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THERAPEUTIC APPROACHES FOR MANAGEMENT OF LEAD EXPOSURE FROMEMBEDDED LEAD METAL FRAGMENTS

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by

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ABSTRACT

Therapeutic Approaches for Management of Lead Exposure from Embedded Lead Metal Fragments

By Shannon Twardy

Elevated blood lead levels from retained lead fragments is a significant but insufficiently recognized public health risk for which there are no well-accepted management guidelines. It is estimated that there are 67,000 non-fatal firearm injuries a year in the U.S., and 42,000 U.S. veterans with retained lead metal fragments from bullet or improvised explosive injuries. To investigate the efficacy of succimer chelation to manage elevated lead levels in patients with embedded lead fragments, an *in vitro* experiment was done to determine if *meso*-2,3-Dimercaptosuccinic acid (DMSA) or ethylenediaminetetraacetic acid calcium disodium salt ($CaNa_2EDTA$) directly increase the release of lead from a solid lead pellet in an artificial extracellular fluid (aECF) cell-free system. Also, a rodent model of elevated lead levels from embedded lead pellets was established using stable lead isotope tracer methodologies to differentiate lead chelated from tissues or from embedded lead pellets. Results from the *in vitro* study show that DMSA and $CaNa_2EDTA$ significantly mobilized lead from a solid lead fragment in an aECF environment. The *in vivo* results show that 5 days of oral succimer chelation

(Chemet, 50 mg/kg/day) significantly reduced blood and tissue lead, as expected, though blood and tissue lead levels rebounded within 1 week post-chelation to levels comparable to the vehicle group. During the initial (day 1) stage of chelation where the greatest blood lead reductions and increases in urine lead excretion occurred, there was no evidence of the pellet lead isotopic signature in the blood or urine, indicating that embedded pellet lead was not mobilized in the first day of chelation. However, lead from the embedded pellet environment was mobilized to the blood and urine with continued chelation (beyond day 1) and during the post-chelation blood lead rebound period. These findings indicate that prolonged succimer chelation in patients with elevated blood lead levels from embedded lead fragments may not produce lasting reductions in blood or tissue lead levels and may in fact increase mobilization of fragment lead into blood and tissues. Thus, the risks of succimer chelation in subjects with embedded lead fragments, which may represent an inexhaustible internal source of lead exposure, may outweigh the benefits of transiently reducing blood lead levels.

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INTRODUCTION

Centers for Disease Control and Prevention (CDC) have stated that there is no safe level of lead in the body and considers a blood lead level >5 ug/dL as elevated (Centers for Disease Control and Prevention, 2021). Over several decades, increasing regulations of common sources of lead exposure have led to a 93.6% decrease in average blood lead levels to 0.92 ug/dL in adults in the United states as of 2016 (Dignam, Kaufmann, Lestourgeon, & Brown, 2019, Centers for Disease Control and Prevention, 2019). Preventative measures to reduce lead exposures from occupational and environmental sources have been successfully implemented, and in cases when blood lead becomes elevated, well-accepted management practices exist to remove the patient from the source of lead exposure and, if clinically indicated, undergo chelation therapy to reduce blood lead levels (Kim et al., 2015; Kosnett et al., 2007; National Institute for Occupational Safety and Health, 2018).

Embedded lead fragments in the soft tissue resulting from firearm-related injuries or projectiles from blast devices are an overlooked source of potential elevated blood lead levels for which there is no standardized treatment. This is a significant public health concern considering that there are ~67,000 nonfatal firearm injuries in the U.S. each year, and that it is estimated that 42,000 veterans have retained metal fragments due to combat-related injuries (Fowler, Dahlberg, Haileyesus, & Annest, 2015; Gaitens, Condon, Squibb, Centeno, & McDiarmid, 2017). There are many factors that influence whether

or not a fragment may leach lead and contribute to elevated blood lead levels, including the number, surface area and composition of the fragments, their location in the body, time since injury, and age and metabolic state of the patient (Apte, Bradford, Dente, & Smith, 2019; Centeno et al., 2014; Mcquirter, 2004; Nickel, Steelman, Sabath, & Potter, 2018). Importantly, a retained fragment is recognized by the body as a foreign object, prompting immune cells to eventually encapsulate the fragment in fibrotic scar tissue to isolate it from the rest of the body, as it would do to an implanted medical device (Apte et al., 2019; Mcquirter, 2004; McQuirter et al., 2001; Riehl, Sassoon, Connolly, Haidukewych, & Koval, 2013; Weiss, Lee, Feldman, & Smith, 2017). However, many case studies have reported associations between elevated blood lead levels and retained fragments in the soft tissue in humans, thus challenging the assumption that metal fragments remain inert in the body (Apte et al., 2019; Farrell, Vandevander, Schoffstall, & Lee, 1999; Grasso, Blattner, Short, & Downs, 2017; Nguyen et al., 2005; Nickel et al., 2018; Weiss, 2012; Weiss, Tomasallo, et al., 2017).

Studies have used stable lead isotope methodologies to show that embedded lead fragments can serve as a source of elevated blood lead levels. In one human case study, retained extra-articular bullet fragments were surgically removed from a patient with a blood lead level of >200 µg/dL, and isotopic analysis of lead from the bullet fragments was consistent with the lead isotopic signature of the patient's blood lead, demonstrating that the

embedded fragments were the source of lead poisoning (Weiss, Lee, et al., 2017). In another study, canines were implanted with fragments isotopically enriched in ^{208}Pb or ^{206}Pb to determine their impact on blood lead levels, depending on whether the fragments were intra- versus extra-articular (i.e., within or outside of a bone joint space). Lead from extra-articular fragments were found to contribute to elevated blood lead levels, but to a lesser extent than intra-articular fragments, indicating that the internal location of the fragments near joints or areas of regular motion may also be an important factor influencing risk of lead poisoning (Manton & Thal, 1986).

More broadly, stable lead isotope tracer methodologies are well-established approaches to differentiate sources of exposure from multiple possible lead sources, and they have been utilized to evaluate the efficacy of treatments for lead poisoning in humans, primates, and rodent studies. In humans, Smith et al. (1994) assessed the absorption of lead across the GI tract after treatment with succimer using stable ^{204}Pb tracer to differentiate lead given orally during the study versus other sources of lead. A similar approach was used in a primate model of moderate childhood lead poisoning to evaluate GI tract absorption of oral ^{206}Pb tracer during succimer treatment (Cremin, Luck, Laughlin, & Smith, 2001). In another primate study, a stable ^{204}Pb tracer methodology was used to evaluate the efficacy of succimer in reducing lead in blood, liver, and skeletal tissue from short- versus long-term lead exposure (D. R. Smith, Woolard, Luck, & Laughlin, 2000). In rodents,

stable ^{204}Pb tracer was used to analyze the potential redistribution of endogenous lead to the brain during treatment with $CaNa_2EDTA$ (Seaton, Lasman, & Smith, 1999). Thus, stable lead isotopic methodologies are well-suited and well-supported for evaluating chelation treatment efficacy for elevated lead levels.

Although treatment guidelines for environmental and occupational sources of lead exposure are well-established, there are no standardized management guidelines for patients with embedded lead metal fragments that contribute to elevated lead levels (Gaitens et al., 2020). For example, for environmental or occupational sources of lead poisoning, guidelines indicate that the patient first be removed from the source of exposure, then other treatment options such as chelation therapy may be considered if indicated (Kosnett et al., 2007). However, in cases of embedded fragments, removal of the lead exposure source is often not possible or not recommended (Gaitens et al., 2020; Hill, Edwards, & Bowyer, 2001; Kane, Kasper, & Kalinich, 2009). It is most common that embedded fragments are not removed at the time of injury due to standard surgical guidance against aggressive surgical removal in order to minimize collateral tissue damage and risk of infection (Kane et al., 2009). As a result, hundreds of fragments can remain embedded in extra-articular tissues, and are assumed to be encapsulated and inert, which they often are not (Cyrus et al., 2011). While case studies have shown success in surgical removal of fragments with and without prior chelation therapy, the

risks and overall morbidity of surgery alone vary on a case by case basis and often outweigh the benefits of removing the fragments (Kane et al., 2009; Riehl et al., 2013; Weiss, Lee, et al., 2017). This suggest that other treatment alternatives should be considered.

Here, we addressed the efficacy of succimer chelation to manage lead poisoning in patients with embedded lead fragments. Specifically, succimer mobilization of lead from solid lead pellets was investigated in an *in vitro* cell-free system and in a rodent model. Physiological and 10x-physiological concentrations of two common chelating agents, *meso*-2,3-Dimercaptosuccinic acid (DMSA) or ethylenediaminetetraacetic acid calcium disodium salt ($CaNa_2EDTA$), were combined with a solid lead pellet in artificial extracellular fluid to determine if chelators can mobilize lead from a solid lead fragment *in vitro*. To test mobilization of lead with lead pellet signature during oral succimer (Chemet) chelation *in vivo*, an animal model was developed using stable ^{206}Pb isotope tracer to produce elevated tissue lead levels with a lead isotopic signature different from that of embedded lead pellets, to establish a simple two-endmember mixing model. As a result, we were able to calculate the relative percentage of each endmember (i.e., embedded lead pellets versus tissues with a ^{206}Pb-enriched isotopic signature) in the measured blood, urine, and tissue prior to and over the course of succimer chelation, and the ensuing post-chelation rebound period.

METHODS

Overview of experimental approaches

An *in vitro* experiment was done to determine if *meso*-2,3-Dimercaptosuccinic acid (DMSA) or ethylenediaminetetraacetic acid calcium disodium salt ($CaNa_2EDTA$) directly increase the release of lead from a solid lead pellet in an artificial extracellular fluid (aECF) cell-free system. Subsequently, a rodent model of retained lead fragments was used to test if succimer (DMSA) chelation mobilized lead from embedded pellets over 5 days of oral succimer chelation or over an ensuing 3 week blood lead rebound period. For the rodent study, a stable lead isotope tracer approach was used to distinguish lead mobilized from embedded fragments (lead pellets) versus tissues with distinctly different lead isotopic signatures. Blood, urine, and tissue samples were collected over time and analyzed for lead concentrations and isotopic compositions, and a simple two-endmember mixing model used to calculate the relative amount of embedded pellet lead contained in tissues.

In vitro study to determine the chelation of lead from a solid lead pellet in an artificial extracellular fluid system.

Physiological and 10x-physiological concentrations of DMSA or $CaNa_2EDTA$ were added to artificial extracellular fluid (aECF) containing a solid lead pellet. Aliquots of the aECF solutions were serially sampled after 30 minutes, 1, 5, 24, and 72 hours and analyzed for lead concentration to

determine the release of lead from the pellets into the aECF. The aECF was composed of Dulbecco's Phosphate Buffer Saline (DPBS, ThermoFisher Scientific), since it has an ionic composition and buffered pH range which closely resembles the aECF reported by McNay & Sherwin (2004). Prior to the experiment, aECF was degassed by vacuum and sonicator bath for 15 minutes. Physiological concentrations of chelators were estimated based on a single clinically recommended dose for succimer (10 mg/kg body weight) or edetate ($CaNa_2EDTA$, 500 mg/m^2) and estimating the amount of chelator (DMSA or $CaNa_2EDTA$) in the liquid weight portion of the body. Physiological concentrations of DMSA and $CaNa_2EDTA$ were estimated to be 0.274 mM and 0.181 mM, respectively. Ten-times physiological concentrations of DMSA and $CaNa_2EDTA$ were prepared by dissolving 30.00 mg DMSA or 40.56 mg $CaNa_2EDTA$ in degassed aECF. Solutions were thoroughly mixed then degassed again for 5 minutes. The 10x physiological DMSA solution was adjusted to pH 6.97 with 4% NaOH (the 10x-$CaNa_2EDTA$ solution required no pH adjustment). Solutions were then diluted 10X with degassed aECF to produce estimated physiological concentrations of DMSA and $CaNa_2EDTA$. The pH of all final aECF solutions with or without added chelators were between 6.91 and 7.08. At the initiation of the experiment, physiological and 10x- physiological concentrations of chelators were added to polyethylene tubes with lead pellets (1 mm x 2 mm, range 0.017 -0.019 g, >99.99% Pb, Sigma-Aldrich, St Louis, MO). Controls were aECF with chelators but without

lead pellet (aECF + chelators), and aECF with lead pellet but without chelators (aECF + pellet, no chelator). Sample tubes were immediately capped with rubber septum stoppers for the duration of the experiment. Solution aliquots were removed by syringe through the septum and the removed aECF volume was replaced with nitrogen gas via a second N_2 gas filled syringe to maintain hypoxic conditions and avoid DMSA oxidation in solution. Sample tubes were placed on a shaker at 37 °C between aliquots. The pH of solutions were recorded after the 72 hour aliquot and were between 6.64 and 6.97 for all groups.

Animal model of elevated blood lead levels from embedded lead pellets to determine pellet lead mobilization during and after succimer chelation.

All animal procedures were approved by the institutional IACUC (protocols Smitd2002) and adhered to National Institutes of Health guidelines set forth in the Guide for the Care and Use of Laboratory Animals.

Pilot study to determine embedded lead pellet load

In order to determine the appropriate load of lead pellets to embed in the gastrocnemius muscle to generate appropriately elevated blood lead levels in our animal model, two or four lead pellets (1 mm x 2 mm, >99.99% Pb, Sigma-Aldrich, St Louis, MO) per gastrocnemius muscle (four or eight pellets per animal total) were implanted into 10 Long Evans rats (Charles River,

Hollister, CA; n=5 per pellet load). Blood samples were collected over a period of 7 weeks from the lateral saphenous vein (see below) for lead concentration analyses. Those results show that blood lead levels significantly increased from background levels (~0.12 ng/mL) to ~8 ng/mL and ~14 ng/mL for the four and eight pellet load groups, respectively, by the first blood sampling period at 1 week post-pellet implant (Supplemental Figure 1). Blood lead levels reached asymptotes of ~11 ng/mL and ~18 ng/mL for the four and eight pellet load groups, respectively, within 3 weeks post-implant. Based on these findings, a four pellet load (two per gastrocnemius muscle) was selected for the main chelation study.

Pellet Implantation Surgery - Lead pellets were implanted into the gastrocnemius muscle using a modified procedure described by Castro et al. (1996). Briefly, rats were anesthetized by administration of 3.5% isoflurane at 4 L/min oxygen flow rate in an induction chamber and transferred to a nose cone and anesthesia was maintained on 2.5% isoflurane at 0.4 L/min. Fur surrounding the incision site overlying the gastrocnemius muscle was shaved and disinfected with 70% isopropyl alcohol and betadine. Using aseptic technique, a 2-3 cm incision was made in the skin over the gastrocnemius muscle of each hind leg. Using a 16-gauge needle and specially designed stainless steel plunger, two sterile lead pellets were implanted into the lateral side of the gastrocnemius muscle of both the left and right leg (four pellets

implanted per animal). A dose of analgesic Marcaine HCl (0.1 mL, 0.5% Bupivacaine) was administered into the incision, and the incisions were sealed with tissue adhesive (VetBond, 3 M Corp, St Paul, MN). Rats were monitored following surgery until ambulatory (typically within 3 – 5 min.). The surgical sites were examined daily for 5 days for signs of inflammation or infection and then weekly thereafter for the duration of the study.

Blood Sampling - Following lead pellet implantation, blood samples were collected (within subject) after 1, 3, 5, and 7 weeks via the lateral saphenous vein (n=10). Briefly, area was clipped and cleaned with 70% isopropyl alcohol then Milli-Q™ ultra-pure water. Saphenous vein was pricked with a sterile 22G stainless steel needle. Blood (~0.1 mL) was collected with plastic capillary tubes (Innovative Med Tech, Blue Island, IL), dispensed into acid cleaned microcentrifuge tubes, and stored frozen.

Succimer chelation study in animals with embedded lead pellets

To determine if pellet lead is mobilized during or after succimer chelation, an animal model of elevated lead levels from embedded lead pellets was established using stable lead isotope tracer methodologies. Two isotopically distinguishable endogenous sources of lead (elevated tissue vs embedded lead pellets) were established and allowed to contribute to elevated blood lead levels. Succimer or vehicle was administered over 5 days, followed

by a 3 week blood lead rebound period. An overview of the two-endmember stable lead isotope mixing model, and the experimental treatment timeline is summarized in Figure 1A, B.

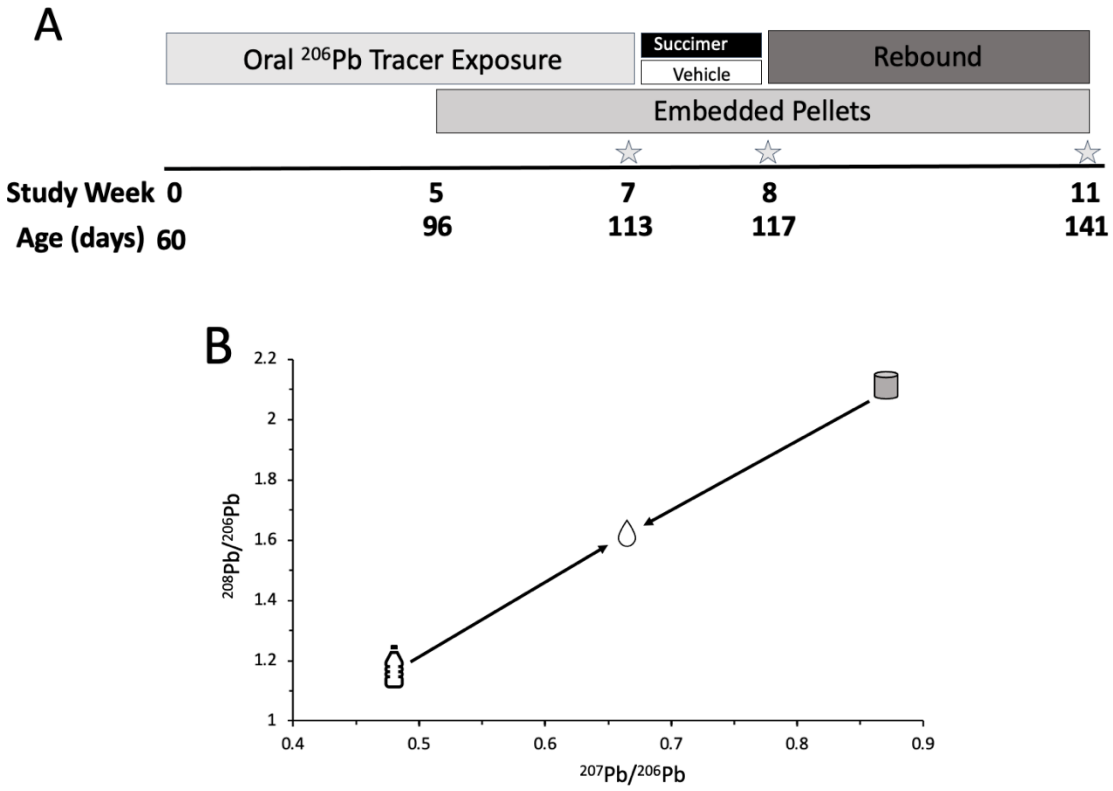


Figure 1. Establishment of a two-endmember mixing model of ^{206}Pb drinking water and embedded lead pellets. (A) Timeline of ^{206}Pb drinking water and embedded lead pellet exposures followed by chelation and the post-chelation rebound period. Drinking water enriched in ^{206}Pb was administered 5 weeks prior to lead pellet implantations. Both sources of lead exposure were allowed to contribute to blood lead levels for 2 weeks prior to chelation. Chelation began study week 7 and lasted for 5 days and was followed by a 3 week rebound period. Stars indicate tissue samples were collected pre-chelation, post-chelation, and post-rebound. (B) Conceptual two-endmember mixing plot of ^{206}Pb drinking water (^{207}Pb/^{206}Pb = 0.4756 ± 0.0002) and embedded lead pellet (^{207}Pb/^{206}Pb = 0.8708 ± 0.0002) exposure contributions to blood lead isotopic signatures prior to the start of chelation.

Animals -Forty postnatal day 60 Long Evans rats (n = 20 male, 20 female) were obtained from Charles River (Hollister, CA). Rats were pair-housed with the same sex throughout the study in polycarbonate cages with filter tops and bedding. Animal rooms were maintained at 21 ± 2 ◦C with 30–70% humidity and a 12:12 hr light:dark cycle throughout the study. Rats were fed a standard rat chow (Harlan Teklad rodent chow #2018). Rats were provided food *ad libitum* between 10 AM and 6 PM. Food was removed between 6 PM and 10AM because 24 hour access to food significantly restricted uptake of oral lead from drinking water (D. Smith, Bayer, & Strupp, 1998).

Oral lead ^{206}Pb-enriched drinking water to establish distinguishable lead isotopic compositions between elevated lead in tissues versus embedded lead pellets - Given that chelation is only indicated in cases of elevated body lead burdens, an animal model of elevated lead levels due to embedded pellets was established. Since the embedded lead pellets had a common lead isotopic signature (^{207}Pb/^{206}Pb = 0.8708 ± 0.0002) that was not sufficiently different from environmental background lead (^{207}Pb/^{206}Pb = 0.8338-8453) (Church et al., 2008; Finkelstein et al., 2012), and given the need to establish distinguishable lead isotopic signatures in the tissues with elevated lead levels versus the embedded lead pellets, rats were exposed via drinking water to elevated lead enriched in ^{206}Pb starting 5 weeks prior to implantation of the

lead pellets. Exposure to elevated ^{206}Pb-enriched drinking water was also necessitated because background blood and tissue lead levels in the study animals prior to drinking water lead exposure or embedding of lead pellets were too low (0.04 ng/mL blood, 0.46 ng/g liver, 1.18 ng/g kidney) to provide reliable ^{207}Pb/^{206}Pb ratios by magnetic sector inductively coupled plasma (ICP) high-resolution mass spectrometry., however ^{206}Pb-enriched drinking water was administered *ad libitum* at a target level of 5 µg/mL to all chelation study rats (n=40) beginning on PND 63 and ended 1 day prior to the start of chelation (PND 50). Leaded drinking water was enriched to 36% ^{206}Pb (natural abundance = 24%) and was prepared in batches of 5 L from a 5 mg/mL stock solution. The 5 mg/mL ^{206}Pb-enriched stock solution was prepared by dissolving 117 mg $Pb(CH_3CO_2)3H_2O$ (>99.99%, Sigma Aldrich, St Louis, MO) with 12.6 mL of an 890 µg/mL ^{206}Pb-enriched solution (99.66% ^{206}Pb enriched, National Institute of Standards and Technology, Gaithersburg, MD) in 0.2 N quartz-distilled HNO_3, and brought to a final volume of 15 mL with 0.2 N HNO3 in Milli-Q ultrapure water. The stock solution was thoroughly vortexed then diluted 1000X with Milli-Q^{TM} water in a low density polyethylene (LDPE) carboy. Water in the carboy was thoroughly mixed before being dispensed into low density polyethylene cage bottles. Cage water bottles were weighed and refilled two to three-times weekly and water intake per cage was recorded, and animals' daily lead intake per kg body weight was estimated based on weekly measured body weights of the two rats housed per cage. Elevated ^{206}Pb-

enriched drinking water target concentrations were monitored weekly via inductively coupled plasma-optical emission spectroscopy (ICP-OES). Measured $^{207}Pb/^{206}Pb$ of elevated ^{206}Pb-enriched drinking water was 0.4756 ± 0.0002 (mean ± SD, n=3) via inductively coupled plasma (ICP) high-resolution mass spectrometer (ICP-MS, ThermoScientific Element XR).

Embedded pellets procedure - After 5 weeks of ^{206}Pb-enriched drinking water exposure, all rats (n=40) were implanted with a total of four lead pellets (two per gastrocnemius muscle) based on findings from the pilot study. Pellet implantation followed the same procedure as the pilot study (see above). Rats retained embedded pellets for the complete duration of the study. Blood samples were collected weekly prior to chelation following the procedure described above.

Succimer or vehicle chelation for 5 days - One day prior to the start of chelation, rats were transferred to unleaded drinking water and randomly assigned to one of three subgroups, based on time of sacrifice: pre-chelation baseline (n=8), post-chelation (n=8 vehicle, 8 succimer), and post-rebound (n=8 vehicle, 8 succimer). One day prior to the start of chelation, blood and urine samples were collected from all animals, and pre-chelation rats were sacrificed via CO_2 asphyxiation to establish baseline tissue lead levels; blood, liver, and kidney were collected from the pre-chelation animals, as described

below. The remaining animals were randomly assigned to succimer or vehicle treatment groups, balanced by sex.

Succimer was obtained as pharmaceutical Chemet (DMSA). The daily oral succimer dose of 50 mg/kg body weight was administered in two equally divided doses (25 mg/kg body weight/dose) given 7 hours apart (9:00 AM and 4:00 PM) for 5 days. Immediately prior to dosing, Chemet was dissolved in apple juice (vehicle) to a stock concentration of 17.2 mg Chemet/mL apple juice and orally administered via 20-gauge stainless steel gavage needle (Popper and Sons, Inc.) in volumes ranging 0.39 mL – 0.70 mL, depending on body weight (range 0.237 kg – 0.480 kg).

Blood and urine samples were collected from animals (n=8/treatment group, balanced by sex) longitudinally every other day during the 5 days of chelation. For this, blood and urine samples were collected within 2 hours following the second dose on chelation day 1, 3, and 5. Another subset of animals (n=8/treatment group) were longitudinally sampled weekly following the end of chelation and throughout the 3 week post-chelation blood lead rebound period [blood and urine were collected from these animals 19 hours following the final succimer or vehicle dose (post-chelation), and then weekly until sacrifice (post-rebound)]. Blood (0.1-0.3 mL) was collected from the lateral saphenous vein as described above, and urine (0.3 – 1.3 mL) was collected using acid cleaned polycarbonate metabolic cages, as described

elsewhere (D. R. Smith & Flegal, 1992). Samples were immediately frozen until analysis.

Tissue Collection - Animals (n=8/treatment group) were sacrificed 21 hours after the final chelation dose (post-chelation) to determine tissue lead concentrations and isotopic compositions immediately following chelation. The remaining animals (n=8/treatment group) were sacrificed 3 weeks after the final chelation dose (post-rebound). All tissue sampling was conducted using trace metal-clean procedures. Dissecting instruments (stainless steel) were cleaned prior to each dissection and rinsed frequently with Milli-Q water. Blood, liver, kidney, and muscle tissue surrounding the lead pellets were collected from all animals. For blood, a ~2 mL sample of whole blood was collected using an acid cleaned 3 cc polypropylene syringe fitted with stainless steel 16G needle via cardiac puncture from surgically exposed hearts and dispensed into low-lead microtainer vacutainers (EDTA anticoagulant). Kidney, liver, and hind legs were surgically removed and immediately transferred to plastic bags and frozen.

Analytical

Blood, urine, and tissue processing

All sample collection, storage, and laboratory-ware (i.e., Teflon, polyethylene, polypropylene, and stainless steel) were acid-cleaned using

established procedures (Flegal and Smith, 1992; Smith et al., 1992). Processing of all samples from the *in vitro* cell-free study and animal model study were conducted under trace-metal–clean HEPA-filtered air (Class-100) conditions using clean techniques (Smith et al. 1992). Acids used in sample processing and analyses were quartz double distilled and water was ultra-pure grade (18 MΩ-cm^2). Blood, liver, kidney, and hind legs were thawed and processed for analyses. For blood, samples were gently mixed and a ~0.25 mL aliquot transferred to a pre-weighed Teflon vial and evaporated to dryness. For hind legs, gastrocnemius muscle tissue (~0.25 g) with embedded pellets were dissected free, the lead pellets removed, and the remaining muscle tissue stored. Liver, kidney, and gastrocnemius muscle tissue were rinsed with 0.2N HNO_3 and Milli-Q, transferred to pre-weighed Teflon vials, and dried at 65 °C to a constant weight. All blood and tissue samples were digested for 8 hours in hot 16N HNO_3, evaporated to dryness, and redissolved in 1N HNO_3 for analysis. For urine analysis, samples were thawed and a 50 µL aliquot removed for creatinine analysis. The remaining urine sample was acidified with 16N HNO_3 to pH < 2.

Determination of lead concentrations and isotopic compositions

Sample aliquots from the *in vitro* cell-free study were diluted into 1 N HNO_3, ^{205}Tl was added as an internal standard, and lead concentrations measured via magnetic sector inductively coupled plasma mass spectrometry

(ICP-MS) (Thermo Element XR ICP-MS, Waltham, MA, USA), measuring masses ^{208}Pb and ^{205}Tl. The analytical limit of detection for the *in vitro* samples was 0.006 ng/mL. Lead concentration and lead isotopic composition analyses in the biological sample digestates from the animal study were performed via ICP-MS in multi-isotope analog mode, measuring masses ^{206}Pb, ^{207}Pb, and ^{208}Pb, with ^{205}Tl used as an internal standard. External lead concentration standardization was via certified standards (Spex Industries Inc., Edison, NJ, U.S.) National Institute of Standards and Technology (NIST) Standard Reference Materials (SRM) 955A (bovine blood) and NIST SRM 2670a (urine) were used to evaluate procedural accuracy. The average analytical detection limit for lead concentration measurements in the biological samples was 0.002 ng/mL, while the average measurement accuracy was 90.4% and 95.9%, based on lead recoveries in the 955A (blood) and 2670a (urine) SRMs, respectively. Lead isotopic composition analyses were performed simultaneously with the lead concentration analyses, with samples bracketed by NIST 981 standards (common lead isotopic SRM) within ICP-MS runs to correct the measured sample isotope abundances based on the measured versus expected NIST 981 isotopic abundances. Measurement precision (2 x relative standard deviation, 2RSD) for sample ^{207}Pb/^{206}Pb isotopic ratios was 0.17% [range 0.10% - 0.26%], based on repeated measurements of biological samples across runs.

Urine creatinine analysis

Urinary creatinine levels were quantified using a commercial kit (#5007, Cayman Chemical, Ann Arbor, MI), following the manufacturer's instructions.

Isotopic composition normalization and % pellet calculation

The isotopic compositions of the ^{206}Pb-enriched drinking water and the embedded lead pellets represent the two-endmembers of a two-endmember mixing model. Samples (tissue, blood, urine) receiving lead contributions only from these two endmembers will fall along a mixing line defined by the endmembers, and the linear distance along that mixing line will represent the relative contributions of each endmember to the tissue. Therefore, the amount of one endmember, here the amount of pellet lead, can be determined from ^{206}Pb/^{207}Pb ratios of the two endmembers and sample. For each rat, ^{207}Pb/^{206}Pb ratios were used to calculate the relative percent pellet lead using the equation below.

i.e., Calculation for % pellet lead:

$$\%\ pellet\ lead = \frac{\left|\left(\frac{^{207}Pb}{^{206}\mathrm{Pb}}\right)_{sample} - \left(\frac{^{207}Pb}{^{206}Pb}\right)_{water}\right|}{\left|\left(\frac{^{207}Pb}{^{206}\mathrm{Pb}}\right)_{pellet} - \left(\frac{^{207}Pb}{^{206}Pb}\right)_{water}\right|}\ x\ 100$$

Where $\left(\frac{^{207}Pb}{^{206}\mathrm{Pb}}\right)_{sample} =$

$lead\ isotopic\ signature\ of\ sample\ from\ individual\ rat$

$\left(\frac{^{207}Pb}{^{206}\mathrm{Pb}}\right)_{water} =$

$lead\ isotopic\ signature\ of\ ^{206}Pb\ enriched\ drinking\ water$

$\left(\frac{^{207}Pb}{^{206}\mathrm{Pb}}\right)_{pellet} =$

$lead\ isotopic\ signature\ of\ embedded\ lead\ pellet$

Statistics

Data were analyzed using ANOVA models, as described below. Specific group contrasts were performed using Tukey's multiple comparison tests or Wilcoxon tests, if data did not satisfy parametric assumptions. P-values < 0.05 were considered statistically significant for all tests. All analyses were conducted using JMP (SAS Inst., 16th ed. 2021). For the animal chelation study, the overall effects of treatment group and time (i.e., treatment

duration) were evaluated using a mixed model repeated measures ANOVA, with treatment (succimer, vehicle) as the between-subjects factor, time as the within-subjects factor, and rat as the random effect. Post hoc contrasts using Tukey's test were performed if the corresponding ANOVA results for the main effects (or their interaction) were significant.

RESULTS

DMSA and CaNa2EDTA mobilize lead from a solid lead fragment *in vitro*.

In order to investigate if metal chelating agents can mobilize lead from solid lead metal fragments, DMSA and $CaNa_2EDTA$ at estimated physiological (274 µM and 235 µM, respectively) and 10x-physiological concentrations (2740 µM and 2350 µM, respectively) in a physiological pH (range 6.91-7.08) artificial extracellular fluid (aECF) cell-free system containing a solid lead metal pellet were serially sampled over 72 hours. Appropriate control treatments (aECF with and without lead pellet) were also included. Results show that lead levels in the DMSA + lead pellet treatments were substantially increased in a time and DMSA concentration-related fashion, with levels in the estimated physiological DMSA treatment rising to ~1360 ng/mL by 24 hours, >450-fold above the aECF + pellet treatment (~3 ng/mL). Levels in the 10x-physiological DMSA treatment increased to even higher levels of ~10,100 ng/mL at 24 hours, a 7.5-fold increase above the estimated physiological DMSA concentration treatment (Figure 2).

$CaNa_2EDTA$ also increased lead levels in the aECF in a time and concentration-dependent manner, though lead levels were much lower than the corresponding DMSA treatments. Specifically, lead levels in the physiological and 10x-physiological $CaNa_2EDTA$ + lead pellet treatments were 55.2 ng/mL and 96.6 ng/mL at 24 hours, which were only ~4% and 1% of the corresponding DMSA treatments, respectively (Figure 2).

Leachate lead levels in the aECF + lead pellet treatment (no chelators) were also increased (to ~3 ng/mL) compared to the treatments without lead pellets at 24 hours (i.e., 0.20 ng/mL and 0.30 ng/mL for the aECF and aECF + chelator treatments, respectively). However, lead levels in the aECF + pellet treatment were only a small fraction (<10%) of the lead levels in solutions with the chelators (Figure 2). This modest release of lead from the pellet in aECF with no chelator *in vitro* was expected, however the substantially higher lead levels in the DMSA treatments compared to their $CaNa_2EDTA$ counterparts was somewhat unexpected and is discussed in the Discussion.

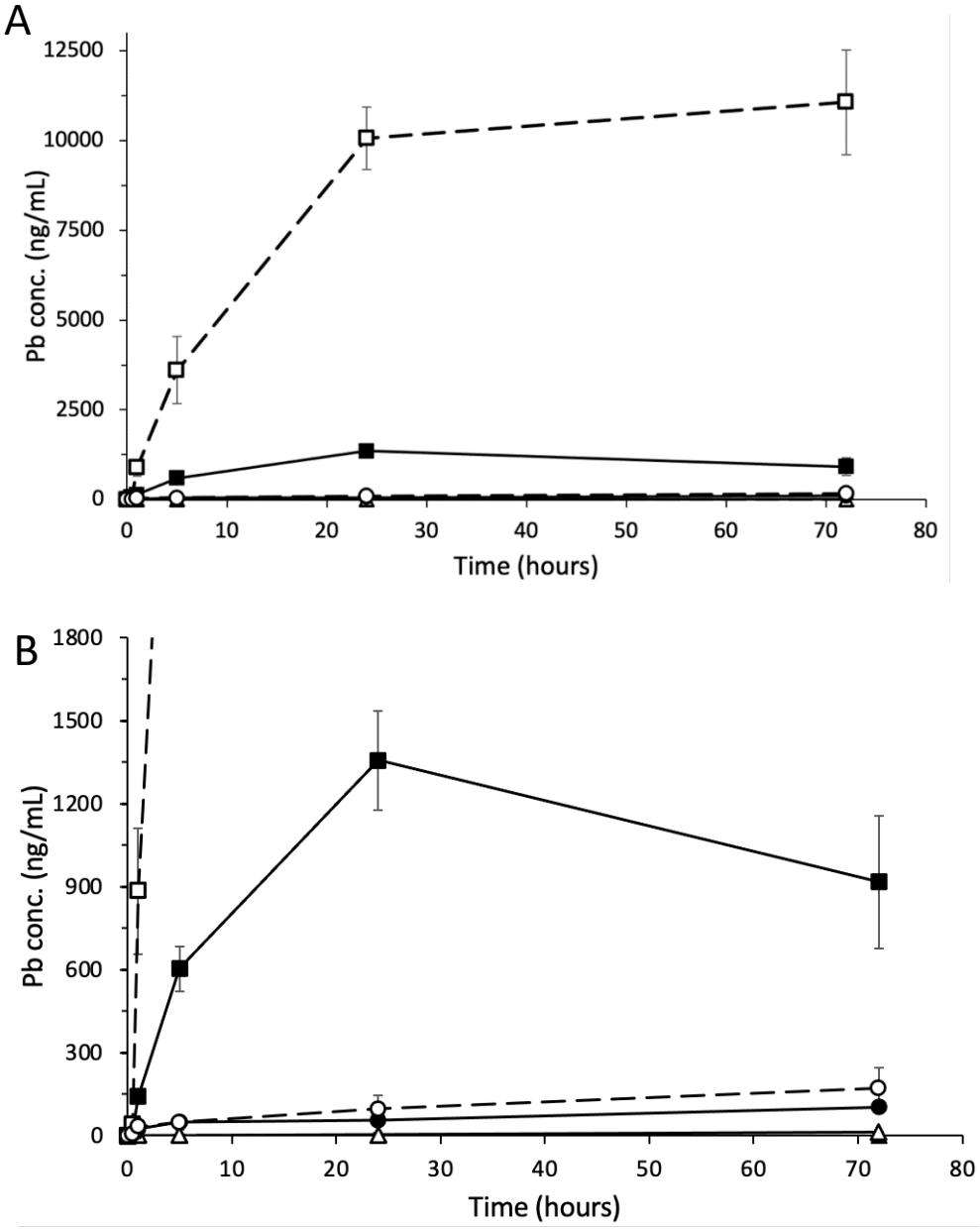


Figure 2. DMSA and $CaNa_2EDTA$ significantly increase mobilization of lead from a solid lead fragment in an aECF cell-free system. (A) Leachate lead concentrations (ng/mL) for each group as a function of time (hours). (B) Same data as in panel A, but with expanded y-axis to better show lower lead level groups. Aliquots were collected at 0, 0.5, 1, 5, 24, and 72 hours. Square and circle symbols represent DMSA and $CaNa_2EDTA$ groups, respectively. Solid lines indicate metal chelator concentration at estimated physiological concentrations for DMSA and $CaNa_2EDTA$ (274 µM and 235 µM, respectively). Dashed lines indicate metal chelator concentration at 10x-physiological concentrations (2740 µM for DMSA and 2350 µM for

$CaNa_2EDTA$). Open and closed triangles represent control groups with and without a lead pellet, respectively. Data are mean ± SD (n=3).

Overview of animal model with oral lead tracer, embedded lead pellets, chelation, and blood lead rebound.

In order to establish different stable lead isotopic signatures of elevated lead levels in tissues versus the embedded pellets so as to distinguish between lead chelated from tissues versus the pellet environment (i.e., pellet or surrounding extracellular fluid and tissue), rats were exposed to two sources of lead with distinct lead isotopic compositions; leaded drinking water enriched in ^{206}Pb ($^{207}Pb/^{206}Pb$=0.4756 ± 0.0002), and the embedded lead pellets ($^{207}Pb/^{206}Pb$=0.8708 ± 0.0002). Two lead pellets were embedded in each of the left and right gastrocnemius muscle (four pellets per rat total), based on results from a pilot study (Supplemental Figure 1). Prolonged exposure of the animals to the ^{206}Pb-enriched drinking water and the embedded lead pellets established a two-endmember lead exposure mixing model so that prior to chelation, animals had an inherent body lead burden in blood and tissues that was ~400-fold higher than background lead levels (i.e., blood lead levels of ~16 ng/mL vs 0.04 ng/mL background), with a lead isotopic composition ($^{207}Pb/^{206}Pb$=0.6047 ± 0.0081) that was intermediate between the oral ^{206}Pb-enriched drinking water and the embedded lead pellets (Supplemental Figure 2A, B).

Succimer chelation significantly reduced blood and tissue lead levels and increased urinary lead excretion.

In order to determine whether succimer chelation increased mobilization of lead from the embedded pellets, animals were treated orally with succimer (50 mg/kg body weight) or vehicle (apple juice) twice a day for 5 days. Exposure to the ^{206}Pb-enriched leaded drinking water stopped 24 hours prior to the first succimer/vehicle dose. Blood lead levels in the vehicle and succimer groups prior to the start of chelation and shortly after cessation of oral lead tracer exposure in drinking water were comparable at ~16 ng/mL (Figure 3A). Mixed model results show that for blood lead concentration, there was a significant main effect of time [$F(4,56) = 104$, $p < 0.0001$], no effect of treatment [$F(1,27) = 0.03$, $p=0.871$), but there was a highly significant interaction of time x treatment [$F(4,56) = 10.9$, $p<0.0001$]. After 1 day of chelation (i.e., two oral doses), blood lead levels in the succimer group were reduced significantly to 4.68 ng/mL ± 0.58 ng/mL (mean ± SE, n=8; $p<0.0001$ vs pre-chelation levels), followed by continued but more modest reductions in blood lead levels through 5 days of chelation (Figure 3A). By comparison, blood lead levels in the vehicle group also declined following the cessation of oral ^{206}Pb exposure, albeit more slowly and to a lesser extent than the succimer group. In the vehicle group, blood lead significantly decreased to 11.8 ng/mL ± 1.17 ng/mL after 1 day of vehicle treatment ($p=0.0024$ vs pre-chelation levels), and to 8.28 ng/mL ± 1.24 ng/mL after 5 days of vehicle ($p<0.0001$ vs pre-chelation levels)

(Figure 3A). As expected, blood lead levels of the succimer group were significantly lower than the vehicle group throughout chelation (p's <0.001 group contrasts within each day).

Pre-chelation urine lead levels were comparable between the two treatment groups at ~30 ng Pb/mg creatinine. Mixed model analyses showed a significant main effect of time [$F(3,38.5) = 6.86$, $p=0.0008$], no effect of treatment [($F(1,47.9)=0.02$, $p=0.880$), but a significant interaction of time x treatment [$F(3,38.5) = 7.27$, $p=0.0006$]. In the succimer group, urine lead levels significantly increased ~10-fold to 307 ng/mg creatinine (± 53.6) after the first day of chelation and remained elevated throughout the 5 days of chelation compared to pre-chelation levels (p's <0.01) (Figure 3A). In contrast, urine lead levels of vehicle treated animals did not change over time relative to pre-chelation levels (p's=1.00). As expected, urine lead levels in succimer-treated animals were significantly higher relative to vehicle-treated animals throughout the 5 days of chelation (p's<0.05).

Pre-chelation liver and kidney lead levels were determined in a subset of pre-chelation baseline animals sacrificed 1 day prior to the start of chelation and were determined to be 41.2 ng/g ± 5.94 ng/g and 710 ng/g ± 58.2 ng/g, respectively (mean ± SE, n = 8) (Figure 3B, C). After 5 days of succimer chelation, liver lead levels were significantly reduced to 15.6 ng/g (± 3.66 ng/g), a 62% reduction relative to pre-chelation lead levels (p=0.0011) (Figure 3B), while kidney lead levels were significantly reduced to 163 ng/g (± 17.2

ng/g), a 77% reduction relative to pre-chelation ($p<0.0001$) (Figure 3C). In the vehicle group, both liver and kidney lead levels also declined (liver to 29.1 ng/g ± 3.79 ng/g, $p=0.265$ versus pre-chelation; kidney to 502 ng/g ± 47.2 ng/g. $p=0.014$ vs pre-chelation levels (Figure 3B, C). Kidney lead levels were significantly lower in the succimer versus the vehicle group post-chelation ($p<0.0001$), while liver lead levels were trending lower in the succimer versus vehicle group (Figure 3B, C).

Notably, lead concentrations in muscle tissue surrounding the embedded pellets were several orders of magnitude higher than liver and kidney levels. Succimer group muscle lead levels were 56,800 ng/g ± 15,900 ng/g whereas vehicle group muscle lead levels were 135,000 ng/g ± 47,000 ng/g (mean ± SE, n=8). Post-chelation lead levels were not significantly different between treatment groups ($p=0.564$, Figure 3D). These findings show that post-chelation significant lead contamination of the tissue surrounding the pellet remains in both treatment groups.

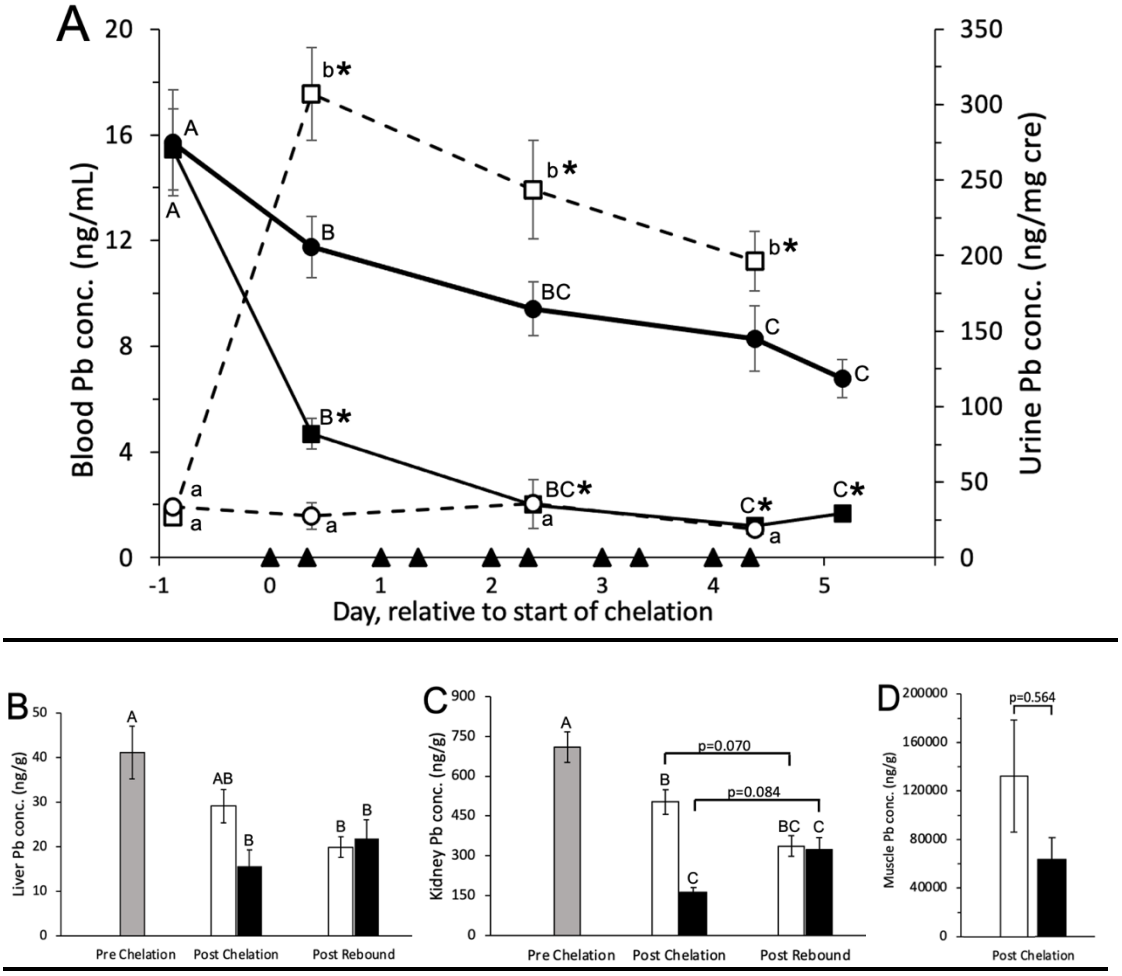


Figure 3. Succimer significantly reduced blood, liver, and kidney lead levels and significantly increased urinary lead excretion. A) Concentration of lead in blood (left y-axis) and urine (right y-axis) over chelation. Triangle symbols on x-axis represent the timing of each oral succimer or vehicle dose. Succimer and vehicle groups are represented by square and circle symbols, respectively. Solid lines represent blood lead concentrations and dashed lines represent urine lead concentrations. Data are mean ± SE (n=8/group for blood, n=5-8/group for urine). Symbols with different superscripts (uppercase for blood, lower case for urine) are statistically different within group ($p < 0.05$), based on Tukey's multiple comparisons test. Asterisks (*) reflect statistical differences between corresponding succimer vs vehicle groups within a time point ($p < 0.05$). B-C) Measured lead concentrations (ng/g dry weight) in the liver (B) and kidney (C) of animals pre-chelation (grey bar), 1 day after the final succimer dose (post-chelation, vehicle=open bars, succimer=black bars), and 3 weeks after the final succimer dose (post-rebound). Bars with different superscripts are statistically different ($p < .05$) based on Tukey's multiple comparisons test. D) Measured concentrations of lead (ng/g dry weight) in the

muscle tissue surrounding the embedded lead pellets of animals sacrificed 1 day after the final succimer dose (post-chelation). Data are mean ± SE (n=8/group). Statistical differences noted based on Wilcoxon test.

The initial stage of succimer chelation does not mobilize lead from embedded pellets, though lead with a pellet isotopic signature is mobilized into blood and urine with continued chelation.

$^{207}Pb/^{206}Pb$ ratios over chelation

To determine if lead from the embedded pellet environment is being mobilized during chelation, blood, urine, and tissue $^{207}Pb/^{206}Pb$ ratios were analyzed over the 5 days of chelation. Prior to the start of chelation, the blood $^{207}Pb/^{206}Pb$ ratios in the succimer and vehicle groups were 0.6253 ± 0.0152 and 0.6003 ± 0.0145, respectively (mean ± SE, n=8/group). Mixed model analyses revealed a significant main effect of time [$F(4,56)=132$, $p<0.0001$], no effect of treatment [$F(1,18.7)=1.48$, $p=0.239$], but a significant interaction of time x treatment [$F(4,56) = 29.0$, $p<0.0001$] (Figure 4A).

The blood $^{207}Pb/^{206}Pb$ isotopic signature in the succimer group changed bi-directionally during chelation, in contrast to the unidirectional change in blood lead isotopic signature of the vehicle group. After 1 day of chelation, the blood $^{207}Pb/^{206}Pb$ signature in the succimer group was significantly reduced to 0.5932 ± 0.0118, shifting away from the $^{207}Pb/^{206}Pb$ signature of the lead pellet to a value lower than the pre-chelation blood ($p=0.0133$ vs pre-chelation value). However, over the course of the remaining days of chelation, the blood $^{207}Pb/^{206}Pb$ signature increased towards the isotopic signature of the

embedded lead pellets, such that by the last day of chelation it ended significantly higher than pre-chelation at 0.6923 ± 0.0134 ($p<0.0001$). Moreover, on the day following the cessation of chelation, the blood $^{207}Pb/^{206}Pb$ signature further increased towards the isotopic signature of the embedded pellets to 0.7544 ± 0.0060, relative to the day prior ($p<0.0001$) (Figure 4A). In contrast, the vehicle group blood $^{207}Pb/^{206}Pb$ signature significantly and unidirectionally increased over chelation, following the cessation of elevated ^{206}Pb-enriched drinking water, and ended at 0.6891 ± 0.0161 after 5 days of vehicle treatment ($p<0.0001$ vs pre-chelation values) (Figure 4A).

Similar to blood, the urine isotopic signature in the succimer group also showed a bi-directional change over chelation, versus a unidirectional change in the vehicle group. There was a significant main effect of time [$F(3,33)=13.9$, $p<0.0001$], no effect of treatment [$F(1,22.7)=0.592$, $p=0.450$], but a significant interaction of time x treatment [$F(3,33) = 12.6$, $p<0.0001$] (Figure 4A). One day following the cessation of elevated ^{206}Pb-enriched drinking water and treatment with succimer, the urine $^{207}Pb/^{206}Pb$ signature in the succimer group significantly decreased from 0.6366 ± 0.0160 (pre-chelation) to 0.5490 ± 0.0100 ($p=0.0003$). Notably, urine $^{207}Pb/^{206}Pb$ ratios at this time point were significantly lower than the corresponding blood $^{207}Pb/^{206}Pb$ ratio ($p=0.014$). Following the first day of succimer treatment (i.e., chelation days 2 – 5), the urine $^{207}Pb/^{206}Pb$ signature increased with continued chelation, with lead

isotopic ratios that were not significantly different from blood (p's > 0.20) (Figure 4A). In comparison, the vehicle group urine $^{207}Pb/^{206}Pb$ ratios unidirectionally increased from pre-chelation (0.6178 ± 0.0154) and tracked closely the blood $^{207}Pb/^{206}Pb$ ratios over the 5 days of chelation, ending at 0.6768 ± 0.0207 (p=0.0017 vs pre-chelation values; Figure 4A). Overall, it is noteworthy that the $^{207}Pb/^{206}Pb$ ratios in the blood and urine of the vehicle group significantly increased towards the pellet isotopic signature during the first initial doses of vehicle treatment (i.e., a positive slope of change with time), then asymptoted out. In contrast, the succimer group blood and urine decreased in $^{207}Pb/^{206}Pb$ ratios over the initial stage (1 day) of chelation (i.e., a negative slope of change), towards the isotopic signature of the ^{206}Pb-enriched drinking water and away from the embedded pellets, and thereafter showed a positive slope of change towards the embedded pellet signature that appears to steepen with continued chelation.

One day following the last chelation dose the tissue (average of liver and kidney) $^{207}Pb/^{206}Pb$ isotopic signature was 0.6915 ± 0.0113 for the succimer group, which was significantly lower than the succimer group blood $^{207}Pb/^{206}Pb$ at the same time point (p=0.0005) (Figure 4A). In contrast, the vehicle group had an average tissue $^{207}Pb/^{206}Pb$ of 0.6756 ± 0.0164, which was not significantly different from the vehicle group post-chelation blood $^{207}Pb/^{206}Pb$ (p=0.451, Figure 4A).

In order to compare the measured post-chelation tissue lead isotopic signature with the (unmeasured) pre-chelation tissue values in the chelated animals, we estimated the chelated animals' pre-chelation tissue lead isotopic signatures using their measured blood $^{207}Pb/^{206}Pb$ ratios and the measured relationship between blood vs kidney and liver $^{207}Pb/^{206}Pb$ ratios in baseline animals (n=8) sacrificed 1 day prior to starting chelation (see Supp. Figure 3A). Based on this, the estimated pre-chelation tissue $^{207}Pb/^{206}Pb$ ratios in the chelated animals was 0.6127 ± 0.0139 and 0.5899 ± 0.0133 for the succimer and vehicle groups, respectively, which were not significantly different ($p=0.657$). However, post-chelation, the tissue $^{207}Pb/^{206}Pb$ ratios for the succimer and vehicle groups (0.6915 ± 0.0113 and 0.6756 ± 0.0164, respectively) were significantly higher than their estimated pre-chelation counterparts for both treatment groups ($p's<0.0001$) (Figure 4A). This is consistent with the higher measured $^{207}Pb/^{206}Pb$ ratios in liver and kidney post-chelation versus levels measured in the pre-chelation baseline animals (0.6068 ± 0.0174 and 0.6046 ± 0.0187 for liver and kidney, respectively (Figure 4B, C).

Following the cessation of ^{206}Pb-enriched leaded drinking water exposure and treatment with succimer or vehicle for 5 days, the liver and kidney $^{207}Pb/^{206}Pb$ ratios significantly increased towards the isotopic signature of the embedded lead pellets. Measured liver $^{207}Pb/^{206}Pb$ ratios in post-chelation succimer (0.6721 ± 0.0114) and vehicle (0.6803 ± 0.0164) animals

were not significantly different from one another (p=0.996), but they were significantly higher than their pre-chelation counterparts (p = 0.044 and p = 0.018, respectively) (Figure 4B). Similarly, kidney $^{207}Pb/^{206}Pb$ ratios in post-chelation animals (0.7109 ± 0.0118 and 0.6708 ± 0.0165 for succimer and vehicle groups, respectively) were higher than their pre-chelation counterparts (p's = 0.0007 and 0.060, respectively), but not different between treatment groups (p=0.451, Figure 4C). Finally, post-chelation muscle tissue surrounding the embedded pellets had $^{207}Pb/^{206}Pb$ ratios of 0.8697 (± 0.0007) and 0.8692 (± 0.0006) in the succimer and vehicle groups, respectively – values that were not significantly different from the embedded pellet $^{207}Pb/^{206}Pb$ ratio of 0.8708 ± 0.0002 (p's>0.10).

Amounts of embedded pellet lead in blood, urine, and tissues over chelation

In order to determine the relative amount of lead in blood, urine, and tissues over chelation that can be attributed to the embedded lead pellets, a simple two-endmember mixing model was used as described above. However, the slight differences between the succimer and vehicle groups in their starting pre-chelation blood and urine $^{207}Pb/^{206}Pb$ ratios, likely due to small differences in ^{206}Pb-enriched leaded drinking water consumption, slightly confounded direct comparison of their isotopic signatures and calculated percent pellet lead over chelation. To address this, the $^{207}Pb/^{206}Pb$ ratios for each animal

over chelation were normalized to an overall pre-chelation average $^{207}Pb/^{206}Pb$ ratio of both treatment groups (see Supp. Figure 4 for details). Normalized $^{207}Pb/^{206}Pb$ ratios were then used to calculate the relative percent pellet lead as described in the Methods.

The relative amount of embedded pellet lead in pre-chelation blood, urine, and tissue for the succimer and vehicle groups was ~35% (Figure 5). As with analyses of the $^{207}Pb/^{206}Pb$ ratios, mixed model analysis of the percent pellet lead in blood over chelation shows a significant main effect of time [$F(4,56)=134$, $p<0.0001$], no effect of treatment [$F(1,18.5)=0.000$, $p=1.00$], but a significant interaction of time x treatment [$F(4,56) = 28.1$, $p<0.0001$]. Following the cessation of ^{206}Pb tracer exposure and treatment with succimer, embedded pellet lead in the blood of the succimer group significantly decreased from 34.7% (± 3.78) pre-chelation to 26.8% (± 2.92) after 1 day of chelation ($p=0.015$; Figure 5). However, over the subsequent days 2 – 5 of chelation, the percent pellet lead in blood significantly increased, such that 1 day following the final succimer dose the blood contained 66.7% (± 1.48) pellet lead, a significant increase relative to pre-chelation levels ($p<0.0001$). In contrast, the vehicle group blood steadily increased over the entire 5 day course of vehicle treatment to 59.0% (± 4.58) 1 day following the last vehicle dose, also a significant increase relative to pre-chelation levels ($p<0.0001$) (Figure 5). One day post chelation, the percent pellet lead in blood of the succimer group was slightly higher than the vehicle group ($p=0.897$) and it is

noteworthy that from chelation day 1 to 1 day post-chelation, the percent pellet lead in blood of the succimer group increased >2-fold more than the vehicle group. Specifically, the percent pellet lead in blood of the succimer group increased ~40% (i.e., from ~27% on chelation day 1 to ~67% 1 day post-chelation), whereas in the vehicle group it only increased ~15% (i.e., ~44% on chelation day 1 to ~59% 1 day post-chelation). This reflects the bi-directional trend of the two stages of lead mobilization during chelation described above in the succimer group blood and urine $^{207}Pb/^{206}Pb$ signatures.

The urine percent pellet lead over chelation followed the same trends as blood in both treatment groups. There was a significant main effect of time [$F(3,31.8)=13.6$, $p<0.0001$], no effect of treatment [$F(1,21.3)=0.000$, $p=0.994$), but a significant interaction of time x treatment [$F(3,31.8) = 12.3$, $p<0.0001$]. In the succimer group, the pellet lead isotopic signature excreted in the urine after 1 day of chelation (16.5% ± 2.49), was significantly lower than pre-chelation urine levels ($p=0.0006$; Figure 5), and was also significantly lower than the percent pellet lead in blood at the same time point ($p=0.025$). On day 5 of chelation, the percent pellet lead in urine of the succimer group increased to 45.8% (± 3.11). In contrast, the percent pellet lead in urine of the vehicle group unidirectionally increased over chelation, ending with 53.5% (± 5.31) pellet lead following the final dose of chelation; this was significantly higher than pre-chelation levels ($p=0.002$; Figure 5).

Notably, at sacrifice 1 day after the final succimer/vehicle dose, the succimer group blood had a significantly higher amount of pellet lead isotopic signature than the corresponding tissue from those animals (51.4% ± 2.85, p=0.0006), while the vehicle group blood and tissue (53.9% ± 4.16) pellet lead isotopic signatures were not measurably different (p=0.428). Additionally, the measured post-chelation tissue for the succimer and vehicle groups increased significantly in percent pellet lead relative to the estimated pre-chelation tissue values (p's<0.0001; Figure 5). Finally, the percent pellet lead in the muscle surrounding the lead pellets were not different between treatment groups (p = 0.780), with an overall average of 99.7% (± 0.10) pellet lead (mean ± SE, n=16, Figure 5).

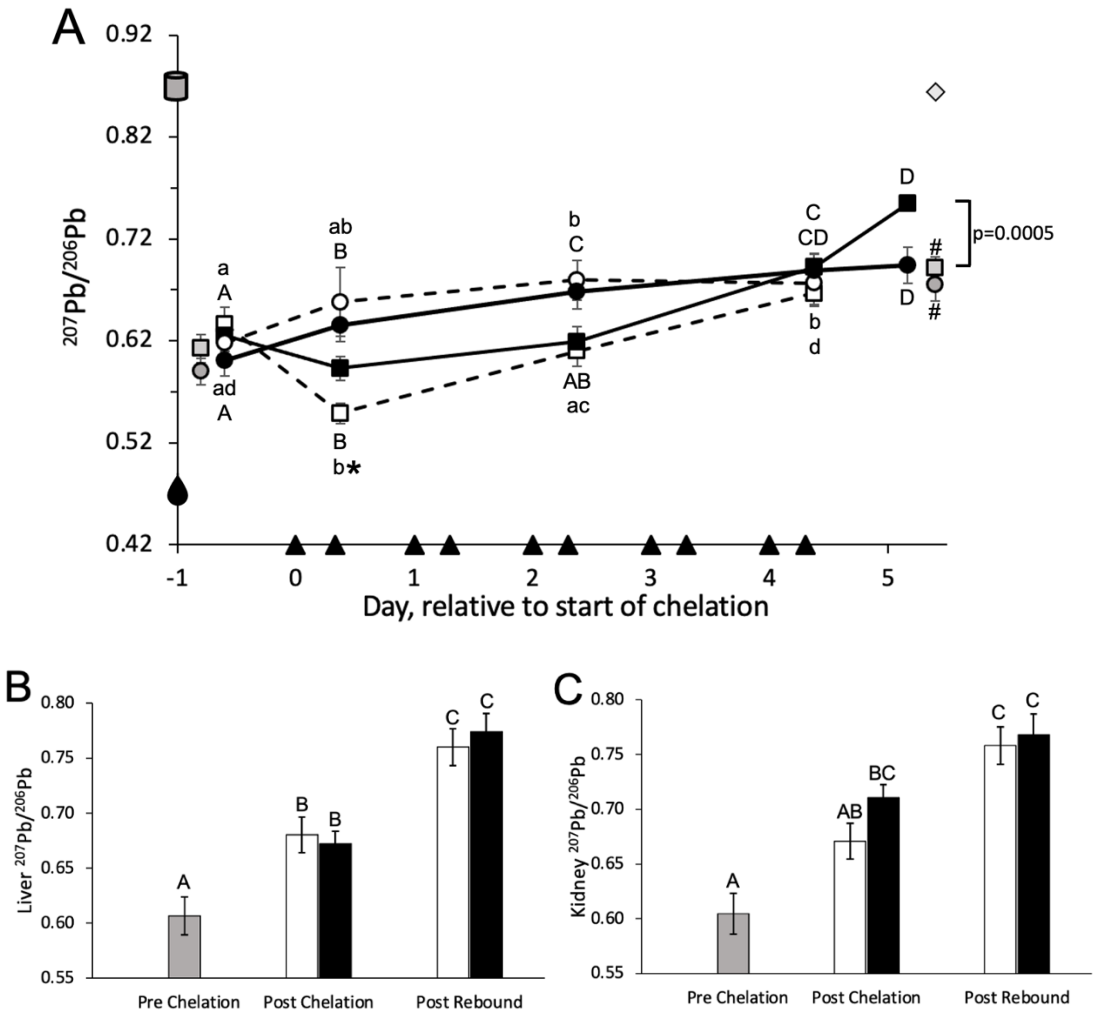


Figure 4. Post-chelation blood, urine, liver, and kidney $^{207}Pb/^{206}Pb$ ratio significantly increased relative to pre-chelation. A) $^{207}Pb/^{206}Pb$ ratios in the blood, urine, and tissue over chelation. Triangle symbols on x-axis represent timing of each oral succimer or vehicle dose. Cylinder and droplet symbol on the y-axis represent the $^{207}Pb/^{206}Pb$ ratio of the embedded lead pellets and ^{206}Pb-enriched drinking water, respectively. Succimer and vehicle groups are represented by square and circle symbols, respectively. Solid lines represent blood and dashed lines represent urine $^{207}Pb/^{206}Pb$ ratios. Grey symbols represent estimated pre-chelation and measured post-chelation tissue $^{207}Pb/^{206}Pb$ ratios. Diamond denotes $^{207}Pb/^{206}Pb$ ratio of muscle tissue surrounding the embedded lead pellets. Data are mean ± SE (n=8/group for blood, n=5-8/group for urine, n=16 for muscle). Symbols with different superscripts (uppercase for blood, lower case for urine) are statistically different within group ($p < 0.05$), based on Tukey's multiple comparisons test. Asterisks (*) reflect statistical differences between corresponding succimer vs vehicle groups within a time point ($p < 0.05$), B-C). Measured $^{207}Pb/^{206}Pb$

ratios in the liver (B) and kidney (C) of animals pre-chelation (grey bar), 1 day after the final succimer dose (post-chelation; black bars = succimer, white bars = vehicle), and 3 weeks after the final succimer dose (post-rebound). Data are mean ± SE (n=8/group). Bars with different superscripts are statistically different ($p < .05$) based on Tukey's multiple comparisons test.

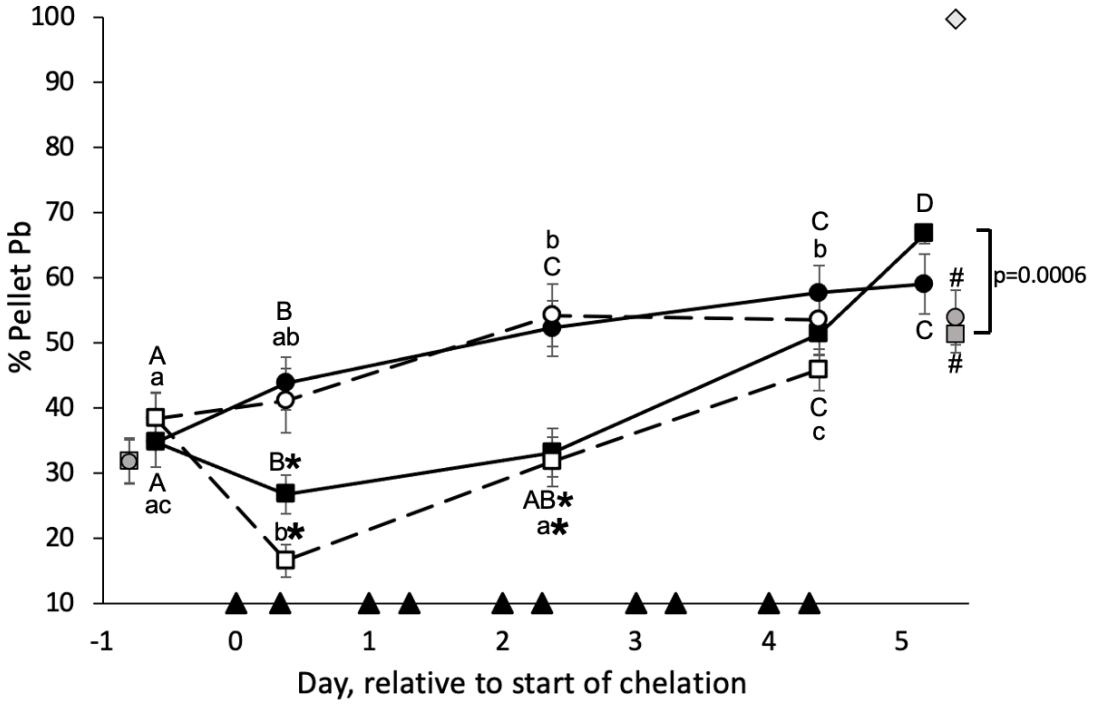


Figure 5. Percent pellet lead isotopic signature in blood and urine of succimer group bidirectionally decreases then increases over chelation, while vehicle group pellet signature unidirectionally changes. Percent pellet lead isotopic signatures in blood, urine, and tissues over chelation calculated using normalized $^{207}Pb/^{206}Pb$ ratios and a two-endmember mixing model (see text). Triangle symbols on x-axis represent the timing of each oral succimer or vehicle dose. Succimer and vehicle groups are represented by square and circle symbols, respectively. Solid lines represent blood and dashed lines represent urine. Grey symbols represent estimated pre-chelation and measured post-chelation tissue percent pellet lead. Diamond denotes percent pellet lead in muscle tissue surrounding the embedded lead pellets. Data are mean ± SE (n=8/group for blood, n=5-8/group for urine, n=16 for muscle). Symbols with different superscripts (uppercase for blood, lower case for urine) are statistically different within group ($p < 0.05$), based on Tukey's multiple comparisons test. Asterisks (*) reflect statistical differences between corresponding succimer vs vehicle groups within a time point ($p < 0.05$). Pound key (#) reflects statistical difference between pre- and post-chelation tissue within a treatment group ($p < 0.05$).

Blood lead, but not urine lead levels transiently increase over the post-chelation rebound period.

In order to determine if lead with an isotopic signature from the pellet environment is mobilized over the post-chelation rebound period, blood and urine samples from a subset of animals were collected prior to chelation, and weekly for 3 weeks following the end of chelation. Pre-chelation, blood lead concentrations in the succimer and vehicle groups were 17.6 ng/mL (± 2.24) and 16.7 ng/mL (± 1.36), respectively. Following chelation, blood lead concentrations in the succimer and vehicle groups were significantly different at 2.99 ng/mL (± 0.61) and 11.5 ng/mL (± 0.87), respectively ($p<0.0001$; Figure 6). Mixed model analyses showed there was a significant main effect of time [$F(3,41)=21.3$, $p<0.0001$] and treatment [$F(1,20.8)=68.9$, $p<0.0001$], and a significant time x treatment interaction [$F(3,41) = 67.8$, $p<0.0001$]. One week after the end of chelation (i.e., ~2 weeks after the start of the 5 day chelation regimen) the succimer group exhibited a rebound in blood lead concentrations, as evidenced by a significant increase in blood lead levels from 2.99 ng/mL (± 0.61) post-chelation to 7.23 ng/mL (± 0.77) 1 week later ($p<0.0001$, Figure 6). In contrast, blood lead levels in the vehicle group continued to decline from 11.5 ng/mL (± 0.87) post-chelation to 8.82 ng/mL (± 0.73) post-rebound ($p = 0.0002$). As a result of the blood lead rebound in the succimer group, within 1 week post-chelation lead levels between the succimer and vehicle groups were no longer significantly different ($p=0.77$, Figure 6). Similarly, urine lead

levels of both treatment groups decreased significantly in the first week of the post-chelation rebound period, from ~20 ng Pb/mg creatinine to ~2 ng Pb/mg creatinine (p's<0.0001), such that urine lead levels of the succimer and vehicle groups were no longer significantly different (p=0.945, Figure 6).

During the rebound period, liver lead levels in the succimer group non-significantly increased to 21.8 ng/g (± 4.30), whereas liver lead levels non-significantly decreased in the vehicle group to 19.9 ng/g (± 2.31), relative to post-chelation (p's>0.5). The succimer group kidney lead levels trended to increase to 324 ng/g (± 43.4), whereas the vehicle group trended to decrease to 337 ng/g (± 38.3), relative to post chelation (p's = 0.084 and 0.070, respectively). After the 3 week rebound period, liver and kidney lead levels were not significantly different between succimer and vehicle groups (p's>0.99; Figure 3B).

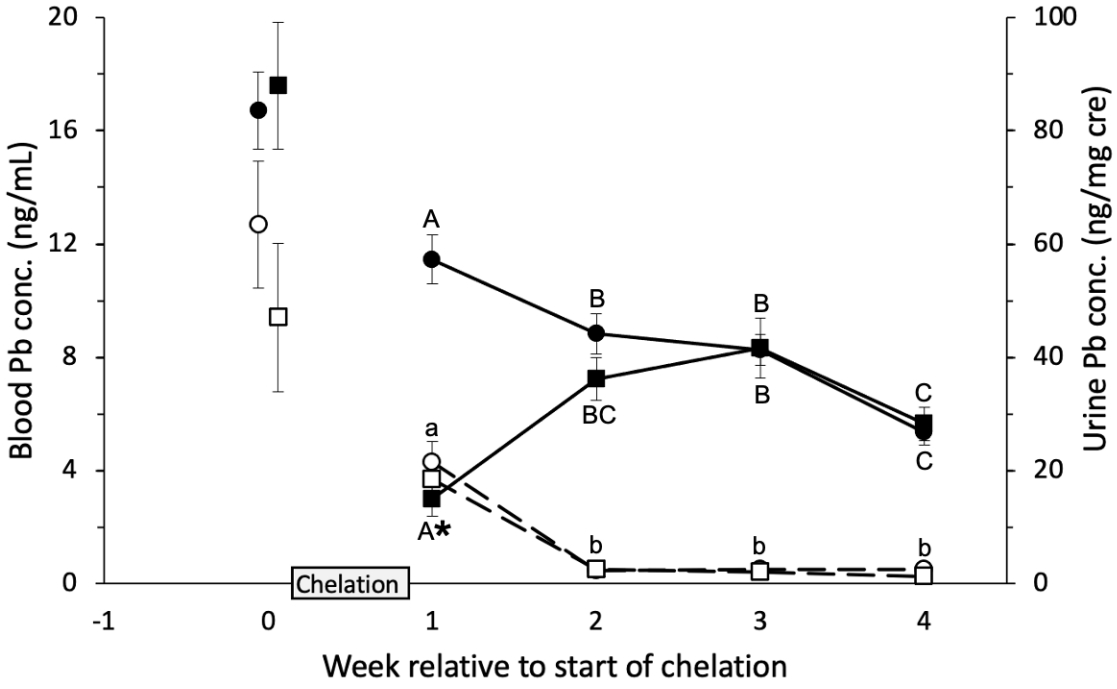


Figure 6. Blood and urine lead levels of succimer treated animals were not different from vehicle treated animals one week after the end of chelation. Concentration of lead in blood (left y-axis) and urine (right y axis) over time. Grey bar on x-axis represents 5 days of chelation with succimer or vehicle. Succimer and vehicle treated animals are represented by square and circle symbols, respectively. Solid lines represent blood lead concentrations and dashed lines represent urine lead concentrations. Data are mean ± SE (n=8/group and time point for blood, n=5-8/group for urine). Symbols with different superscripts (uppercase for blood, lower case for urine) are statistically different within group ($p < 0.05$), based on Tukey's multiple comparisons test. Asterisks (*) reflect statistical differences between corresponding succimer vs vehicle groups within a time point ($p < 0.05$).

Lead with a pellet isotopic signature increases in blood, urine, and tissues over the post-chelation rebound period.

For both treatment groups, blood and urine $^{207}Pb/^{206}Pb$ ratios increased over the 3 week post-chelation rebound period. Mixed model analysis of blood $^{207}Pb/^{206}Pb$ ratio shows a significant main effect of time [$F(3,40)=76.1$, $p<0.0001$], no effect of treatment [$F(1,14.8) = 1.25$, $p=0.282$], but a significant

interaction of time x treatment [$F(3,40) = 3.19$, $p=0.034$]. The greatest increase in blood and urine $^{207}Pb/^{206}Pb$ ratios occurred during the first week of the rebound period, with no measurable changes thereafter. In the succimer group, blood $^{207}Pb/^{206}Pb$ ratios increased towards the lead isotopic signature of the pellet, from 0.7332 (± 0.0155) post-chelation to 0.7622 (± 0.0181) 1 week later ($p<0.0001$, Figure 7). Similarly, in the vehicle group blood $^{207}Pb/^{206}Pb$ ratios also significantly increased from 0.7049 (± 0.0188) post-chelation to 0.7484 (± 0.0159) 1 week later ($p<0.0001$). Urine $^{207}Pb/^{206}Pb$ ratios followed similar trends as their respective blood samples for both groups. Mixed model analysis of urine $^{207}Pb/^{206}Pb$ ratio shows a significant main effect of time [$F(3,32.3)=29.2$, $p<0.0001$], no effect of treatment [$F(1,16.1) = 0.710$, $p=0.412$], but a significant interaction of time x treatment [$F(3,32.3) = 3.10$, $p=0.041$]. Specifically, the succimer group urine $^{207}Pb/^{206}Pb$ ratios increased from 0.7283 (± 0.0180) post-chelation to 0.7456 (± 0.0203) 1 week later. In the vehicle group, urine $^{207}Pb/^{206}Pb$ ratios also increased significantly from 0.7000 (± 0.0179) post-chelation to 0.7599 (± 0.0101) one week later ($p < 0.0001$). Over the subsequent 2 weeks of the post-chelation rebound period the succimer and vehicle blood and urine $^{207}Pb/^{206}Pb$ ratios were not significantly different (p's>0.95)

At the time of sacrifice following the 3 week rebound period, liver $^{207}Pb/^{206}Pb$ ratios in the succimer and vehicle groups significantly increased to 0.7741 (± 0.0164) and 0.7601 (± 0.0167), respectively, relative to their post-

chelation counterparts (p's = 0.0005 and 0.0087, respectively), but were not different between groups (p = 0.970, Figure 4B). Similarly, kidney $^{207}Pb/^{206}Pb$ non-significantly increased to 0.7682 ± 0.0186 in the succimer group (p=0.133), but significantly increased to 0.7582 ± 0.0172 in the vehicle group (p=0.006) relative to post-chelation, and were not different between groups (p = 0.993; Figure 4C). Mixed model analysis of the tissue $^{207}Pb/^{206}Pb$ ratios (average of liver and kidney) shows a significant main effect of time [$F(1,14)=667$, $p<0.0001$], no effect of treatment [$F(1,14.5) = 0.646$, $p=0.434$], but a significant effect of time x treatment [$F(1,14)=21.3$, $p=0.0004$]. Estimated post-chelation tissue $^{207}Pb/^{206}Pb$ ratios for the succimer and vehicle groups was 0.6651 (± 0.0194) and 0.6853 (± 0.172), respectively, and following the 3 week rebound period average tissue $^{207}Pb/^{206}Pb$ ratios significantly increased in the succimer and vehicle group to 0.7712 (± 0.0174) and 0.7592 (± 0.0168), respectively (p's <0.0001, mean ± SE, n=8/group) (Figure 7).

The relative (%) amount of lead in the blood, urine, and tissue over the rebound period that can be attributed to embedded lead pellets was calculated using the normalized $^{207}Pb/^{206}Pb$ ratios for each animal, as noted above and described in the Methods (see Supplement Figure 5 for details). As with analyses of the $^{207}Pb/^{206}Pb$ ratios, mixed model analysis of the percent pellet lead in blood over the post-chelation rebound period shows a significant main effect of time [$F(3,40)=75.3$, $p<0.0001$], no effect of treatment [$F(1,14.8) = 1.96$, $p=0.182$], but a significant interaction of time x treatment [$F(3,40) = 3.11$,

p=0.037]. One week after the cessation of chelation therapy (i.e., ~2 weeks after the start of chelation), embedded pellet lead in the blood increased in both treatment groups relative to post-chelation. One day after the end of chelation, blood lead of the succimer group contained 66.4% (± 3.96) pellet lead and significantly increased to 73.8% (± 4.61) after 1 week ($p<0.0001$; Figure 8). A similar significant increase in embedded pellet lead in the blood was observed in the vehicle group, increasing from 57.3% (± 4.78) post-chelation to 68.4% (± 4.11) 1 week later ($p<0.0001$).

Mixed model analysis of embedded pellet lead in urine shows a significant main effect of time [$F(3,32.3)=29.9$, $p<0.0001$], no effect of treatment [$F(1,15.9) = 1.04$, $p=0.323$], but a significant interaction of time x treatment [$F(3,32.3) = 3.12$, $p=0.040$]. In the succimer group, urine pellet lead increased from 65.8% (± 5.01) post-chelation to 70.3% (± 4.78) 1 week later ($p=0.226$). Similarly in the vehicle group, urine increased from 57.0% ± (4.02) pellet lead post-chelation to 72.6% ± (4.45) pellet lead 1 week later ($p<0.0001$).

Additionally, the relative amount (%) of pellet lead in the averaged tissue increased relative to the estimated post-chelation values for both treatment groups (Figure 8). In the succimer group, post-chelation tissue contained 47.7% (± 5.10) pellet lead and significantly increased to 74.7% ± 4.69% pellet lead by the end of the 3 week rebound period ($p<0.0001$), which was 4 weeks after the start of chelation. Similarly, in the vehicle group, the post-rebound tissue had a greater percentage of pellet lead relative to estimated post-

chelation tissue, increasing from 55.3% (± 4.61) to 74.5% (± 4.59) pellet lead (p<0.0001).

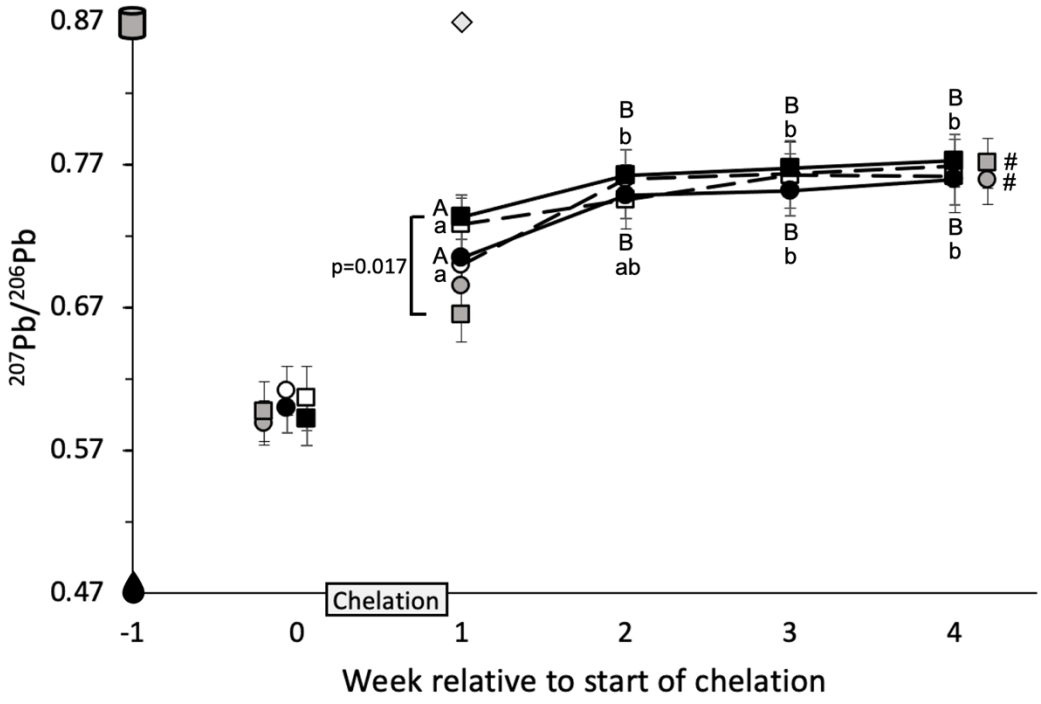


Figure 7. Blood, urine, and tissue $^{207}Pb/^{206}Pb$ ratios increase, towards the lead isotopic composition of the lead pellet, over the course of the rebound period. Measured $^{207}Pb/^{206}Pb$ ratios in the blood and urine over the rebound period. Grey bar on x-axis represents 5 days of chelation with succimer or vehicle. Succimer and vehicle groups are represented by square and circle symbols, respectively. Solid lines represent blood $^{207}Pb/^{206}Pb$ rations and dashed lines represent urine $^{207}Pb/^{206}Pb$. ratios. Grey symbols represent estimated pre-chelation and post-chelation tissue $^{207}Pb/^{206}Pb$ ratios and measured post-rebound $^{207}Pb/^{206}Pb$ ratios. Diamond denotes $^{207}Pb/^{206}Pb$ ratios of muscle tissue surrounding the embedded lead pellets. Data are mean ± SE (n=8/group for blood, n=5-8/group for urine). Symbols with different superscripts (uppercase for blood, lower case for urine) are statistically different within group ($p < 0.05$), based on Tukey's multiple comparisons test. Asterisks (*) reflect statistical differences between corresponding succimer vs vehicle groups within a time point ($p < 0.05$). Pound key (#) reflects statistical difference between post-chelation and post-rebound tissue within a treatment group ($p < 0.05$).

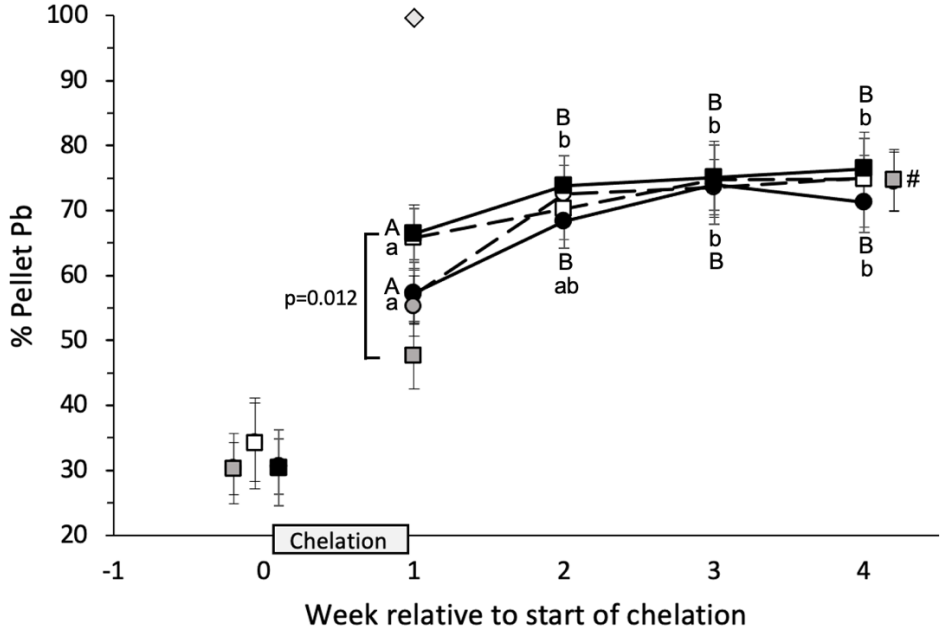


Figure 8. Relative amount of pellet lead in the blood, urine, and tissue, significantly increased during the rebound period in both treatment groups. Percent pellet lead isotopic signatures in blood, urine, and tissues over the rebound period calculated using normalized $^{207}Pb/^{206}Pb$ ratios and a two-endmember mixing model (see text). Grey bar on x-axis represents 5 days of chelation with succimer or vehicle. Succimer and vehicle groups are represented by square and circle symbols, respectively. Solid lines represent blood and dashed lines represent urine. Grey symbols represent estimated pre-chelation and post-chelation tissue and measured post-rebound. Symbols with different superscripts (uppercase for blood, lower case for urine) are statistically different within group ($p < 0.05$), based on Tukey's multiple comparisons test. Asterisks (*) reflect statistical differences between corresponding succimer vs vehicle groups within a time point ($p < 0.05$). Pound key (#) reflects statistical difference between post-chelation and post-rebound tissue within a treatment group ($p < 0.05$).

DISCUSSION:

Risk of lead poisoning from retained lead bullet fragments in gunshot victims is a significant, though insufficiently recognized and understood public health threat (Gaitens et al., 2020; Weiss, Tomasallo, et al., 2017). In the U.S. it is estimated that 42,000 veterans have retained metal fragments from bullet or explosive debris injuries suffered during combat (Gaitens et al., 2017). Additionally, approximately 67% of the 100,000 annual firearm injuries in the U.S. are non-fatal, suggesting that the past decade has produced over half a million victims with retained lead metal fragments (Fowler et al., 2015). While there are well-established management guidelines for treating lead poisoning from environmental exposure sources, including chelation therapy, there are no standard practices for managing lead poisoning arising from embedded lead metal fragments (Gaitens et al., 2020; Kane et al., 2009; Nickel et al., 2018). In particular, it remains unclear whether chelation is an effective treatment for lead poisoning arising from retained lead fragments, and whether chelation removes lead from retained fragment(s) with possible remobilization to other tissues (Gaitens et al., 2020). Our findings show that in a cell-free aECF system, DMSA significantly increased the release of lead from solid lead fragments in a time and DMSA-concentration dependent manner ($CaNa_2EDTA$ did as well, but to a much lesser extent). In our animal model, during the first stage of chelation (1 day), succimer mobilized lead with an isotopic signature towards the ^{206}Pb-enriched drinking water (i.e., non-pellet

lead) into the blood and urine, however during a second stage of chelation (beyond day 1), the amount of embedded lead pellet signature in the blood and urine increased, steepening with each day of continued chelation. Following the end of chelation, blood lead levels in the succimer group rebounded, such that they were no longer different from the vehicle-treated group. Overall, these findings suggest that succimer chelation may not be efficacious for reducing longer-term risk of lead poisoning from embedded lead fragments.

DMSA and $CaNa_2EDTA$ mobilize lead from a solid fragment *in vitro*

Our findings clearly show that DMSA (and to a lesser extent $CaNa_2EDTA$) substantially increased the release of lead from a solid fragment in a concentration and time-dependent manner in a cell-free system at physiological pH (Figure 2). This suggests that succimer chelation in patients with embedded lead fragments may increase the release of lead from the embedded fragments into the body, potentially increasing the remobilization of lead to other lead-sensitive tissues. The much greater ability of DMSA versus $CaNa_2EDTA$ to mobilize lead from the fragments *in vitro* was unexpected, but may be explained by the different ionization chemistries of the functional groups participating in the chelator-Pb coordination bonds in DMSA (-SH and $–COO^-$) versus $CaNa_2EDTA$ ($–COO^-$ and NH_3) (Zhang, Qin, Deng, & Wells, 2017). In the pH neutral aECF in this study, the DMSA thiol groups (pK_a =

9.32) are nearly 100% protonated, whereas the carboxyl groups (pK_a = 3.37) are nearly 100% deprotonated. Given DMSA's high binding affinity for lead (Ka = 1 x $10^{17.4}$ M^{-1}), the reduced thiols of DMSA would readily give up their protons upon coordinating with lead in the solid metal (Harris, Chen, Stenback, & Shah, 1991). This may decrease the pH in the aECF solution at the fragment-solution interface, and thereby accelerate solubilizing lead from the fragment into the aECF solution. In contrast, $CaNa_2EDTA$ carboxylic acid functional groups (pK_a of 3.37) are already deprotonated at the neutral pH of the aECF, so that coordination with lead would not lead to functional group deprotonation or lowering of the pH at the solution-lead fragment interface.

While our findings clearly show that succimer can increase release of lead from solid lead fragments in aECF, it is not clear whether similar processes may occur *in vivo*. Previous studies in humans have shown that succimer is readily metabolized *in vivo*, with the majority (>85%) of an oral succimer dose excreted in urine as mixed disulfide complexes with biomolecules, with relatively little succimer existing as the parent compound (Dart & Hurlbut, 1991; Rivera, Zheng, Aposhian, & Fernando, 1989), suggesting that parent reduced succimer may not be contributing significantly to the chelation of lead *in vivo*.

Stable lead isotopic approaches to distinguish sources of lead exposure

In our rodent model, drinking water enriched in stable ^{206}Pb was used to isotopically label elevated lead in blood and tissues prior to the implantation of the lead pellets, in order to establish elevated tissue lead levels with an isotopic lead signature distinguishable from lead in the embedded pellets. Prior to onset of chelation, animals had blood lead isotopic compositions that were intermediate between the two sources of lead exposure, i.e., intermediate between the leaded drinking water and embedded lead pellets (Supp. Figure 2B). As a result, we were able to determine the relative percentage of lead from each exposure source in blood, urine, and tissue samples prior to and over the course of succimer chelation, and the ensuing post-chelation rebound period.

Similar stable lead isotope tracer approaches have been used in other studies to demonstrate that embedded fragments may constitute significant endogenous sources of lead exposure, and to distinguish embedded fragments from exogenous (outside the body) exposure sources. In one human case study of elevated blood lead levels from embedded fragments in the soft tissue, stable lead isotope methodologies were used to differentiate environmental versus embedded fragment sources of lead exposure in order to establish a direct association between blood lead isotopic composition and fragment lead isotopic composition (Weiss, Lee, et al., 2017). In a canine model of intra-articular versus embedded lead fragments, implantation of ^{208}Pb

or ^{206}Pb isotopically-enriched lead fragments into the knee joint or muscle were used to show that intra-articular lead fragments have a greater effect to elevate blood lead levels than extra-articular fragments (Manton & Thal, 1986).

Lead from the embedded pellet environment is mobilized with continued chelation

Our results show that, as expected, oral succimer chelation significantly reduced blood and tissue lead levels, and increased urinary lead excretion, consistent with past studies investigating the efficacy of succimer chelation therapy to reduce body lead burdens (Bradberry, Sheehan, & Vale, 2009; Friedheim, Graziano, Popovac, Dragovic, & Kaul, 1978; Graziano et al., 1992; Rogan et al., 2001; D. R. Smith et al., 2000; D. Smith & Strupp, 2013). Previous studies in primates have shown chelation therapy to be effective in reducing blood and tissue lead levels, but chelation was only somewhat more effective than removal from the source of lead exposure alone (D. R. Smith et al., 2000), similar to what was shown in the present study. In humans, succimer chelation significantly reduced blood lead concentrations and significantly increased urine lead excretion in adults with blood lead levels >50 µg/dL (Bradberry et al., 2009). In our study, liver and kidney lead levels were also significantly reduced (Figure 3B/C), consistent with other studies in primates (D. R. Smith et al., 2000).

Importantly, our findings also show that chelation and urinary elimination of lead occurred in two apparent ‘stages’ over the 5-day chelation period in this study. In the first stage (1 day), succimer did not appear to mobilize lead from the pellet environment into blood and urine, but did mobilize lead from the pellet environment during the second, more prolonged stage of chelation (beyond day 1), relative to the vehicle group. More specifically, during the first apparent stage of chelation, succimer readily increases mobilization of body lead into blood and urine towards the isotopic signature of the ^{206}Pb-enriched drinking water, and away from the lead pellet signature, while at the same time producing a significant reduction in blood lead levels and an increase in urinary lead elimination (Figures 3A, 4A, 5). Further, the percent pellet lead in the blood and urine of the succimer group decreased to levels below the estimated pre-chelation tissue percent pellet lead, suggesting that lead in a sub-compartment of the tissue that contained relatively more ^{206}Pb-enriched drinking water lead (and less pellet lead), was more readily chelated by succimer than other lead sub-compartments (see Figure 9). This suggestion that succimer may target more labile sub-compartments of lead in the initial stage of treatment is also supported by the significantly lower percent pellet lead isotopic signature in urine relative to blood, knowing that urine lead reflects soluble chelated lead, whereas whole blood lead contains both soluble chelated lead in plasma and a majority of lead complexed within the cellular fraction of blood, particularly erythrocytes.

In contrast, during an apparent second stage of chelation (i.e., beyond day 1, and a period where the isotopic signature of the vehicle group asymptotes out), the succimer group blood and urine isotopic signature begins to increase towards the isotopic signature of the embedded pellets, with a steepening of the rate of increase with continued chelation. One explanation for this is that after the first stage of chelation, the readily accessible lead in sub-compartments of extracellular fluid, blood, and tissue may have become relatively depleted, whereas the lead levels in the pellet environment remained extremely high (~96,000 ng Pb/g post-chelation) with an isotopic signature ($^{207}Pb/^{206}Pb$=0.8694) measurably indistinguishable from the embedded pellets ($^{207}Pb/^{206}Pb$=0.8708). Thus, lead from compartments of higher concentrations, such as lead in the pellet environment, may be mobilized to compartments of lower concentrations, such as the continuously chelated extracellular fluid and blood compartment in an effort to re-establish equilibrium (see Figure 9). This, taken together with the sustained significant elevation in urinary lead excretion over chelation suggests increased mobilization of lead from the pellet environment with continued chelation.

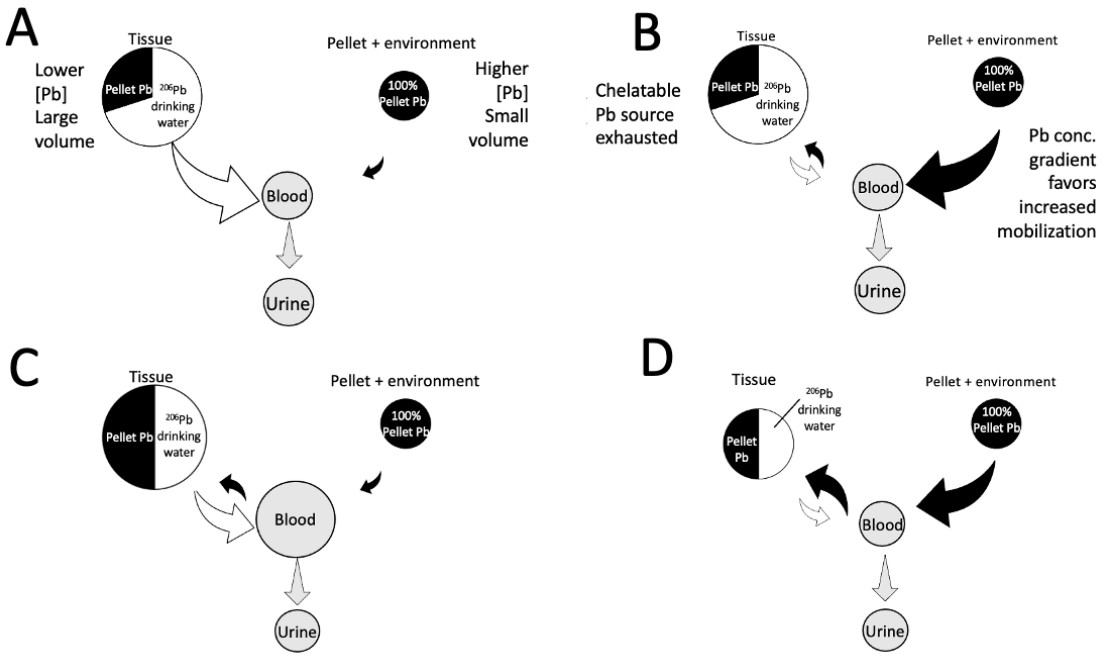


Figure 9. Proposed explanation of lead mobilization during chelation and during the post-chelation rebound. (A) Lead mobilization during the initial stage (1 day) of succimer chelation. The pellet environment is extremely high in lead, concentrated in a spatially small volume of the body. Although the lead in the rest of the body tissue has a relatively lower concentration, it occupies a greater volume of the body. Thus, the lead in the tissue may be a more easily mobilizable source of lead that succimer readily has access to during the initial doses of chelation. (B) Lead mobilization during the second stage of chelation (beyond day 1) of continued succimer chelation. After the initial doses of chelation, the easily mobilizable source of lead in the tissue may have been exhausted. After the initial doses, blood lead levels significantly declined to establish a steeper concentration gradient. This steeper concentration gradient and exhaustion of easily mobilizable lead may mobilize lead from the high lead pellet environment to the blood compartment of lower lead level. (C) Lead mobilization in the vehicle group during the post-chelation "rebound" period. After removal from one source of lead exposure (^{206}Pb drinking water), blood and tissue lead levels continue to decrease over the post-chelation rebound period. Blood and tissue lead levels in the vehicle group were higher than the succimer group and did not produce as steep of a concentration gradient. Although percent pellet lead was not different between groups, because tissue lead levels continued to decrease, this suggests there was a net movement of lead out of the tissue. (D) Lead mobilization in the succimer group during the post-chelation "rebound" period. Treatment with succimer disrupted body lead equilibrium by significantly reducing blood and tissue lead levels. This may produce a steep concentration gradient that may mobilize lead from the pellet

or its high concentration environment in order to re-establish this body lead equilibrium. Although percent pellet lead was not different between groups, because tissue lead levels increased, this suggests there was a net movement of pellet lead into the tissue.

Succimer did not produce lasting reductions in blood or tissue lead levels following chelation

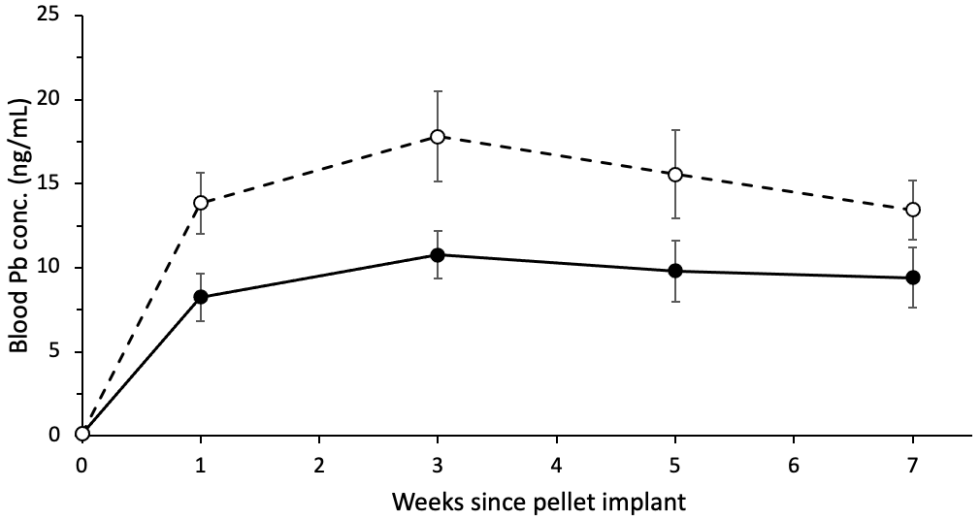
In this study, in which the embedded pellet source of lead exposure remained throughout the study, blood and to a lesser extent tissue lead levels decreased with chelation, but then increased post-chelation, such that there was no significant difference in lead levels between the succimer and vehicle groups by 1 week post-chelation (Figure 6). This indicates that there was a continued net movement of lead out of the tissues and into blood in the vehicle group, but a net movement of lead into the blood and tissues of the succimer group (i.e., rebound) (Figures 6, 7, 8). Further, over this post-chelation period the tissue lead isotopic signatures increased towards the isotopic signature of the embedded pellets in both treatment groups. These findings suggest succimer chelation did not produce prolonged (>1 week) reductions in tissue lead levels because of increased remobilization of lead from the pellet environment into the blood and subsequently into the tissues during the rebound period. Prior clinical and primate studies have shown that subjects removed from their exogenous sources of lead exposure and then chelated

with succimer, readily available lead in the plasma and extracellular fluid excreted via the urine and feces, resulting in lower lead concentrations in these compartments, which in turn favors re-equilibration of lead from the tissue into the plasma and extracellular fluid compartments during the rebound period (Rogan et al., 2001; D. R. Smith et al., 2000).

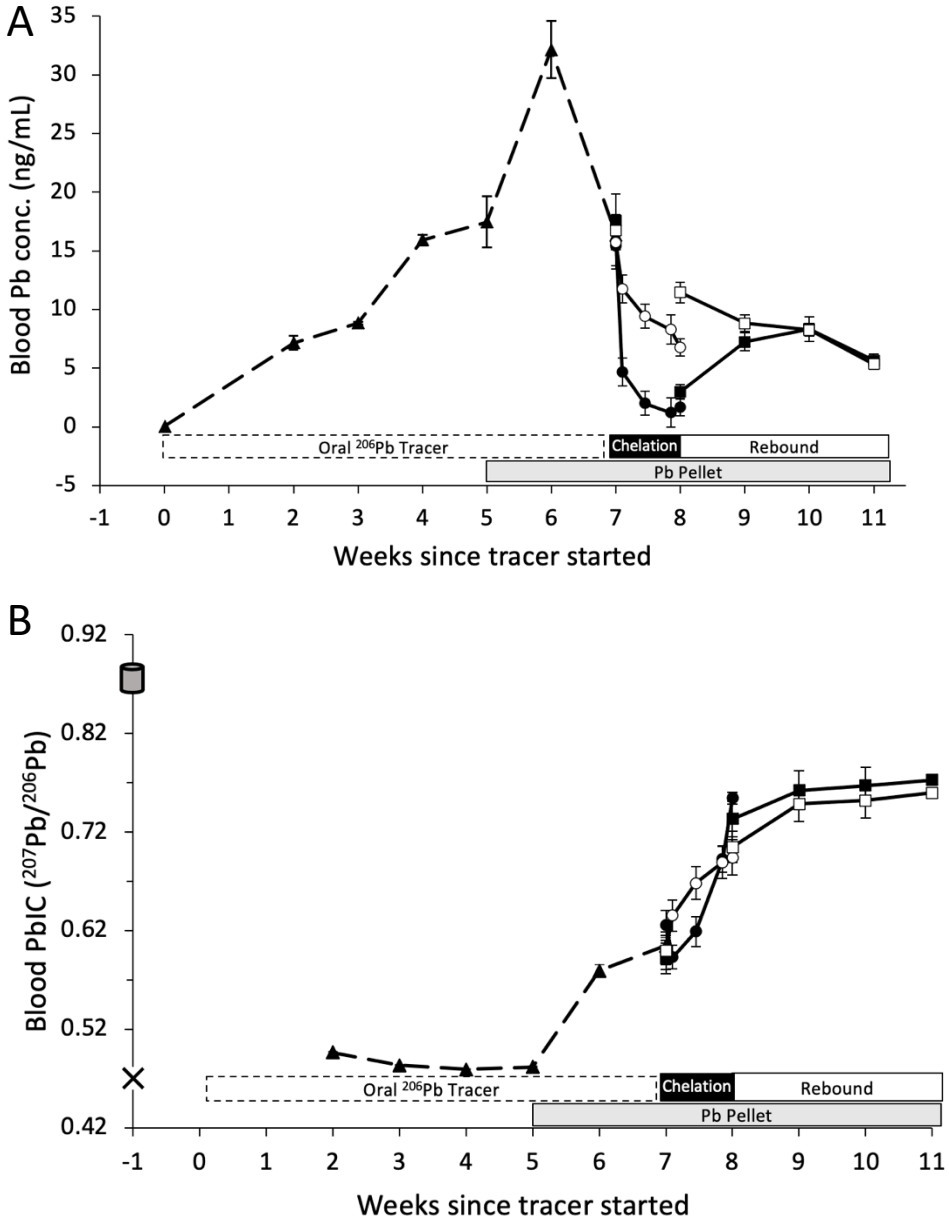
More generally, concern remains that embedded metal fragments pose a risk of leaching toxic metals into the body and producing detrimental impacts on human health (Gaitens et al., 2017, 2020). As a result, it has been recommended that patients with embedded metal fragments should be bio-monitored for elevated metal concentrations in blood and urine to allow for early intervention if elevated levels do occur (Gaitens et al., 2020). Biomonitoring of 14 different metals in a group of war-injured Iraq and Afghanistan veterans found that 44.7% of veterans with retained fragments had one or more elevated metal concentrations in their urine (Gaitens et al., 2017). In humans and rodents, tissue metal distributions and health outcomes from embedded fragments containing depleted uranium, tungsten, nickel, and/or cobalt have been reported (Kalinich et al., 2017; Kalinich, Vergara, & Emond, 2008; Leggett & Pellmar, 2003; Pellmar, 1999; Squibb & McDiarmid, 2006). While toxicities from other common metals found in embedded fragments are still being defined, lead toxicity is well characterized and treatment options for elevated lead levels from embedded lead fragments should be explored.

In conclusion, our results suggest that lead from embedded pellets (or their immediate environment) was mobilized during and after treatment with succimer in a rodent model. Prior to this study, short term chelation was cautiously recommended (Gaitens et al., 2020). However in light of our findings, treatment with succimer chelation in patients with embedded lead fragments may not be efficacious because it may not produce lasting reductions in blood or tissue lead levels. It remains a risk that succimer chelation may remobilize lead from embedded fragments or the fragment environment in the course of re-establishing body lead equilibrium post-chelation. The blood and tissue lead level rebound post-chelation, seemingly from the remobilization of embedded pellet lead, may provide some explanation of the diverse outcomes in human case studies of chelation in patients with embedded lead fragments, including the inability of chelation to sustainably reduce blood lead levels or the emergence of health effects from lead poisoning (Cristante, De Souza, Barros Filho, Oliveira, & Marcon, 2010; Cyrus et al., 2011; de Madureira et al., 2009; Grasso et al., 2017; Stromberg, 1990). Thus, the risks of chelation therapy in patients with embedded lead fragments may outweigh the potential benefits (Gaitens et al., 2020).

Supplemental Figures:

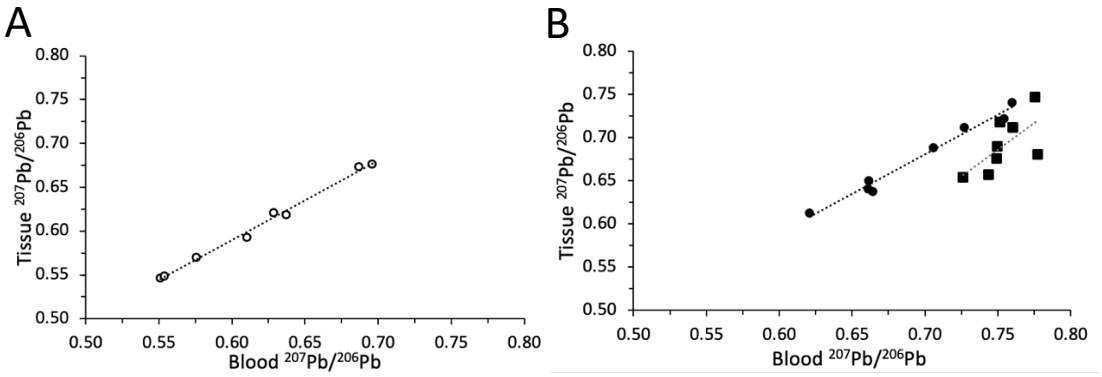


Supplemental Figure 1: Lead pellet implantation increased blood lead levels in dose-dependent manner. Rats received either a moderate (n=4) or high (n=8) lead pellets, divided evenly between the left and right gastrocnemius muscles in alternating directions. Blood lead levels were monitored over 7 weeks. Black line with filled symbols represents the moderate pellet load group and dashed line with open symbols represents the high pellet load group. A moderate pellet load was chosen because the blood lead increase that was comparable to the blood lead levels produced by ^{206}Pb-enriched leaded drinking water at 5 ppm. Data are mean ± SE (n=5/group).

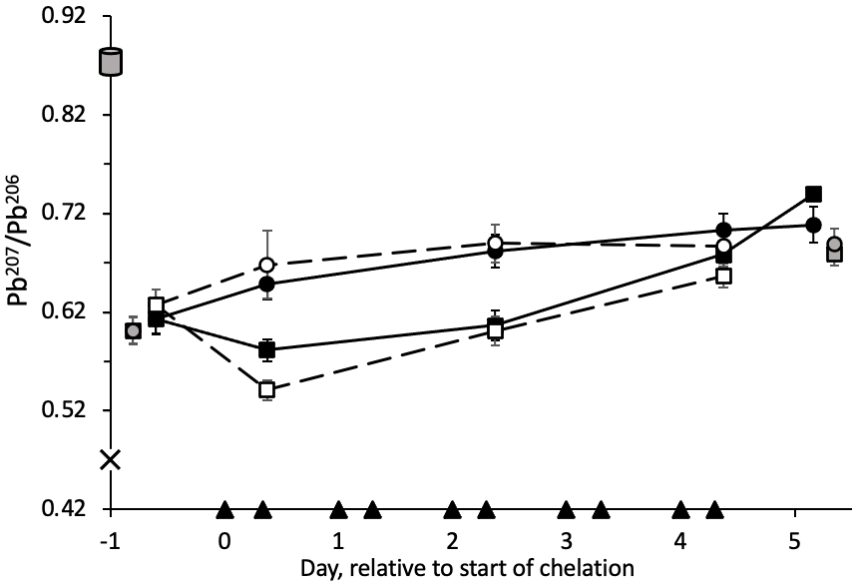


Supplemental Figure 2: Overview of blood lead concentrations and $^{207}Pb/^{206}Pb$ isotopic signature in blood over the course of oral tracer exposure, lead pellet implantation, chelation, and post-chelation rebound. Blood lead concentrations (A) and lead isotopic signatures (B) over the course of the study. Elevated ^{206}Pb-enriched drinking water was about 5 ppm (range 4.08 ppm-7.43 ppm) and $^{207}Pb/^{206}Pb$ was 0.4756 ± 0.0002 (Mean ± SD, n=3) as is represented on the y-axis as a bold "X". Embedded lead pellets $^{207}Pb/^{206}Pb$ ratio was determined to be 0.8708 ± 0.0002 (Mean ± SD, n=5). Typical measurement error is ± 0.0014. Dashed bar at the bottom of the

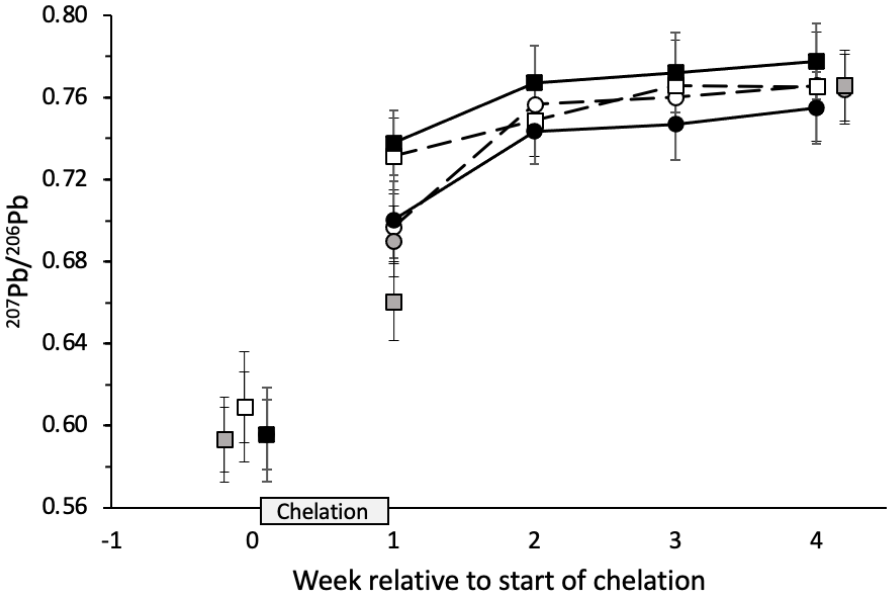
graph indicates elevated ^{206}Pb-enriched oral exposure period from study week 0 to week 7. Grey bar at the bottom of the graphs indicates embedded lead pellets. Black bar indicates chelation took place for 5 days between week 7 and week 8. Following chelation, the rebound period began and lasted three weeks as indicated by the white bar. Dashed line on both graphs represents averages of all rats regardless of subset and treatment group. At week 7, animals were separated into subsets (pre-chelation, post-chelation, or post-rebound) and treatment groups (succimer or vehicle). Post-chelation and post-rebound subsets are represented by circle and square symbols, respectively. Succimer or vehicle treated animals are represented by closed or open symbols, respectively.



Supplemental Figure 3: Relationship of Blood ^{207}Pb/^{206}Pb and tissue ^{207}Pb/^{206}Pb. Blood, liver, and kidney ^{207}Pb/^{206}Pb ratios were measured at time of sacrifice. Within animal, liver and kidney ^{207}Pb/^{206}Pb were not measurably different, however there were slight differences between blood ^{207}Pb/^{206}Pb. Average ^{207}Pb/^{206}Pb ratios of liver and kidney are plotted for each animal. A regression plot was generated to establish a relationship between blood ^{207}Pb/^{206}Pb ratio and tissue ^{207}Pb/^{206}Pb ratios for (A) baseline and (B) post-chelation animals to estimate tissue ^{207}Pb/^{206}Pb ratios prior to and immediately following chelation. A) Regression plot of blood and tissue ^{207}Pb/^{206}Pb ratios in baseline animals (n=8). Slope was 0.912 with an intercept of 0.0424 and R=0.997. B) Regression plot of blood and tissue ^{207}Pb/^{206}Pb ratios in succimer animals (squares) and vehicle animals (circles). Slope for succimer regression was 1.25 with an intercept of -0.248 and R=0.657. Slope for vehicle regression was 0.917 with an intercept of 0.039 and R=0.989.



Supplemental Figure 4: Adjusted blood, urine, and tissue $^{207}Pb/^{206}Pb$ during chelation. Blood, urine, and tissue pre-chelation $^{207}Pb/^{206}Pb$ ratios were normalized to an intermediate starting value between succimer and vehicle groups within each sample type. Triangle symbols on x-axis represent the timing of each oral succimer or vehicle dose. Succimer and vehicle treated animals are represented by square and circle symbols, respectively. Solid lines represent blood $^{207}Pb/^{206}Pb$ ratios and dashed lines represent urine $^{207}Pb/^{206}Pb$ ratios. Grey symbols represent estimated pre-chelation tissue $^{207}Pb/^{206}Pb$ ratios and measured post-chelation $^{207}Pb/^{206}Pb$ ratios. Data are mean ± SE (n=8/group for blood, n=5-8/group for urine).



Supplemental Figure 5: Adjusted blood, urine, and tissue $^{207}Pb/^{206}Pb$ during the rebound period. Blood, urine, and tissue pre-chelation $^{207}Pb/^{206}Pb$ ratios were normalized to an intermediate starting value between succimer and vehicle groups within each sample type. Grey bar on x-axis represents 5 days of chelation with succimer or vehicle. Succimer and vehicle groups are represented by square and circle symbols, respectively. Solid lines represent blood $^{207}Pb/^{206}Pb$ ratios and dashed lines represent urine $^{207}Pb/^{206}Pb$ ratios. Grey symbols represent estimated pre-chelation and post-chelation tissue $^{207}Pb/^{206}Pb$ ratios and measured post-rebound tissue $^{207}Pb/^{206}Pb$ ratios. Data are mean ± SE (n=8/group for blood, n=5-8/group for urine).

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