. To 5 ml of the decanted supernatant was added an equal volume of a CaCl_2 (90 mM)- NaHSO_4 (1.6 mM) solution, and the resulting mixture was vortexed for 30 seconds and stored at 4°C for 2 hours to precipitate proteins. The resulting solution was filtered through a 0.45- μm membrane before HPLC analysis (described later). To increase the sensitivity of the previously described method, the supernatants of different tissue samples (liver, heart, brain, and kidney) were also concentrated. Each supernatant (5 ml) was evaporated to dryness in a Speed Vac (Savant, Hicksville, NY). To the precipitate was added 500 μl acetonitrile, and the mixture was vortexed for 1 minute, after which the CaCl_2 - NaHSO_4 solution

Fig. 3. Changes in BW of juvenile Japanese quail gavaged with CL-20 for 5 days (subacute study). Exposure schedule is shown in Figure 2. Each value (expressed as percent of control, which was set at 100%) is the average $\pm$ SEM ($n = 6$ to 10 birds). *Value is statistically different from the control ($p \leq 0.05$). The average BWs of the control group ($g \pm$ SEM) ($n = 6$ birds) for study days 2, 3, 4, 5, 10, and 14 were 98 ± 8 , 106 ± 6 , 116 ± 7 , 126 ± 8 , 172 ± 8 , and 202 ± 9 , respectively

was added as described previously. For the control recovery studies, whole organs (liver, brain, spleen, or heart) were taken from non-treated quail and spiked with varying concentrations of CL-20 before being immediately frozen at -80°C for 24 hours. The organs were then thawed in cold acetonitrile, and CL-20 was extracted as previously described.

Extraction of CL-20 from the Test Diet

Acetonitrile extractions of test diet were also performed using the USEPA Method No. 8330A (USEPA 1998). One ml 1.6 mM NaHSO_4 prepared in water was added to 2 g feed and vortexed for 10 seconds. To this was added 10 ml (for feed containing 10 mg CL-20/kg feed) or 20 ml acetonitrile (for feed containing 100 to 1000 mg CL-20/kg feed), before vortexing for 1 minute.

HPLC Analysis

In brief, a ThermoFinnigan chromatographic system composed of a model P4000 pump, a model AS1000 injector, and a model UV6000LP photodiode-array detector ($\lambda = 230$ nm) was used. A Supelcosil LC-CN column (250 $\times$ 4.6 mm, 5- μm particles; Supelco, Bellefonte, PA) was used for separation with a column heater set at 35°C . The isocratic mobile phase consisted of methanol and water (70/30 v/v) delivered at 1.0 ml/min. The sample volume injected was 50 μl with a 14-minute run time. The limit of quantification was 0.05 mg/L. Relative SD for the instrument precision was $<1.3\%$ for concentrations ≥ 0.5 mg CL-20/L and 7.5% for a concentration of 0.05 mg CL-20/L.

Statistical Analysis

The data were evaluated by two-way and repeated measures analysis of variance (ANOVA). Differences between exposure groups and their respective controls were considered significant when $p \leq 0.05$ using the Dunnett's test. Statistical analysis was performed using JMPIN software (version 4; SAS Institute, Cary, NC).

Fig. 4. Somatic index of selected organs of juvenile Japanese quail gavaged with CL-20 for 5 days followed by 10 days of vehicle only (no CL-20). Data are expressed as mean $\pm$ SD ($n = 44$ birds). Study design is shown in Figure 2. *Value is statistically different from the control ($p \leq 0.05$)

Results

Subacute Study

No clinical or overt toxic symptoms were observed during the 5-day exposure period to CL-20. The change in average weight gain (expressed as the percent of respective control) for each exposure group is shown in Figure 3. Data indicate that a dose-dependent decrease in BW gain occurred during the first 5 days of the study ($p \leq 0.05$). On the second study day, the CL-20 quail exposure groups ≥ 964 mg/kg BW/d gained less weight than the control group ($p \leq 0.05$); however, this effect was not detected by the end of the study (day 14 or 10 d of no CL-20 exposure). Initial BWs before exposure were not significantly different between groups (data not shown).

Liver weights were significantly increased in the highest dose group (5304 mg/kg BW/d), whereas other organ weights (brain and spleen) were not significantly different between CL-

Table 1. Selected plasma biochemical parameters of adult Japanese quail exposed to CL-20 by gavage for 5 days followed by 10 days of exposure with no CL-20

Parameters	CL-20 exposure groups (mg CL-20 kg BW/d) ^a					
	0	307	964	2439	3475	5304
PO ₄ (mmol/L)	3.22 ± 0.21 ^b	2.89 ± 0.26	2.91 ± 0.26	2.53 ± 0.21	2.73 ± 0.24	2.96 ± 0.39
TP (g/L)	19.2 ± 3.6	22.0 ± 2.6	22.3 ± 2.9	18.4 ± 2.9	20.8 ± 2.8	21.63 ± 3.25
Glu (mmol/L)	16.3 ± 1.0	17.0 ± 0.7	16.7 ± 1.0	16.4 ± 1.2	17.1 ± 1.3	16.36 ± 1.45
Cre (μmol/L)	20.0 ± 2.6	18.6 ± 3.2	20.3 ± 4.0	18.2 ± 5.4	21.8 ± 4.4	25.00 ± 3.16*
Na (mmol/L)	143.9 ± 0.8	145.5 ± 1.0	144.0 ± 0.8	144.6 ± 2.7	149.5 ± 1.6	151.7 ± 2.70*
ALP (IU/L)	592 ± 327	711 ± 253	1056 ± 301	485 ± 396	328 ± 275	509 ± 230
ALT (IU/L)	4.25 ± 1.50	4.20 ± 1.30	5.00 ± 0.82	4.80 ± 0.84	4.50 ± 1.05	4.50 ± 0.76
Uric (μmol/L)	201.3 ± 53.3	239.2 ± 78.1	269.0 ± 134.8	195.0 ± 37.6	216.0 ± 101.8	300.6 ± 68.5
AMY (IU/L)	336 ± 71	460 ± 190	377 ± 197	178 ± 60	436 ± 136	377 ± 193
AST (IU/L)	101.3 ± 11.7	87.3 ± 10.0	94.0 ± 10.1	107.2 ± 21.9	95.5 ± 13.5	101.9 ± 11.8
Chol (mmol/L)	5.32 ± 0.74	4.06 ± 0.91	5.17 ± 1.52	5.63 ± 2.50	4.67 ± 0.98	5.38 ± 1.44
Dbili (μmol/L)	1.25 ± 0.24	0.95 ± 0.21	0.78 ± 0.31	1.40 ± 0.50	0.83 ± 0.27	1.16 ± 0.49
Tbili- (μmol/L)	2.8 ± 0.5	2.6 ± 1.6	4.0 ± 1.2	2.2 ± 1.3	2.3 ± 1.1	3.5 ± 0.9
GGT (IU/L)	6.5 ± 4.5	8.8 ± 2.3	8.0 ± 1.4	8.4 ± 0.9	7.5 ± 2.2	10.0 ± 2.2
LDH (IU/L)	59.8 ± 13.0	48.8 ± 8.4	47.0 ± 9.5	49.2 ± 12.8	41.0 ± 12.7	56.4 ± 28.8
TG (mmol/L)	1.84 ± 0.20	1.42 ± 0.32	2.05 ± 0.91	2.04 ± 0.52	1.49 ± 0.36	2.06 ± 0.78
Mg (mmol/L)	0.15 ± 0.50	0.20 ± 0.01	0.28 ± 0.05	0.11 ± 0.14	0.10 ± 0.04	0.13 ± 0.7

ALP = Alkaline phosphatase.

ALT = Alanine aminotransferase.

AMY = Amylase.

AST = Aspartate aminotransferase.

Chol = Cholesterol.

CL-20 = Hexanitrohexaazaisowurtziane.

Cre = Creatinine.

Dbili = Billirubin.

GGT = γ glutamyltransferase.

Glu = Glucose.

LDH = Lactate dehydrogenase.

Tbili = Total Billirubin.

TG = Triglycerides.

TP = Total proteins.

^a Measured concentrations of CL-20 intake by gavaged quails.^b Data are expressed as mean ± SD (*n* = 4 to 8 birds/exposure group).* Exposure group is significantly different from control using Dunnett's test (*p* ≤ 0.05).

20-treated and control groups (Fig. 4). Increases in plasma sodium and creatinine levels were found in birds treated with 5304 mg CL-20/kg BW/d compared with controls (Table 1). Hematologic parameters (heterophil-to-lymphocyte ratios and hematocrit) in the subacute study were not statistically different between exposure groups compared with controls (data not shown).

Subchronic Study

No clinical or overt toxic symptoms were observed during the 42-day exposure period in this study. Aggressive behavior was noted in one individual who was subsequently isolated from the control group. Another bird from a different control group had to be killed because of injuries inflicted by an aggressive control bird that was subsequently removed from the triplicate. These observations were not related to exposure. Based on feed consumption (feed consumed per number of individuals in each dose group) and mean BW, the calculated daily doses

to quail consuming the CL-20 diet were 0, 0.96, 10, and 94 mg CL-20/kg BW for the measured 0, 11, 114, and 1085 mg CL-20/kg feed exposure groups, respectively. Throughout this study, no differences in feed consumption or BW were observed between exposure groups (data not shown). Clinical chemistry analysis of quail blood taken from the subchronic study (Table 2) revealed that the AST level was increased by 1.25 times in the birds of the highest measured feed dose level (1085 mg CL-20/kg) relative to controls (*p* ≤ 0.05).

Embryo weight was found to decrease significantly and in a dose-dependent manner (Fig. 5). Based on the measured exposure concentration, the unbounded lowest observed adverse effect level for this effect was 11 mg/kg feed. The unintentional evaporation of the preserving medium from some of the storage vials caused a dehydration of the embryos, thus making these samples unsuitable for further analysis. Embryos in vials that still contained formalin were analyzed. In the 114 mg/kg feed exposure group, two of the six embryos had multiple (cranial and facial) deformities, whereas in the highest dose group (1085 mg/kg feed), three of the nine embryos experienced beak curvatures, possible mid-brain

Table 2. Selected plasma biochemical parameters of adult Japanese quail fed CL-20 for 42 days

Parameters	CL-20 exposure groups (mg CL-20/kg feed) ^a			
	0	11	114	1085
PO ₄ (mmol/L)	2.87 ± 0.63 ^b	2.56 ± 0.39	3.03 ± 0.84	2.11 ± 0.71
TP (g/L)	31.6 ± 4.0	30.1 ± 2.3	33.5 ± 1.7	28.3 ± 3.2
Glu (mmol/L)	15.1 ± 0.99	15.4 ± 1.29	14.5 ± 0.8	14.4 ± 0.9
Cre (μmol/L)	30.3 ± 9.6	32.4 ± 4.7	24.4 ± 6.1	27.0 ± 9.6
Na (mmol/L)	144.4 ± 3.6	142.3 ± 1.3	139.8 ± 3.5	142.3 ± 1.3
ALT (IU/L)	5.16 ± 1.60	4.83 ± 2.22	6.00 ± 1.63	6.50 ± 1.51
Uric (μmol/L)	213.4 ± 58.7	303.0 ± 147.0	136.8 ± 71.2	232.3 ± 131.5
AMY (IU/L)	507 ± 179	368 ± 222	643 ± 304	472 ± 154
AST (IU/L)	133.3 ± 11.6	144.8 ± 23.5	154.6 ± 27.4	167.2 ± 23.2 *
Chol (mmol/L)	6.27 ± 2.06	4.84 ± 1.76	7.39 ± 3.85	4.63 ± 1.34
Tbili (μmol/L)	7.65 ± 1.97	6.94 ± 1.45	8.12 ± 1.39	6.98 ± 0.96
GGT (IU/L)	5.88 ± 1.64	6.57 ± 1.13	6.13 ± 2.41	6.43 ± 1.90
LDH (IU/L)	54.8 ± 59.0	31.3 ± 27.0	34.8 ± 51.0	74.2 ± 39.0
TG (mmol/L)	11.7 ± 2.4	9.8 ± 2.7	10.8 ± 2.2	9.2 ± 2.7
Mg (mmol/L)	1.63 ± 0.33	1.36 ± 0.23	1.48 ± 0.29	1.34 ± 0.25
K (mmol/L)	2.9 ± 0.5	3.2 ± 0.3	2.7 ± 0.3	2.9 ± 0.4
Cl (mmol/L)	110.1 ± 4.2	107.8 ± 3.9	107.1 ± 3.5	110.3 ± 3.2

ALT = Alanine aminotransferase.

AMY = Amylase.

AST = Aspartate aminotransferase.

Chol = Cholesterol.

CL-20 = Hexanitrohexaazaisowurtzitane.

Cre = Creatinine.

GGT = γ -glutamyltransferase.

Glu = Glucose.

LDH = Lactate dehydrogenase.

Tbili = Total bilirubin.

TG = Triglycerides.

TP = Total proteins

Uric = Uric acid.

^a Measured concentration in feed using USEPA Method 8330A.^b Data are expressed as mean ± SD ($n = 7$ to 9 birds group).* Exposure group is significantly different from control using Dunnett's test ($p \leq 0.05$). **Fig. 5.** Effects of 42 days of dietary exposure to CL-20 on Japanese quail embryo weights. Data are mean embryo weight ± SD ($n =$ number of embryos evaluated). *Value is statistically different from the control ($p \leq 0.05$)

enlargement, and classic one-sided development with microphthalmia. These CL-20 exposure-related deformities were not observed in the control group; however, two of the nine

control embryos showed very slight ocular asymmetric deviations. No decrease in the rate of development was detected; all embryos were at stages 30 to 31 (Hamburger and Hamilton 1951). In addition, CL-20 exposure tended to decrease the number of eggs laid per female bird ($0.48 < p < 0.99$ for the different treatment groups using the Dunnett's two-sided t test for multiple comparisons) compared with controls (Fig. 6).

CL-20 Recovery from Tissue

CL-20 was not detected in the plasma or selected organs (brain, spleen, heart, and liver) of quail treated with CL-20 (data not shown), despite the excellent recovery of the chemical (99% to 105%) using different spiked tissues (Figs. 7 and 8). This method was specific for CL-20 detection only.

Discussion

Few acute and subchronic effects were found from oral CL-20 exposure to quail in this study. However, our 42-day subchronic study indicated that CL-20 exposure led to significant

Fig. 6. Effects of 42 days of dietary exposure to CL-20 on the mean number of eggs produced per hen. These exposure effects were not significant compared with controls ($p > 0.05$)

decreases in embryo weight without a corresponding effect on developmental stage. This would suggest an effect on energy metabolism rather than a decrease in the rate of embryonic development *per se*. Although we have evidence that CL-20 exposure caused abnormal developmental effects, these results should be considered preliminary until a more rigorous teratologic evaluation is done using increased number of replicates. Bhushan *et al.* (2004a, 2004b) reported the formation of nitrite NO_2^- , nitrous oxide N_2O , ammonia, formic acid, ammonium, or glyoxal from the enzymatic biodegradation of CL-20 by the flavin containing enzymes, nitroreductase, and salicylate 1-monooxygenase. Quail have been found to possess both phase I and II biotransformation enzymes (Gregus *et al.* 1983). It is possible that quail may possess similar enzymes capable of degrading CL-20 and that the presence of the resulting degradation products may be related to the observed CL-20 toxicity in our present studies. A preliminary survey of the avian toxicity literature failed to identify studies characterizing the toxic effects of the latter CL-20 degradation products to quail. To further characterize the reported effect of embryo malformation by CL-20 and gain more information about the mechanism of CL-20 toxicity (direct and indirect effects of CL-20 on the embryo), *in ovo* approaches are suggested.

Earlier studies have shown that exposure to the monocyclic nitramine explosive RDX causes central nervous system disturbances in birds (northern bobwhite), terrestrial salamanders, and mammals (rats and miniature swine) (Schneider *et al.* 1976; Levine *et al.* 1981; Gogal *et al.* 2003; Johnson *et al.* 2004). Rats injected intraperitoneally with 500 mg RDX/kg BW had seizures before death. Miniature swine treated intravenously with RDX showed convulsions from 12 to 24 hours after exposure. A recent study reported that red-backed salamanders (*Plethodon cinereus*) exposed to 5,000 mg RDX/kg soil in laboratory microcosms for 28 days showed signs of neuromuscular effects including lethargy, convulsions, rolling and gaping motions, and hyperactivity as well as marked weight loss (Johnson *et al.* 2004). In contrast to these earlier studies using RDX, none of the CL-20 treated birds in our present studies exhibited signs of neurotoxicity.

Fig. 7. Recovery of CL-20 using spiked tissue samples ($\triangle$ = brain; $\diamond$ = spleen; $\blacksquare$ = heart) and modified USEPA Method No. 8330A. R^2 value was determined by linear regression using least-squares method

Fig. 8. Recovery of CL-20 from spiked liver tissue using modified USEPA Method No. 8330A.

Earlier studies by Gogal *et al.* (2003) showed that RDX in a water vehicle was lethal to the northern bobwhite at a single dose (≥ 187 mg RDX/kg BW) after 72 hours exposure. In our subacute study, CL-20 caused no mortality in adult Japanese quail gavaged with up to 5304 mg CL-20/kg BW. These data suggest that CL-20 is not as lethal to galliform species as RDX. Physicochemical differences between RDX (aqueous solubility at 20°C = 42.58 mg/L, $\log K_{ow}$ = 0.90) and CL-20 (aqueous solubility at 20°C = 3.16 mg/L, $\log K_{ow}$ = 1.92) (Monteil-Rivera *et al.* 2004) may favor the absorption or bioavailability of RDX relative to CL-20 and may help to explain the differences in acute toxicity.

Increased liver-to-BW ratios were observed in the highest dose group (5304 mg CL-20/kg BW/d) at 10 days post-CL-20 exposure, whereas the spleen- and heart-to-BW ratios were not significantly altered compared with controls. This effect contrasts with the earlier results showing decreased liver-to-BW ratios observed for northern bobwhite exposed to RDX in feed (Gogal *et al.* 2003). Birds exposed to RDX for 14 days caused

significant increases in hematologic parameters such as heterophil counts and the heterophil-to-lymphocyte ratio as well as an increase in PCV (Gogal *et al.* 2003). In contrast, our study using CL-20 did not show an effect of exposure on the heterophil-to-lymphocyte ratio or PCV, suggesting that CL-20 is not an immunotoxicant or a mitogen, at least not during this period of exposure. It is possible that immunologic effects may have occurred but were reversed during our exposure schedule that allowed 10 days of no exposure to CL-20.

In conclusion, although CL-20 has certain structural similarities to the monocyclic nitramine RDX (Fig. 1), this polycyclic nitramine does not appear to demonstrate the same profile of toxicities as RDX in quail. Our present study has provided basic toxicity data and suggests that further research should be carried out to study the developmental effects of CL-20 in birds.

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