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DEPARTMENT OF THE AIR FORCE
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1 May 2001

MEMORANDUM FOR USEPA
ATTN: ANNIE JARABEK
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FROM: DEIRDRE MAHLE
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SUBJECT: Consultative Letter, AFRL-HE-WP-CL-2001-0001, Hormone and Perchlorate Data from Cross-Fostering Study

1. This consultative letter is an update from the CL (AFRL-HE-WP-CL-2000-0043) submitted in Oct 2000. Additional perchlorate data in dam and pup tissues have been included in this letter (Attachment 1). Attachment 2 contains the poster presented at the 40th Annual Meeting of the Society of Toxicology (*The Toxicologist*, 60(1):227-228, 2001).

2. Litters from timed pregnant Sprague-Dawley rats were cross-fostered at birth and euthanized at PND 10. Two separate groups (Surrogate and Donor) of timed pregnant, Sprague-Dawley rats were received on day 2 of gestation (GD 2). Immediately upon arrival, the Surrogate rats were separated into two sub-groups. Half were placed on perchlorate treated drinking water at a dose of 1.0 mg/kg-day. The second, untreated half were given control water. The Donor group of GD 2 rats was received a few days after the Surrogate group. Immediately upon arrival, the Donor rats were separated into two sub-groups exactly the same as the Surrogate rats. When the Surrogate dams delivered, the litters were standardized to eight pups. The dams remained on either the perchlorate treated or control water. At parturition of the untreated Donor group, the entire litter was removed and placed with an exposed Surrogate dam whose own litter had been removed just prior to crossing. As each of the perchlorate-exposed Donor dams delivered, the entire litter was removed and placed with an untreated Surrogate dam whose own litter had been removed just prior to the crossing. The Donor dams and original Surrogate litters were euthanized by CO_2 inhalation. A set of untreated dams and perchlorate-exposed dams from the

Donor group remained with their own litters to serve as 'true control' and 'true exposed'. All dams remained on control or perchlorate treated drinking water for the duration of the study. The day of crossing was designated as PND 1 (postnatal day 1). On PND 2, the crossed litters were randomly standardized to eight pups (four male and four female). At PND10, dams and pups were euthanized by CO_2 inhalation. Dam serum, thyroid and skin and pup serum, skin and gastric contents were collected. Male and female pup sera were pooled within litters. Serum was analyzed for TSH, T_3, T_4 and perchlorate. All other tissues were analyzed for perchlorate concentration.

3. The data for serum hormone levels in dam, female pups and male pups are in Tables 1, 2 and 3 of Attachment 1, respectively. TSH was significantly elevated in both lactationally exposed groups for dam and male and female pups when compared to the control groups. Female pup serum TSH was significantly higher than male pup serum TSH. T_4 in female pup serum from exposed dams was significantly lower than control female pups and male pups from the same exposure group.

4. Tissue levels of perchlorate are in Attachment 2. Perchlorate levels in blood and skin were significantly higher in the lactationally exposed group than the true exposed dams. Little change was seen in thyroid perchlorate levels between the two exposed groups. In male and female pups, the concentration of perchlorate in all tissues tended to be greater in the lactationally exposed group than the true exposed group, except male pup gastric contents. Differences between the true exposed and the lactation only exposed groups were significant for male and female pup skin and female pup gastric contents ($p \leq 0.05$). Within the true exposed group, female pups had significantly higher levels of perchlorate in the gastric contents than male pups.

5. For further information, contact me at (937) 255-5150 ext. 3183, (937) 255-1474 (fax) or at deirdre.mahle@wpafb.af.mil.

Deirdre A Mahle

DEIRDRE MAHLE
Operational Toxicology Branch

Attachments:
1. Serum hormone data
2. Society of Toxicology presentation

1st Ind, AFRL/HEST 1 May 2001

MEMORANDUM FOR US EPA
ATTN: MS. ANNIE JARABEK

This letter report has been coordinated at the branch level and is approved for release.

RICHARD R. STOTTS, DVM, Ph.D.
Chief,
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Table 1. Serum Thyroid Hormones for Maternal Rats at PND 10.

	True control	Control dams with exposed litters	Exposed dams with control litters	True exposed
TSH (ng/ml)	7.26 ± 0.55	7.23 ± 0.73	8.54 ± 0.68	8.64 ± 0.86
T_4 (μg/dl)	2.55 ± 0.23	2.64 ± 0.26	2.46 ± 0.25	2.32 ± 0.19
T_3 (ng/dl)	83.51 ± 7.23	83.5 ± 6.63	78.17 ± 7.51	79.67 ± 7.79

Data are mean ± s.d., n = 6-7.

Table 2. Serum Thyroid Hormones from Pooled Female Pups at PND 10.

	True control pups	Exposed female pups with control dams	Control female pups with exposed dams	True exposed pups
TSH (ng/ml)	5.64 ± 0.33	6.29 ± 0.72	8.47 ± 0.82	8.53 ± 0.89
T_4 (μg/dl)	2.61 ± 0.21	2.54 ± 0.26	2.18 ± 0.18	2.03 ± 0.23
T_3 (ng/dl)	79.24 ± 5.07	84.1 ± 11.62	74.23 ± 8.68	76.90 ± 8.05

Data are mean ± s.d., n = 6-7

Table 3. Serum Thyroid Hormones from Pooled Male Pups at PND 10.

	True control pups	Exposed male pups with control dams	Control male pups with exposed dams	True exposed pups
TSH (ng/ml)	5.55 ± 0.58	5.47 ± 0.59	6.32 ± 0.56	6.32 ± 0.64
T_4 (μg/dl)	2.55 ± 0.24	2.58 ± 0.28	2.47 ± 0.19	2.35 ± 0.26
T_3 (ng/dl)	80.34 ± 7.56	80.25 ± 7.01	76.91 ± 6.39	76.88 ± 6.13

Data are mean ± s.d., n = 6-7

Thyroid Hormonal Changes in Cross-Fostered Sprague-Dawley Rat Litters Exposed to Perchlorate

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Abstract

Ammonium perchlorate (NH_4ClO_4) salt is used as an oxidizer in rocket fuel. Because of its water solubility it is a common groundwater contaminant that readily dissociates to NH_4^+ and ClO_4^-. The ClO_4^- ion competes with I^- for uptake into the thyroid causing a reduction in thyroid hormone production. It is unclear what effect *in utero* and lactational exposure to ClO_4^- has on pup hormone production. Pregnant Sprague-Dawley rats were given either control or perchlorate (1 mg/kg-day) treated drinking water beginning on gestation day 2. Immediately after birth half of the control litters were placed with treated dams and half of the treated litters were placed with the control dams. True control and true exposed litters remained with their own dams. Dam serum and thyroid and pooled male and female pup serum were collected on postnatal day 10 and analyzed for ClO_4^-, TSH, T_3 and T_4. At PND 10 the treated male and female pup serum level of ClO_4^- from control dams was not detectable as compared to 0.50 and 0.54 ug/ml for the true exposed male and female pups, respectively. Control pups receiving ClO_4^- through lactation had serum levels of 0.54 ug/ml and 0.56 ug/ml for male and female pups, respectively. Female pups receiving ClO_4^- from milk had significantly lower levels of T_4 than true control pups and pups exposed to perchlorate in utero only. Male pups from all groups statistically had the same T_4 level. TSH levels in female pups exposed lactationally were significantly increased as compared to controls. Serum levels of TSH increased from 5.5 ng/ml in control male pups to 6.3 ng/ml in male pups receiving ClO_4^- from milk. Understanding the effect of in utero and lactational exposure to ClO_4^- will provide additional data for PBPK models to improve the health risks from exposure to perchlorate.

Introduction

Because of its use as a rocket fuel oxidizer and its water solubility, ammonium perchlorate has become a problematic contaminant in groundwater sources. Ammonium perchlorate readily dissociates into NH_4^+ and ClO_4^-. The perchlorate anion, ClO_4^-, competes with iodide for uptake in the thyroid gland (Wolff, 1998). The Na^+/I^- symporter located in the membrane of the thyroid follicular cells transports iodide across the membrane (Carrasco, 1993; Eskandari, 1997). Tyrosyl residues of thyroglobulin (Tg) are iodinated and, coupled with thyroglobulin, form thyroxine (T_4) and triiodothyronine (T_3). Since ClO_4^- is similar in molecular size and charge to iodide, it inhibits the uptake of I^- by the symporter (Goldman and Stanbury, 1973). This competitive inhibition can lead to hypothyroidism.

T_4 is primarily released into the blood by proteolysis of Tg. T_4 is enzymatically deiodinated to T_3 by 5'-deiodonase (Greenspan, 1994; Taurog, 1991; Wolff, 1998). The hypothalamus detects low blood levels of thyroid hormone and releases thyrotropin releasing hormone (TRH) which stimulates the pituitary to release thyroid stimulating hormone (TSH). TSH stimulates uptake of iodide across the Na^+/I^- symporter and the secretion of T_4 and T_3. Ammonium perchlorate has been shown to inhibit the uptake of iodide into adult rat thyroid

(Caldwell et al, 1995; Fisher et al, 2000). In these rat studies serum TSH was elevated and serum levels of T_4 and T_3 were decreased.

It is unclear what effect in utero and lactational exposures to ClO_4^- have on pup hormone production. Thyroid hormones play a critical role in the early development of the rat pup. Developmental hypothyroidism has been shown to cause brain cell disorganization and to delay puberty in rodents (Bakke et al, 1976; Clos et al, 1974). Hypothyroidism caused by post natal (lactational) exposure to PCBs resulted in malformation of cochlear structures and functional hearing loss in rats (Crofton et al, 2000). At 18 days of gestation the rat fetus has a functional pituitary-thyroid axis and is beginning to secrete its own thyroid hormones (Eguchi et al, 1980). The fetus is still dependent on the mother for iodine, which passes through the placenta (Roti et al, 1983; Brown-Grant, 1961). Diminished levels of iodide in the dam from perchlorate exposure could reduce the amount of iodide available to the fetus through the placenta. After birth the pup is still dependent on the lactational transfer of iodide from the mother for hormone synthesis. Because of similarities between the perchlorate ion and iodide, it is reasonable to assume that perchlorate will be sequestered in the mammary tissue, reducing the amount of iodide available to the pup or that perchlorate would be transferred to the pup through the milk.

The information generated from the cross-fostering studies can provide important data for the development of physiologically based pharmacokinetic models to expand what is known about the risks associated with perchlorate exposure.

Materials and Methods

Two separate groups (Surrogate and Donor) of timed pregnant, Sprague-Dawley rats were purchased from Charles Rivers Laboratories and were received on day 2 of gestation (GD 2). Immediately upon arrival, the Surrogate rats were separated into 2 sub-groups. Half were placed on perchlorate treated drinking water at a dose of 1 mg/kg/day. The second, untreated half were given control, reverse osmosis water. The Donor group of GD 2 rats was received a few days after the Surrogate group. Immediately upon arrival, the Donor rats were separated into 2 sub-groups exactly the same as the Surrogate rats. When the Surrogate dams delivered, the litters were culled to 8. The dams remained on either the perchlorate treated or control water. As each of the untreated Donor dams delivered, the entire litter was removed and placed with an exposed Surrogate dam whose own litter had been removed just prior to crossing. As each of the perchlorate-exposed Donor dams delivered, the entire litter was removed and placed with an untreated Surrogate dam whose own litter had been removed just prior to the crossing. The Donor dams and original Surrogate litters were sacrificed by CO_2 inhalation. A set of untreated dams and perchlorate exposed dams from the Donor group remained with their own litters to serve as 'true control' and 'true exposed'. Table 1 shows the resulting treatment groups.

All dams remained on control or perchlorate treated drinking water for the duration of the study. The day of crossing was designated as PND 1 (postnatal day 1). On PND 2 the crossed litters were randomly culled to 8 (4 male and 4 female). At PND10 dams and pups were sacrificed by CO_2 inhalation. Dam serum, thyroid and skin and pup serum, skin and gastric contents were collected. Male and female pup sera were pooled within litters. All tissues were analyzed for perchlorate by HPLC using the method described in Yu et al (2000). Hormone assays for TSH, T_3 and T_4 were conducted using radio-immunoassay (RIA) kits following the manufacturer's instructions. Thyroid hormone kits were purchased from Diagnostic Product Corp. (Los Angeles, CA), and the TSH kit was obtained from Amersham Corp. (Arlington Heights, IL).

Statistical analysis was performed using an LSD test ($p \leq .05$). Paired comparison P-values are from 2-tailed paired t-tests.

Table 1. Experimental design for cross-fostered litters.

Group ID	Exposure from Dams	
	Prenatal	Postnatal
True control (Ctrl/Ctrl)	Water	Water
True exposed (ClO_4/ClO_4)	1 mg/kg/day ClO_4	1 mg/kg/day ClO_4
Ctrl/ClO_4	Water	1 mg/kg/day ClO_4
ClO_4/Ctrl	1 mg/kg/day ClO_4	Water

Results

Body Weight

Maternal body weights from GD 2 to PND 10 are shown in Figure 1. There was no statistical difference ($p \leq .05$) between the control groups and the ClO_4 exposed groups, suggesting no maternal toxicity from the perchlorate exposure. Male and female pup weights were measured from PND 3 to PND 10 (Figures 2a, b). There were virtually no differences between male and female pup body weight. The 2 cross fostered litters (Ctrl/ClO_4 and ClO_4/Ctrl) were significantly lower in body weight than the true control and the true exposed for both male and female pups. In fact, the ClO_4/Ctrl and the Ctrl/ClO_4 groups weighed 17.8 ± 3.6% and 25.0 ± 3.5% less than the true control group, respectively (male and female, time weighted average). The male and female pups from the true exposed group only weighed 4.0 ± 2.3% less than the true control (time weighted average).

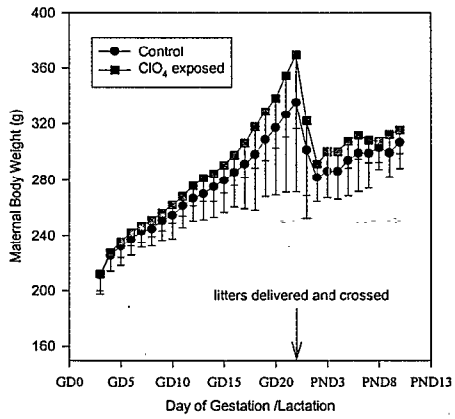

Figure 1. Body weights of control and perchlorate exposed maternal rats from gestation day 2 to post natal day 10.

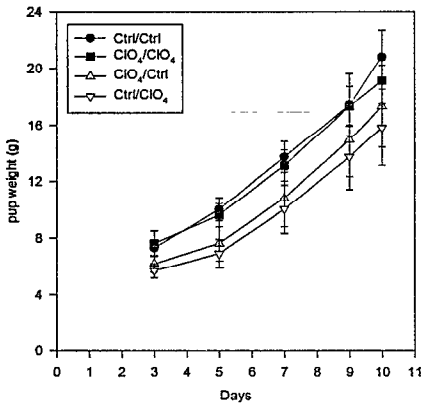

Figure 2a. Body weights of male pups from all exposure groups from postnatal day 3 to 10.

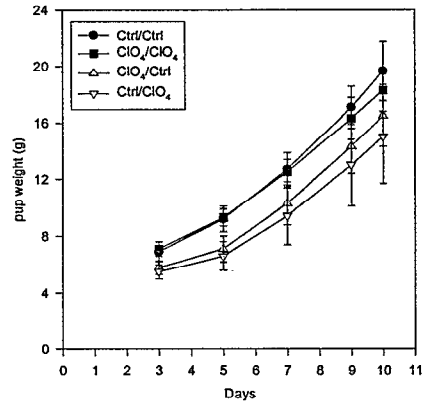

Figure 2b. Body weights of female pups from all exposure groups from postnatal day 3 to 10.

Disposition of Perchlorate

Table 2 shows the tissue levels of perchlorate in maternal rats at PND 10. As expected there were no detectable tissue levels of perchlorate in either of the untreated dam groups. Perchlorate levels in blood and skin were significantly higher in the Ctrl/ClO_4 group than the ClO_4/ClO_4 dams. Serum perchlorate was 0.53 ± 0.07 μg/ml for the Ctrl/ClO_4 group whereas the ClO_4/ClO_4 group serum perchlorate was 0.41 ± 0.03 μg/ml. Skin perchlorate levels were 1.12 μg/ml and 1.44 μg/ml in the ClO_4^-/ClO_4^- group and the Ctrl/ClO_4^- group, respectively. Little change was seen in thyroid perchlorate levels between the 2 exposed groups.

Table 2. Tissue levels of perchlorate in maternal rats on day 10 of lactation in cross fostered groups.

	True Control (Ctrl/Ctrl)	True Exposed (ClO_4/ClO_4)	Exposed dam/ untreated litter (Ctrl/ClO_4)	Untreated dam/ exposed litter (ClO_4/Ctrl)
Serum, μg/ml	ND*	0.41 ± 0.03	0.53 ± 0.07	ND
Thyroid, μg/g	ND	13.94 ± 1.5	15.07 ± 1.7	ND
Skin, μg/g	ND	1.12 ± 0.09	1.44 ± 0.16	ND

*ND – not detected

Mean ± standard deviation

In male and female pups the concentration of perchlorate in all tissues tended to be greater in the Ctrl/ClO_4 group than the true exposed (ClO_4/ClO_4) group, except male pup gastric contents (Table 3). Differences between the true exposed and the Ctrl/ClO_4 groups were

significant for male and female pup skin and female pup gastric contents ($p \leq .05$). Within the true exposed group female pups had significantly higher levels of perchlorate in the gastric contents than male pups. Levels of perchlorate in the true control and ClO_4/Ctrl groups were not detectable, as expected.

Table 3. Tissue levels of perchlorate in male and female pups.

	True Control (Ctrl/Ctrl)	True Exposed (ClO_4/ClO_4)	Exposed dam/ untreated litter (Ctrl/ClO_4)	Untreated dam/ exposed litter (ClO_4/Ctrl)
Serum, ug/ml				
Male	ND*	0.50 ± 0.05	0.54 ± 0.06	ND
Female	ND	0.54 ± 0.05	0.56 ± 0.07	ND
Skin, ug/g				
Male	ND	1.35 ± 0.16	1.90 ± 0.21	ND
Female	ND	1.43 ± 0.16	1.97 ± 0.23	ND
Gastric contents, ug/g				
Male	ND	3.39 ± 0.37	3.62 ± 0.39	ND
Female	ND	4.13 ± 0.44	3.68 ± 0.40	ND

*ND – not detected

Mean ± standard deviation

Thyroid Hormones and TSH

T_3: The levels of T_3 maternal serum and male and female pup serum were nearly the same for all dose groups (Fig 3). The true control and Ctrl/ClO_4 groups had lower but statistically insignificant levels of T_3.

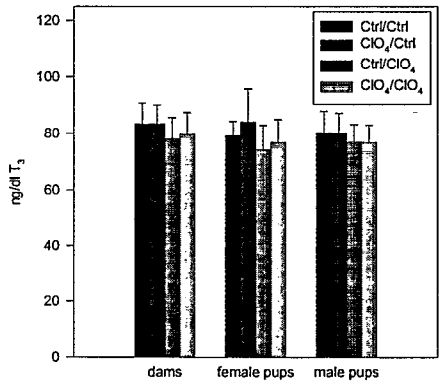

Figure 3. Serum levels of T_3 in dam and male and female pup at post natal day 10. *Significant at $p \leq .05$.

T_4: For the dams and the male pups T_4 levels were not substantially different between dose groups (Fig 4). Female pups, however, had significantly decreased levels of T_4 in both the true exposed group and the Ctrl/ClO_4 group, as compared to both true control and the ClO_4/Ctrl group. Additionally, female pups from the Ctrl/ClO_4 group had significantly lower T_4 levels than male pups from the same group.

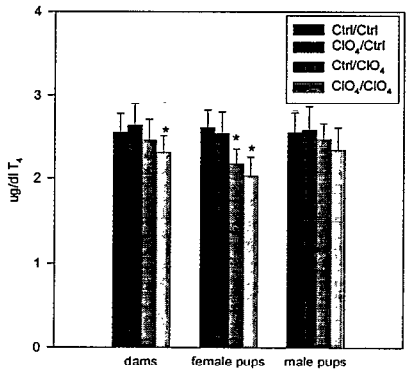

Figure 4. Serum levels of T_4 in dam and male and female pups at post natal day 10. *Significant at $p \leq .05$.

TSH: TSH levels in male pup serum were elevated significantly in both lactationally exposed groups (ClO_4/ClO_4, Ctrl/ClO_4) as compared to the control groups (Ctrl/Ctrl and ClO_4/Ctrl)(Fig 5). However, the dams and female pups from the same exposed groups had substantially elevated TSH levels in serum as compared to the same control groups. Although both male and female pups had elevated serum TSH, the female pup serum TSH was significantly higher than in male pup.

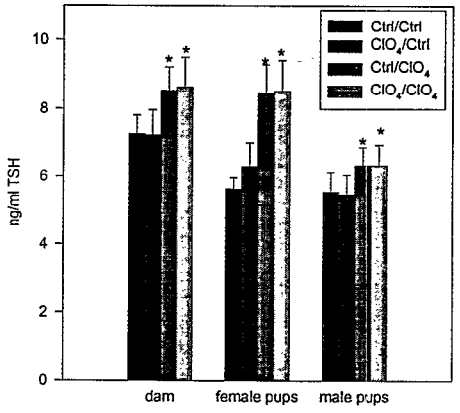

Figure 5. Serum levels of TSH in dam and male and female pups

at post natal day 10. *Significant at $p \leq .05$.

Summary

- Perchlorate exposure of 1 mg/kg-day during gestation did not appear to cause maternal toxicity.
- Male and female pups from the Ctrl/ClO_4 and ClO_4/Ctrl groups had significantly lower body weights from PND3 to PND10. This decrease in body weight does not correlate with serum T_4 and TSH levels. However, this may be a result of litters being crossed to surrogate dams.
- Perchlorate cleared readily by PND10 from male and female pups that received *in utero* only exposure to perchlorate.
- *In utero* and lactational exposure to perchlorate did not affect serum T_3 levels in all groups.
- Female pups appear more sensitive to perchlorate exposure than male pups. Although both male and female pups had similar serum perchlorate levels at PND 10, T_4 and TSH were more affected in female pups than male pups.

Animal Use

All studies involving live animals were conducted under a program of animal care accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International and in accordance with the "Guide for the Care and Use of Laboratory Animals", National Research Council (1996).

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