PROPOSAL

BIOAVAILABILITY OF LEAD FROM SOILS:

PHASE I, PARTS I & II: DOSING METHODS AND ANIMAL MODEL

Collaborative Research Contract between
Louisiana State University
School of Medicine in New Orleans (LSUSMNO) &
E.I. Du Pont de Nemours & Company, Inc. (DU PONT)

SUBCHRONIC ORAL TOXICITY: 30-DAY FEEDING STUDY IN MALE RATS & MINI-PIGS WITH LEAD

SURGICAL RESEARCH PROJECT NO. 101

PROTOCOL

Louisiana State University School of Medicine
in New Orleans IACUC Approval # 909

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ABSTRACT:

Multimedia exposure of children to lead is recognized as a health problem of international proportions. Ingestion of soil lead and dust incidental to hand to mouth activity presents one of the principal direct pathways for exposure to non-dietary lead in areas with significant soil contamination. Environmental lead contamination is derived from a variety of sources including: lead-based housepaint, auto emissions, smelter emissions, wind-blown tailings or mining wastes which have been used for fill in residential developments and/or land-fills. This phase of study seeks to determine which animal model and dose level provide the best measure of soil-lead bioavailability, transport from the intestinal lumen to the blood stream.

OBJECTIVES

The overall objectives of this study are: 1) to gain a more thorough understanding of the chemical and physical characteristics of soil that influence lead bioavailability and 2) to develop scientifically and legally defensible determinations of soil lead bioavailability in site soils to support the development of site-specific cleanup goals. The proposed research uses soils with a wide range of lead concentrations, test feeds animals a diet mixed with the soil, and assesses lead bioavailability using biomarkers.

As currently conceived this study will proceed in three related phases. The objectives of this first phase are the determination of critical aspects of study design (e.g. animal model selection and concentration effects) that will be used to tailor the definitive protocol for the second and third phases involving site soils from various locations throughout the DU PONT corporation.

This first phase of study will evaluate the bioavailability of lead in soil when incorporated into nutritionally adequate purified AIN 76 basal diet and fed to male rats (Harlan Sprague Dawley and male mini-pigs (Charles River Yucatan Minipig YU) for 30 days. The oral route of administration was selected because it is the potential route of human exposure.

SPONSOR AND TEST FACILITY

This study is sponsored by E.I. du Pont de Nemours & Co., Inc., Wilmington, Delaware. The study will be conducted in the Department of Surgery, Louisiana State University, School of Medicine in New Orleans, in accordance with all applicable Good Laboratory Practice (GLP) standards (40 CFR, Part 792).

STUDY DESIGN

The experimental design is as follows:

Task 1 (Concentration Effects and Lead Acetate vs. Lead Nitrate for Control Lead Normalization Study: holds soil constant, varies Pb Concentration and Pb salt form)

Control Soil + PbAcO Group #	Rats/Group	Control Soil + $Pb(NO_3)_2$ Group #	Rats/Group	Pb-Soil Pb-Soil Group #	Rats/Group	Acetate, Nitrate & Soil Pb Conc.
1	5	2	5	3	5	<30 ppm (Control)
4	10*	5	5	6	5	~375 ppm
7	5	8	5	9	5	~750 ppm
10	5	11	5	12	5	~1500 ppm
13	5	14	5	15	5	~3000 ppm

Task 2 (Animal Model Comparison Study; holds Pb constant @ ~1000 ppm, varies animal model)

Group #	Rats/Group	Group #	Mini-pigs/Group	Dietary Concentration (ppm)
16	10*	17	5	<30 ppm Soil + PbAcO at 1000 ppm
18	5	19	5	1000 ppm

* 5 rats in groups 4 & 16 and the mini-pigs in group 17 will be bled at 3, 7, 14, 21, 28 and 30 days; Feeding and testing may continue, if blood lead levels do not plateau; this will require contract amendment to provide for additional animal care & testing per diem costs.

The schedule for study functions is as follows:

Function	Approximate Study Day		
	Pretest	15	30
Soil Lead Concentration	Analyzed	-	Analyzed
Diet Preparation	<- Once ----------		---------->
Diet Sampling	a	b	-
Clinical Sign Observation	<----------	Daily	---------->
Body Weights	<----------	Daily	---------->
Food Consumption	<----------	Daily	---------->
Feces Collection	<----------	Daily	---------->
Feces Analysis	<----------	Weekly	---------->
Blood lead Levels	<- c -- c ------	c ---- c	-----c-c->

a Sampled for homogeneity and concentration at start of study
b Sampled from feeders for concentration analysis during study
c Orbital bleeds on Groups 4 & 16 (rats) and venipuncture bleeds on Group 17 (mini-pigs) at 3, 7, 14, 21, 28 & 30 days; cardiac blood from all other animals at sacrifice, 30 days.

MATERIALS AND METHODS

A. Test Material

The test material will be supplied by the sponsor, DU PONT. The test material will be assigned a LSU-Surgery Test Material Number (LSU-Surg TM #) and LSUSMNO will provide a chain of custody for the test material. The concentration of lead in the diet/soil mixtures will be confirmed by analyses at the beginning and end of the study, during diet mixing and from feeders during the study.

B. Test Species

One hundred fifteen (115) male Sprague Dawley rats, approximately 21 days of age, with body weights ranging from 40 to 65 grams will be acquired from Harlan Sprague Dawley, Indianapolis, IN. Rodents will be delivered in Harlan climate-controlled trucks to the LSU-Surgery laboratory once a week. The Hsd: SD rat has been selected on the basis of extensive experience with this strain and its suitability with respect to longevity, hardiness, sensitivity, and low incidence of spontaneous diseases.

Eleven (11) male Yucatan mini-pigs, approximately 30 days of age, with body weights ranging from 3 to 6 kilograms will be acquired from Charles River Laboratories, Wilmington, MA. Mini-pigs will be crated and shipped to New Orleans by air freight. The weanling Charles River MINIPIG YU has been selected on the basis of its low body weight and its suitability as a model of soil-lead ingestion by children and with respect to longevity, hardiness, sensitivity, and low incidence of spontaneous diseases.

C. Animal Husbandry

Each rat will be housed, singly in filter-top polycarbonate plastic cages to prevent test animal exposure to environmental lead and with cage cleaning and feeding conducted in a laminar-air-flow hood, to protect laboratory personnel from lead exposure. Raised-wire mesh floors will be used to prevent coprophagy. HEPA-filtered inflow-outflow filter-top caging systems (Thoren, Allentown or Lab Products), which can be adjusted to maintain positive or negative cage air pressures, will be utilized to contain lead spiked diets and to protect the test animals from environmental exposure to lead. Individual cage racks will be relocated within the room weekly and cages on the racks will be repositioned every 2 weeks.

Each minipig will be housed singly in air-tight isolation cages (Plas Labs) to prevent test animals exposure to environmental lead and with daily cage cleaning, feeding, diet & animal weighing conducted in a laminar-air-flow station (Standard Safety Equipment Co., Palatine, IL hood, to protect laboratory personnel and other test animals from lead exposure. Raised-floors will be used to prevent coprophagy and aid in feces collection. HEPA-filtered inflow-outflow filter-top caging systems, which can be adjusted to maintain positive or negative cage air pressures, will be utilized to contain lead spiked diets and to protect the test animals from environmental exposure to lead. Individual cage racks will be relocated within the room daily,

Animal rooms will be targeted at a temperature of 23 ± 2°C (rats) and 25 ± 5°C (mini-pigs, See Appendix III) and relative humidity of 50 ± 25%. Animal rooms will be artificially illuminated (fluorescent light) on a 12-hour light/dark cycle.

All rats and mini-pigs will be provided test diets and deionized water ad libitum. During the pretest period (see Section D), all rats will be fed Teklad 5% basal diet and all mini-pigs the Ziegler 5% basal diet. During the test period, all rats and mini-pigs will be fed the diet of their respective treatment group (see Section F).

LSU-Surgery Laboratory has an animal health monitoring program which consists of periodic food and water analyses for contaminants and sampling freshly washed cages and cage racks for bacteria. This program is monitored and administered by the LSUSMNO laboratory animal veterinarian. This program will be applied to this study.

D. Pretest Period

Upon arrival at LSU-Surgery Laboratory, all rats and mini-pigs will be removed from shipping cartons and housed three per cage (rats) one per cage (mini-pigs), in a quarantine room. The rats and mini-pigs will be:

o quarantined for a minimum of six days.
o identified permanently with a subcutaneous microchip; American Veterinary Identification Devices, Inc. (AVID), and cage identification;
o weighed daily; and,
o observed with respect to weight gain and any gross signs of disease or injury.

The rats and mini-pigs will be released from quarantine by the laboratory animal veterinarian or her designee on the bases of body weights and clinical signs of all rats and mini-pigs at the end of the pretest period.

Rats and mini-pigs that die or are euthanized *in extremis* during the pretest period will be necropsied to check for the presence of disease. Dependent upon these findings, further diagnostic procedures will be employed at the discretion of the study director or the laboratory animal veterinarian.

E. Assignment to Groups

Animals will be assigned during the pre-test period separately for each task. Animals will be assigned to the groups specified in the Study Design.

Each rat and minipig will be housed individually, assigned its own LSU-Surgery animal number, and permanently identified with a microchip placed under the skin (see Appendix I).

Rats and mini-pigs that have not been assigned to a test group may, at the discretion of the study director, be released for use in other studies, or be sacrificed by sodium pentobarbital overdose (100 mg/kg body weight) and discarded without pathological evaluation.

F. Diet Preparation and Analyses

During the test period, rats and mini-pigs in each group (as identified in the Study Design) will be fed the following purified diets: a) RATS - TEKLAD 5% Basal Diet (T5%BD) and b) MINI-MINI-PIGS - ZIEGLER 5% Basal Diet (Z5%BD). These diets will be custom formulated by their respective diet manufacturers so that each diet contains complete normal nutrition for each species when the diets will be diluted (weight/weight) with test soil containing <30, ~375, ~750, ~1500, or ~3000 ppm lead.

LEAD/soil will be added to T5%BD & Z5%BD and thoroughly mixed for a period of time that is adequate to assure homogeneous distribution in the diet. Control diets will be mixed for the same period of time. All diets will be prepared once and refrigerated until used.

Before the beginning of the study, samples will be collected from each concentration of diet prepared with LEAD/soil. These samples will be analyzed to verify concentration and homogeneity in the test diets.

Homogeneity samples will be collected from the top, middle, and bottom of the diet mixer. Samples will be frozen until analyzed. Homogeneity diet samples may also be collected if a new method of diet preparation is used. Also, if a new method of diet preparation is used, it will be documented in the study records.

Stability testing of the soils will not be performed. USEPA publication SW-846, Methods for the Analysis of Solid Waste, specifies a holding time for solid material samples of six months with proper refrigeration for samples being analyzed for lead. Since lead in the samples to be tested in this project is not expected to degrade during the study period, stability testing is not deemed to be necessary.

Extra diet samples may be taken at the discretion of the study director. Whenever extra diet samples are taken, a sample of control diet will be collected and frozen the same day as diet preparation.

Once during the study, samples will be collected from randomly selected feed jars to verify concentration of lead in the feeders. Samples will be collected from every group. Backup samples will be taken on the same day from other feeders in the same group and analyzed if needed. Feeder samples and backup samples will be frozen on the day of collection.

G. Body Weights

All rats and mini-pigs will be weighed once a day unless experimental findings warrant a more infrequent weighing schedule.

H. Food Consumption and Intake of Test Material

The amount of food consumed by each individual test animal each day will be determined daily throughout the study. From these determinations and body weight data, average test animal daily food consumption and intake of test material will be calculated.

I. Fecal Collection and Analyses

Feces will be collected daily as follows: rats & mini-pigs entire fecal output for each test animal will collected and weighed (wet weight). It is expected that the entire rat fecal output (3 to 10 grams) will be collected daily, weighed, and pooled for 7 days. From this pooled sample, total weekly lead excretion will be determined. Mini-pigs will have their fecal output (0.5 to 1.5 kilograms) collected daily, weighed and a representative 25 gram

sample collected for lead analysis.

J. Clinical Observations and Mortality

Cage-site examinations to detect moribund or dead rats and mini-pigs and abnormal behavior and appearance among rats and mini-pigs will be conducted once daily throughout the study. Moribund rats and mini-pigs will be euthanized. Moribund and dead rats and mini-pigs will be given a pathological examination. At every weighing, each rat and mini-pig will be individually handled and examined for abnormal behavior and appearance.

K. Lead Analyses

Samples of soil, diets, and tissues will be analyzed for total lead content according to the schedule specified in the Study Design.

L. Pathological Examination

All rats and mini-pigs that are found dead, accidentally killed, or sacrificed in extremis before the end of the study will be necropsied. After approximately 30 days of continuous feeding, surviving rats and mini-pigs will be sacrificed by methoxyflurane and exsanguination, and necropsied. The order of sacrifice will be random among all treatment groups.

The following tissues will be collected and preserved in formalin from rats and mini-pigs that are found dead, accidentally killed (tissue integrity permitting), sacrificed in extremis, and from those rats and mini-pigs sacrificed by design after 30 days on test:

Stomach
*Liver
Pancreas
Small intestine (duodenum, jejunum, and ileum)
Large intestine (cecum, colon)
Kidney
Brain
Peripheral nerve (sciatic and/or vagus)
*Bone (femur)
Muscle (thigh)
All gross lesions

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*Portion collected for lead analyses

Microscopic evaluation of these tissues will be performed if warranted by experimental findings.

Blood will be collected as indicated in the Study Design and during exsanguination at the final sacrifice. Whole blood will be

analyzed for lead content.

STATISTICAL ANALYSES

Body weights, body weight gains, food consumption, will be analyzed by a one-way analysis of variance. When the test for differences among test group means (the value of the F test statistic) is significant pairwise comparisons between test and control groups will be made using the Dunnett's. test.

Significance will be judged at alpha = 0.05. Other methods will be used- if appropriate, at the time of analyses.

Relative bioavailability will be determined by comparing tissue lead levels from the soil test groups with tissue lead levels from their respective lead acetate and nitrate control groups.

SAFETY AND HOUSEKEEPING

Lead in soil has a low acute oral toxicity. Good housekeeping procedures will be practiced to avoid contamination of diet preparation facilities and potential health hazards. To avoid skin contact, gloves will be worn when handling either the test material or diets. In addition, the test material will be handled in a chemical hood. Diets will be prepared in properly ventilated areas.

RECORDS AND SAMPLE RETENTION

All original records will be retained at LSU-Surgery Laboratory or at the E. I. du Pont de Nemours and Company, Wilmington, Delaware. Preserved wet tissues, paraffin blocks, histological slides, and be retained at LSU-Surgery Laboratory. A portion of test sample will be collected for archive purposes prior to the outset of the study and retained at LSU-Surgery Laboratory.

Any change, addition or deletion to the protocol will be documented as a Protocol Amendment and a copy sent to the sponsor.

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PROTOCOL APPENDIX I

SURGICAL RESEARCH PROJECT NO. 101

ANIMAL IDENTIFICATION MARKING SYSTEM

Rats will be identified by a subcutaneously implanted microchip MC#. The numbering system will be according to the following scheme:

Task 1

Control + $PbAcO_4$		Soil + $Pb(NO_3)_2$		Pb-Soil		
Male Rats	MC#	Male Rats	MC#	Male Rats	Pb-Soil MC#	Acetate, Nitrate & Soil Pb Conc.
1	101-5	2	201-5	3	301-5	<30 ppm (Control)
4	111-10	5	211-5	6	311-5	~375 ppm
7	121-5	8	221-5	9	321-5	~750 ppm
10	131-5	11	231-5	12	331-5	~1500 ppm
13	141-5	14	241-5	15	341-5	~3000 ppm

Task 2 (Animal Model Comparison Study)

Male Rats	MC#	Male Mini-Pigs	MC#	Dietary Concentration (ppm)
16	501-10	17	560-65	<30 ppm Soil + $PbAcO_4$ at 1000 ppm
18	601-5	19	660-65	1000 ppm

PROTOCOL APPENDIX II

SURGICAL RESEARCH PROJECT NO. 101

Study Personnel and Study Dates

Study Function	Study Personnel
Study Director LSUSMNO	James B. Heneghan, Ph.D. Professor, Surgery & Physiology
Consultant Xavier	Howard Mielke, Ph.D. Associate Professor, Pharmacy
Clinical Pathology LSUSMNO	Charmaine Foltz, D.V.M. Lab Animal Veterinarian
Analytical Chemistry LSUSMNO	Cecelia Breaux, B.S., (M.S.) Laboratory Supervisor

Study Dates

Proposed Project Start August 1, 1992

Phase 1: Task 1, Concentration & Control Lead Normalization Study in Rats

Pretest	October 26, 1992
30-Day	November 25, 1992
Scheduled Euthanasia	November 25, 1992

Phase 1: Task 2, Animal Models (Rats vs. Mini-pigs) High and Low Doses

Pretest	February 15, 1993
30-Day	March 17, 1993
Scheduled Euthanasia	March 17, 1993

Proposed Report Dates for DU PONT/LSU-Surgery Studies:

Task 1	February 1, 1993
Task 2	April 5, 1993

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PROTOCOL

Date

James B. Heneghan, Ph.D. (LSUSMNO)
Study Director

<>
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