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Succimer chelation does not produce lasting reductions of blood lead levels in a rodent model of retained lead fragments

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ABSTRACT

Retained lead fragments from nonfatal firearm injuries pose a risk of lead poisoning. While chelation is well-established as a lead poisoning treatment, it remains unclear whether chelation mobilizes lead from embedded lead fragments. Here, we tested whether 1) DMSA/succimer or $CaNa_2EDTA$ increases mobilization of lead from fragments in vitro, and 2) succimer is efficacious in chelating fragment lead in vivo, using stable lead isotope tracer methods in a rodent model of embedded fragments. DMSA was > 10-times more effective than $CaNa_2EDTA$ in mobilizing fragment lead in vitro. In the rodent model, succimer chelation on day 1 produced the greatest blood lead reductions, and fragment lead was not mobilized into blood. However, with continued chelation and over 3-weeks post-chelation, blood lead levels rebounded with mobilization of lead from the fragments. These findings suggest prolonged chelation will increase fragment lead mobilization post-chelation, supporting the need for long-term surveillance in patients with retained fragments.

1. Introduction

Lead-based fragments retained in the body due to firearm or blast injuries represent a significant but understudied source of lead exposure and toxicity. There are an estimated ~86,000 nonfatal firearm injuries in the U.S. each year, and significantly more, though poorly documented, firearm injuries worldwide that result in retained bullet lead fragments in the body(Kaufman et al., 2021). Moreover, there are an estimated 42,000 veterans that have retained metal fragments due to combat-related injuries, with a subset of these cases involving lead-based metal fragments(Centeno et al., 2014; Gaitens et al., 2017, 2020). In one study, veterans with retained bullet fragments were four-times more likely to have elevated urine lead levels compared to those with blast related injuries, likely due to the use of lead-based ammunitions versus heterogeneous metal mixtures from blast projectiles(Gaitens et al., 2017). In another study, about 7% of 210 veterans with blood lead levels ≥ 25 μg/dL treated at US Veteran Health Administration facilities between 2015 and 2021 had retained fragments from occupational or non-occupational firearm related injury(Oda et al., 2021). Depending on the firearm and bullet caliber and type, nonfatal firearm injuries can produce hundreds of lead fragments that are retained in the body, leading to a prolonged and potentially significant source of lead endogenous exposure(McQuirter et al., 2001; McQuirter, 2004; Knott et al., 2010; Centeno et al., 2014; Weiss et al., 2017a; Weiss et al., 2017; Gaitens et al., 2020; Oda et al., 2021; Kershner et al., 2022).

Retained fragments, as with other foreign objects in the body such as medical devices, can promote an inflammatory immune response, often resulting in local fibrosis and fragment encapsulation(Farrell et al., 1999; McQuirter et al., 2001; McQuirter, 2004; Riehl et al., 2013; Centeno et al., 2014; Weiss et al., 2017a; Apte et al., 2019). There is no nationwide reporting of blood lead levels in patients with retained metal fragments, though a number of studies have reported associations between retained lead fragments in the soft tissue and elevated blood lead levels in humans(Nguyen et al., 2005; Grasso et al., 2017; Weiss et al., 2017a; Weiss and Tomasallo, 2017; Nickel et al., 2018; Apte et al., 2019; Kershner et al., 2022). One study reported blood lead levels as high as ~300 μg/dL arising from retained fragments in the soft tissue, while a recent review of retained bullets and lead toxicity found that the majority of patients with retained fragments had clinically elevated blood lead levels(M H Aly, H C Kim, S W Renner, A Boyarsky, M Kosmin, 1993; Kershner et al., 2022). For reference, adult blood lead levels < 3.5 μg/dL are considered normal, while levels > 10 μg/dL are associated with potential toxicity(Kosnett et al., 2007, 2023; Flora et al., 2012; Brent et al., 2017; CDC, 2018; Centers for Disease Control and Prevention, 2021; Skerfving and Bergdahl, 2021). The factors influencing the extent that retained lead fragments release elevated levels of

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lead into the surrounding tissues and circulation are not well understood, though they likely include the number, surface area and composition of the retained fragments, their location in the body, time since injury, and age and metabolic state of the patient (Apte, 2019; Centeno, 2014; Kershner, 2022; McQuirter, 2004; Nickel, 2018).

The limited recognition of the potential health risks posed by elevated body lead levels arising from retained lead fragments may in part be due to the challenge of demonstrating that the retained fragments serve as a significant endogenous source of exposure and elevated blood/body lead levels. In a small number of cases in human and animal studies, stable lead isotope tracer methods have been used to differentiate between embedded fragment lead and lead from other exposure sources(Manton and Thal, 1986; Weiss, Lee et al., 2017a). For example, Weiss et al. used lead isotopic analyses in a patient with an elevated blood lead level (>200 µg/dL) to show that the isotopic signature of a surgically removed extra-articular embedded fragment matched the lead isotopic signature of the patient's blood, demonstrating that the embedded fragment was the source of lead poisoning(Weiss et al., 2017a). In another study, Manton and Thal implanted lead fragments with distinct lead isotopic signatures (i.e., enriched in ^{208}Pb or ^{206}Pb) in intra- and extra-articular areas of the leg in a canine animal model, and found that the intra-articular fragments contributed significantly greater amounts of lead to the blood than the extra-articular fragments (Manton and Thal, 1986).

There are well-developed practices for the management and treatment of elevated lead exposure and lead poisoning in children and adults arising from environmental and occupational sources. The Centers for Disease Control and Prevention recommends aggressive environmental intervention, including removal from the source(s) of exposure, and if clinically indicated, treatment with chelation therapy involving Chemet (succimer, *meso*-2,3-dimercaptosuccinic acid, DMSA) or Versenate (ethylenediaminetetraacetic acid calcium disodium salt, $CaNa_2EDTA$)(Needleman, 2004; Papanikolaou et al., 2005; Gracia and Snodgrass, 2007; Kosnett et al., 2007; Kim et al., 2015; CDC, 2022). Following these general guidelines, patients with retained lead fragments and elevated blood lead levels may be recommended for surgical debridement. However, in cases of retained fragments, surgical removal is often not possible or recommended due to risk of collateral tissue damage and infection(Hill et al., 2001; Kane et al., 2009; Gaitens et al., 2020). Further, the health risks and morbidity associated with surgical removal of retained fragments vary case by case, depending on the number, size, and location of the fragments, and often outweigh the benefits of removing retained fragments(Kane et al., 2009; Riehl et al., 2013; Weiss et al., 2017a; Kershner et al., 2022). As a result, fragments are often retained within subjects for years to decades, where they may present endogenous sources of prolonged lead exposure(Meggs et al., 1994; Farrell et al., 1999; McQuirter et al., 2001; McQuirter, 2004; Cyrus et al., 2011; Weiss et al., 2017a). In these cases, there are no well-accepted guidelines for the medical management of patients with retained fragments, and it is not known whether treatment options such as chelation therapy offer long term benefit. A limited number of case studies have shown that in patients with retained fragments and elevated blood lead levels, chelation therapy can be effective in reducing blood lead levels when prescribed prior to and following surgical removal of fragments(Meggs et al., 1994). However, studies have reported mixed outcomes in patients who were chelated without surgical removal of all retained fragments, with some reporting short term reductions in blood lead levels, while others report more complex long term outcomes of chelation in patients with retained fragments(Cyrus et al., 2011; Weiss et al., 2017b; Yen and Yen, 2021).

While chelation treatment is well-established for reducing blood lead levels in cases of lead poisoning from environmental and occupational sources, it remains less clear whether chelation treatment can be an effective approach for managing lead poisoning arising from retained fragments, given that retained fragments may serve as a substantial source of endogenous lead exposure. To address this, we investigated whether 1) succimer (DMSA) or $CaNa_2EDTA$ was able to mobilize lead from solid lead pellets in an artificial extracellular fluid cell-free system, and 2) oral succimer (Chemet) mobilized lead from embedded pellets in vivo in a rodent model, using a stable ^{206}Pb tracer method to produce elevated tissue lead levels with a lead isotopic signature different from that of the embedded lead pellets. This approach allowed us to determine the relative amount of lead arising from the embedded lead pellets versus tissues with a ^{206}Pb-enriched isotopic signature in the measured blood, urine, and tissues prior to and over the course of succimer chelation, and the ensuing post-chelation rebound period.

2. Methods

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

Estimated physiological or 10X-physiological concentrations of dimercaptosuccinic acid (DMSA, Sigma-Aldrich, ~98%, Lot SLCD1413) or ethylenediaminetetraacetic acid calcium disodium salt ($CaNa_2EDTA$, Sigma-Aldrich, 98%, Lot 69C0415) DMSA or $CaNa_2EDTA$ were added to artificial extracellular fluid (aECF) containing a solid lead pellet. Physiological concentrations of 0.274 mM and 0.181 mM for DMSA and $CaNa_2EDTA$, respectively, were estimated based on the clinically recommended single human dose for succimer (10 mg/kg body weight) or edetate ($CaNa_2EDTA$, 500 mg/m^2), and the estimated chelator concentration in the liquid portion of the body arising from those single doses. 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(McNay and Sherwin, 2004). aECF solutions containing chelators at 1X or 10X physiological concentrations were degassed under vacuum and sonication, and if necessary, pH adjusted with 4% NaOH, and further degassed. 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, 14 mL of degassed aECF solutions of DMSA and $CaNa_2EDTA$ chelators were added to polyethylene tubes with lead pellets (1 mm × 2 mm, range 17 – 19 mg, $>99.99\%$ lead, Sigma-Aldrich, St Louis, MO). Controls were aECF with chelators but without lead pellets (aECF + chelators), and aECF with lead pellets but without chelators (aECF + pellet, no chelator). Upon addition of the aECF/chelator solutions the sample tubes were immediately capped with rubber septums and maintained in a shaker/incubator at 37 ˚C for 72 h. Solution aliquots were serially removed at 30 min, 1, 5, 24, and 72 h of incubation by syringe through the septum, and the removed aECF volume was replaced with nitrogen gas via a second syringe to maintain hypoxic conditions and avoid DMSA oxidation in solution. The pH of solutions were recorded after the 72-hour aliquot and were between 6.64 and 6.97 for all groups. Sample aliquots were acidified with quartz-distilled ultrapure concentrated nitric acid to $\mathrm{pH}<2$ and stored in acid-cleaned polyethylene vials for lead concentration analysis, as described below.

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

Subjects were adult male and female Long-Evans rats (Charles River, Hollister, CA). Rats were pair-housed with the same sex throughout the study in polycarbonate cages with filter tops and bedding within ventilated cage racks. Animal rooms were maintained at 21 ± 2 ∘C with 30–70% humidity and a 12:12 h light:dark cycle throughout the study. Rats were fed a standard rat chow (Harlan Teklad rodent chow #2018) and were provided food ad libitum between 10 AM and 6 PM as a daily timed food restriction to increase uptake of oral lead from drinking water(Smith and Flegal, 1992). All animal procedures were approved by

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the institutional IACUC (protocols Smith2002) and adhered to National Institutes of Health guidelines set forth in the Guide for the Care and Use of Laboratory Animals.

2.2.1. Pilot study to determine embedded lead pellet load

To determine the appropriate load of lead pellets to embed in the gastrocnemius muscle to generate elevated blood lead levels in our animal model, two or four lead pellets (1 mm × 2 mm pellets, generated from 1 mm diameter lead wire, >99.99% lead, Sigma-Aldrich, St Louis, MO) were implanted into the rats' left and right gastrocnemius muscle (i.e., four or eight pellets per animal, n = 5 rats per pellet load) using a modified procedure reported by Castro et al.(Castro et al., 1996). Briefly, rats were anesthetized via inhalation 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 on the left and right legs was shaved and disinfected with 70% isopropyl alcohol and betadine. Using aseptic technique, a 1–2 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 or four sterile lead pellets were implanted into the lateral side of the gastrocnemius muscle of both the left and right leg. Analgesic Marcaine-HCl (0.1 mL, 0.5% Bupivacaine) was administered into the skin 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.

Following lead pellet implantation, blood samples were collected after 1, 3, 5, and 7 weeks via the lateral saphenous vein. Briefly, the sampling area was shaved of fur and cleaned with 70% isopropyl alcohol then Milli-Q™ ultra-pure water. The saphenous vein was pricked with a sterile 22 G stainless steel needle, and blood (~0.1 mL) was collected with plastic capillary tubes (Innovative Med Tech, Blue Island, IL) and dispensed into acid cleaned microfuge tubes, and stored frozen for lead analyses. Results showed 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 Fig. S1). After 7 weeks, blood lead levels reached asymptotes of ~11 ng/mL and ~18 ng/mL (i.e., ~90–150-fold above background) for the four and eight pellet load groups, respectively, within 3 weeks post-implant (Supplemental Fig. S1). Based on these findings, a four pellet load (two per gastrocnemius muscle) was selected for the main chelation study.

2.2.2. Succimer chelation study in animals with embedded lead pellets

2.2.2.1. Overview. 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. For this, two isotopically distinguishable endogenous sources of lead (elevated tissue vs embedded lead pellets) were established in the animals and allowed to contribute to elevated blood lead levels. Subjects were 40 postnatal day 60 Long Evans rats (n = 20 male, 20 female). Oral succimer or vehicle was administered over 5 days, followed by a 3-week blood lead rebound period. For an overview of the experimental treatment timeline, see Supplemental Fig. S2A, B.

2.2.2.2. Oral lead ^{206}Pb-enriched drinking water to establish distinguishable lead isotopic compositions between elevated lead in tissues versus embedded lead pellets –. Several factors drove the experimental design to create markedly distinguishable lead isotopic signatures in the body tissues versus the embedded lead pellets. First, chelation is only indicated in cases of elevated body lead burdens. Second, the embedded lead pellets had a measured ^{207}Pb/^{206}Pb isotopic signature of 0.8708 (± 0.0002), which was not sufficiently different from contemporary background environmental lead (^{207}Pb/^{206}Pb = 0.8338–8453)(Church et al., 2008; Finkelstein et al., 2012) that would serve as lead exposure source to generate elevated tissue lead levels in the subjects. Therefore, animals were exposed orally to lead in drinking water (5 μg Pb/mL) enriched in ^{206}Pb (^{207}Pb/^{206}Pb = 0.4756 ± 0.0002, mean ± SD, n = 3). ^{206}Pb-enriched drinking water was administered ad libitum beginning on PND 63 (5 weeks prior to implantation of the lead pellets) and continued for a total duration of 7 weeks, ending 1 day prior to the start of chelation (PND 112; see Supplemental Fig. S2A, B). ^{206}Pb-enriched drinking water was prepared in batches of 5 L from a 5 mg/mL ^{206}Pb-enriched stock solution. The latter was prepared by dissolving 117 mg $Pb(CH_3CO_2)3\ H_2O$ (>99.99%, Sigma Aldrich, St Louis, MO) in 12.6 mL of an 890 μg/mL ^{206}Pb 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 HNO_3 in ultrapure Milli-Q water. For preparation of the ^{206}Pb-enriched drinking water, the ^{206}Pb-enriched stock solution was diluted 1000X with Milli-Q™ water in a low-density polyethylene (LDPE) carboy, and water in the carboy was thoroughly mixed before being dispensed into LDPE cage water bottles (pH ~ 6). Cage water bottles were refilled two to three-times weekly and water intake per cage was recorded. ^{206}Pb-enriched drinking water target lead concentrations were monitored weekly via inductively coupled plasma-optical emission spectroscopy (ICP-OES, see below).

2.2.2.3. 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 left and right gastrocnemius muscle) at age PND 98, as described above. Embedded pellets were in place for 2 weeks prior to the start of chelation.

2.2.2.4. 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 projected time of sacrifice: 1) pre-chelation baseline (n = 8), 2) post-chelation (n = 8 vehicle, 8 succimer), and 3) post-rebound (n = 8 vehicle, 8 succimer). All groups were balanced by sex. One day prior to the start of chelation, blood and urine samples were collected from all animals. The pre-chelation rats were sacrificed via CO_2 asphyxiation to establish baseline tissue (blood, liver, kidney) lead levels, as described below.

Over the 5-day chelation period, animals were orally (gavage) treated with vehicle or succimer (pharmaceutical Chemet) at a daily dose of 50 mg/kg body weight, administered in two equally divided doses (25 mg/kg body weight/dose) given 7 h apart (9:00 AM and 4:00 PM). 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 a 20-gauge stainless steel gavage needle (Popper and Sons, Inc.) in volumes of 0.39–0.70 mL, depending on body weight.

Blood and urine samples were collected from animals longitudinally over the chelation and post-chelation rebound period. Over chelation, samples were collected within 2 h following the second Chemet or vehicle dose on chelation days 1, 3, and 5 (n = 8/treatment group, balanced by sex). In a second set of animals (i.e., post-rebound group, n = 8/treatment), samples were collected prior to chelation (pre-chelation), one day after completion of chelation (post-chelation), and weekly over the 3-week post-chelation blood lead rebound period until sacrifice. 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 (Smith, Bayer and Strupp, 1998). Samples were stored frozen until analysis.

Tissue samples for lead concentration and isotopic composition analyses were collected from animals' pre-chelation (n = 8), 21 h after the final chelation dose (post-chelation, n = 8/treatment), or 3 weeks

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following the final chelation dose (post-chelation rebound, n = 8/treatment). All tissue sampling was conducted using trace metal-clean procedures, as described elsewhere(Smith et al., 1992; Smith, Bayer and Strupp, 1998). Briefly, 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 mL polypropylene syringe fitted with stainless steel 16 G needle via cardiac puncture from surgically exposed hearts and dispensed into low-lead vacutainers (EDTA anticoagulant). Kidney, liver, and hind legs were surgically removed and immediately transferred to plastic bags and frozen.

2.3. Analytical

2.3.1. 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 (Smith et al., 1992). Processing of all samples was 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 (Milli-Q) was ultra-pure grade (18 MΩ-cm^2). Following thawing, 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 0.2 N HNO_3 and Milli-Q water, transferred to pre-weighed Teflon vials and dried at 65 °C to a constant weight. All blood and tissue samples were digested for 8 h in hot 16 N HNO_3, evaporated to dryness, and re-dissolved in 1 N HNO_3 for analyses. For urine, samples were thawed and a 50 µL aliquot removed for creatinine analysis. The remaining urine sample was acidified with 16 N HNO_3 to pH < 2.

2.3.2. Determination of lead concentrations and isotopic compositions

Sample aliquots from the in vitro cell-free study were diluted with 1 N HNO_3, ^{205}Tl 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, USA). National Institute of Standards and Technology (NIST) Standard Reference Materials (SRM) 955 A (bovine blood) and NIST SRM 2670a (urine) were used to evaluate procedural accuracy for lead concentrations. 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 955 A (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 NIST 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.

2.3.3. Urine creatinine analysis

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

2.3.4. Hind Leg Histology

At time of sacrifice, hind limbs were immersion fixed in 10% neutral buffered formalin for 24–48 h then stored in 70% ethanol at − 20 °C until processing for histological analysis. Transverse sections through the skeletal muscle at the site of lead pellet implantation were collected for histopathologic evaluation. Formalin-fixed tissues were processed, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Tissue sections were evaluated and scored for fibrosis, inflammation (including inflammation type), skeletal muscle atrophy, and cumulative lesion score by a board-certified veterinary pathologist (DMI) at the Comparative Pathology Laboratory, University of California Davis.

2.3.5. 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 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 ^{207}Pb/^{206}Pb ratios of the two endmembers and the sample. For each rat, ^{207}Pb/^{206}Pb ratios were used to calculate the relative percent pellet lead using the following equation:

i.e., Calculation for % pellet lead:

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

Where $\left(\frac{^{207}\text{Pb}}{^{206}\text{Pb}}\right)_{\text{sample}}$ = lead isotopic signature of sample from individual rat.

$\left(\frac{^{207}\text{Pb}}{^{206}\text{Pb}}\right)_{\text{water}}$ = lead isotopic signature of ^{206}Pb-enriched drinking water.

$\left(\frac{^{207}\text{Pb}}{^{206}\text{Pb}}\right)_{\text{pellet}}$ = lead isotopic signature of embedded lead pellet.

2.4. Statistics

Data were analyzed using ANOVA models, as described below. Specific group contrasts were performed using Tukey's multiple comparison tests or Wilcoxon tests (the latter if the 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. Note that while the study was not statistically powered to detect sex differences in the measured outcomes, sex was included in initial models. Results revealed no sex differences in evaluated outcomes (blood and urine lead levels and isotopic ratios); sex was therefore omitted from the revised models. Post hoc contrasts using Tukey's test were performed if the corresponding ANOVA results for the main effects (or their interaction) were significant.

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3. Results

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

To determine if DMSA and $CaNa_2EDTA$ can mobilize lead from solid lead metal fragments in vitro, estimated physiological (274 μM and 235 μM, respectively) and 10x-physiological concentrations (2740 μM and 2350 μM, respectively) concentrations of DMSA and $CaNa_2EDTA$ in a physiological pH aECF cell-free system containing a solid lead metal pellet were incubated at 37 °C and serially sampled over 72 h. Appropriate control treatments (i.e., aECF with and without chelators or lead pellets) were also included. Lead levels in the physiological 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 h, > 450-fold higher than the aECF + pellet treatment (~3 ng/mL) (Fig. 1). Levels in the 10x-physiological DMSA treatment increased to substantially higher levels of ~10,100 ng/mL at 24 h, an ~7.5-fold increase above the estimated physiological DMSA concentration treatment (Fig. 1).

$CaNa_2EDTA$ also increased lead levels in the aECF in a time and concentration-dependent manner, though to a much lesser extent 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 h, respectively, which were ~20-fold and ~30-fold higher than the aECF + pellet control, and only ~4% and 1% of the corresponding DMSA treatments, respectively (Fig. 1). In contrast, aECF lead levels in the aECF + lead pellet control (no chelators) were only modestly increased to ~3 ng/mL at 24 h compared to the treatments without lead pellets at 24 h (i.e., 0.20 ng/mL and 0.30 ng/mL for the aECF and aECF + chelator treatments, respectively).

3.2. Lead chelation in an animal model of embedded fragments

3.2.1. Animal model to distinguish tissue versus embedded pellet lead over chelation

In our rodent model, drinking water enriched in stable ^{206}Pb ($^{207}Pb/^{206}Pb$ mean ± SD = 0.4756 ± 0.0002) was used to isotopically label lead in blood and tissues prior to the implantation of the lead pellets [$^{207}Pb/^{206}Pb$ = 0.8708 (± 0.0002)] to establish elevated tissue lead levels with an isotopic lead signature distinguishable from lead in the embedded pellets. Prolonged exposure of the animals to the ^{206}Pb-enriched drinking water and the embedded lead pellets established a two-endmember lead exposure system of body/tissue lead (^{206}Pb-enriched) and the embedded pellets endmembers, allowing use of a simple two-endmember mixing model to determine the relative (%) amount of each endmember in blood, urine, and tissue over chelation and the post-chelation rebound period(Smith and Flegal, 1992; Smith, Osterloh and Russell Flegal, 1996). Immediately prior to the start of chelation (i.e., 7 weeks after starting exposure to the ^{206}Pb-enriched drinking water and 2 weeks after implantation of the lead pellets), 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 signature ($^{207}Pb/^{206}Pb$ = 0.6047 ± 0.0081) that was intermediate between the oral ^{206}Pb-enriched drinking water and the embedded lead pellets (Supplemental Fig. S2A, B). 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.

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

To determine whether succimer chelation increased lead mobilization 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. Blood lead levels in the vehicle and succimer groups prior to the start of chelation were comparable at ~16 ng/mL (Fig. 2A). Mixed model results show that for blood lead concentration, there was a significant main effect of chelation duration [$F(4,56) = 104$, $p < 0.0001$], no main effect of treatment [$F(1,27) = 0.03$, $p = 0.871$), but there was a highly significant interaction of chelation duration x treatment [$F(4,56) = 10.9$, $p < 0.0001$]. This interaction reflects that 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 (Fig. 2A). By comparison, blood lead levels in the vehicle group also declined following the cessation of oral ^{206}Pb-enriched drinking water exposure, albeit more slowly and to a lesser extent than the succimer group. 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) (Fig. 2A).

Pre-chelation urine lead levels were comparable between the succimer and vehicle treatment groups at ~30 ng Pb/mg creatinine (Fig.

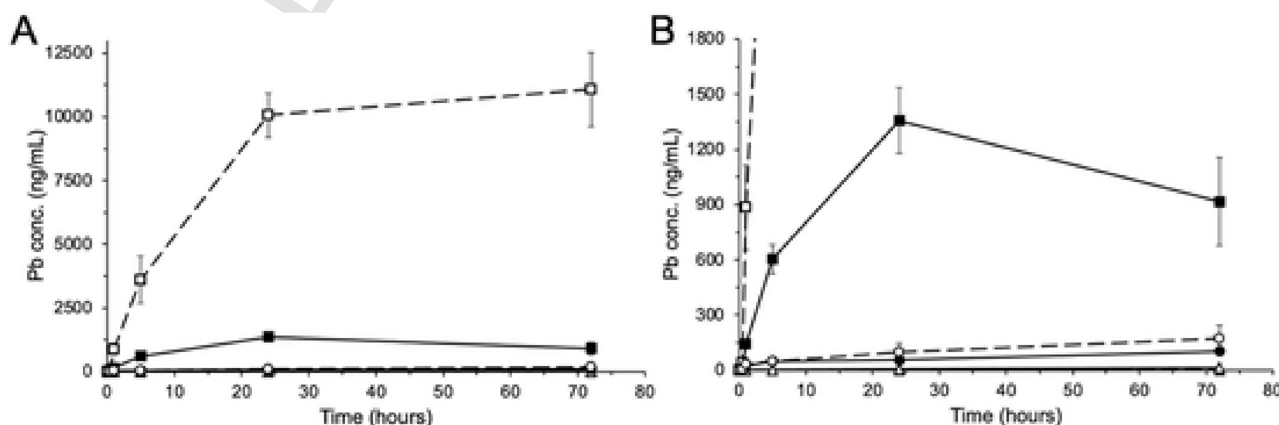

Fig. 1. DMSA and $CaNa_2EDTA$ significantly increase mobilization of lead from a solid lead fragment in an aECF cell-free system. (A) Leachate lead concentrations (ng/mL) for all groups as a function of time (hours). (B) Expanded y-axis of data presented in panel A. Aliquots were collected at 0, 0.5, 1, 5, 24, and 72 h. 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).

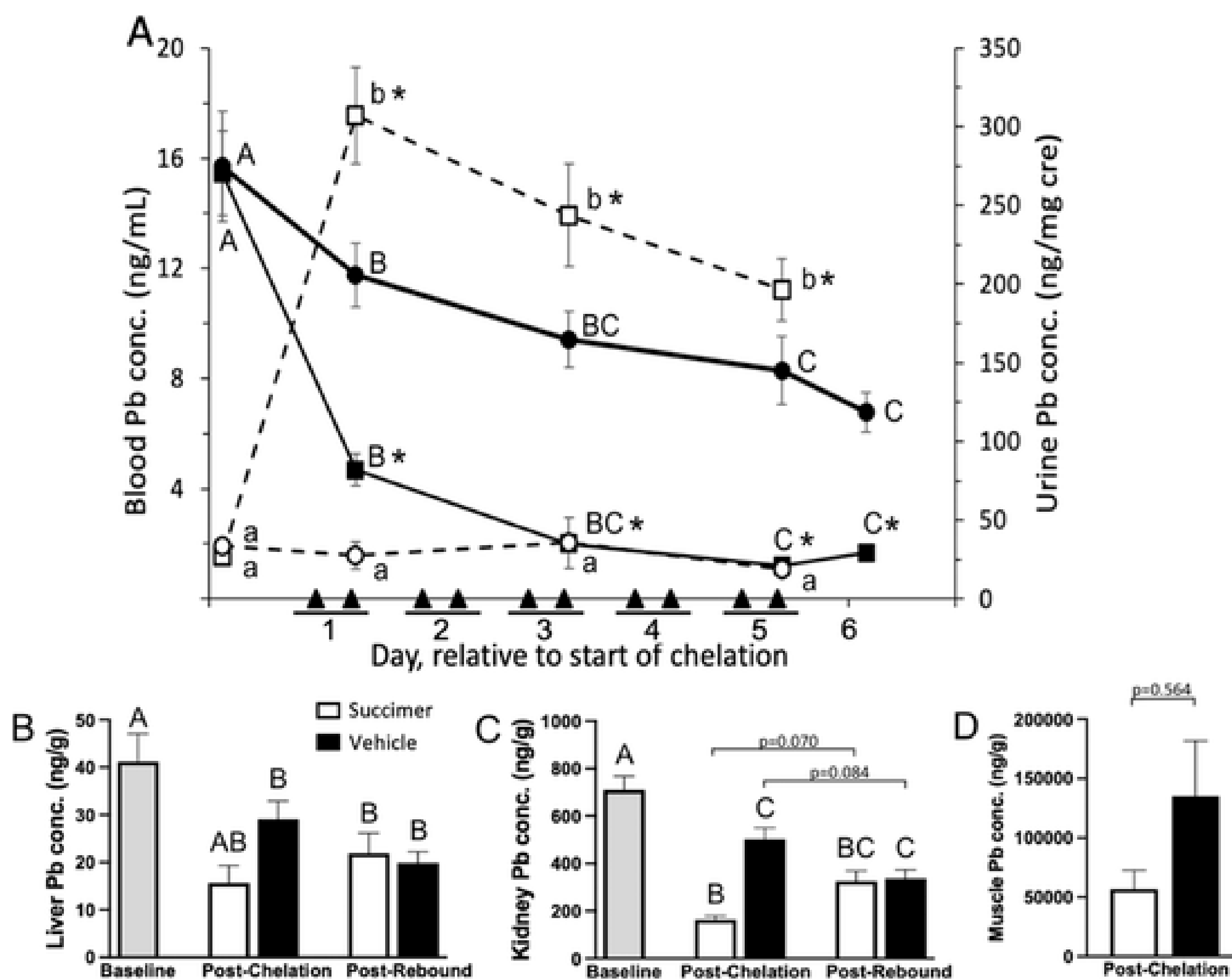

Fig. 2. Blood and tissue lead levels decrease while urine lead levels increase over the 5 days of succimer chelation. (A) Lead concentrations of blood (filled symbols, solid lines) and urine (open symbols, dashed lines) over chelation (succimer = squares, vehicle = circles). The timing of each oral succimer or vehicle dose is indicated on the x-axis. Data are mean ± SE (n = 8/group for blood and tissue, 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 liver and kidney lead concentrations (ng/g dry weight) of baseline group animals pre-chelation, 1 day after the final succimer dose (post-chelation), and 3 weeks after the final succimer dose (post-rebound). Bars with different superscripts are statistically different ($p < 0.05$) 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 post-chelation. Data are mean ± SE (n = 8/group). Statistical differences noted based on Wilcoxon test.

2A). Mixed model analyses showed a significant main effect of chelation duration [$F(3,38.5) = 6.86$, $p = 0.0008$], no main effect of treatment [($F(1,47.9) = 0.02$, $p = 0.880$), but a significant interaction of chelation duration 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 (all p's < 0.01) (Fig. 2A). 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) (Fig. 2A).

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, mean ± SE, n = 8) and 710 ng/g (± 58.2 ng/g), respectively (Fig. 2B, C). After 5 days of succimer chelation, liver lead levels were significantly reduced by 62% (to 15.6 ng/g ± 3.66 ng/g, $p = 0.0011$), while the vehicle group liver lead levels non-significantly decreased by 29% (to 29.1 ng/g ± 3.79 ng/g, $p = 0.265$) (Fig. 2B). Similar trends were observed in kidney lead levels of succimer and vehicle-treated animals (Fig. 2C). Kidney lead levels were significantly lower in the succimer versus the vehicle group post-chelation ($p < 0.0001$).

Notably, lead concentrations in the muscle tissue surrounding the embedded pellets were several orders of magnitude higher than liver and kidney levels (Fig. 2B-D). 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), though the treatment groups were not significantly different from one another ($p = 0.564$) (Fig. 2D). These findings show that there was significant lead contamination within the muscle tissue surrounding the pellet, remaining until post-chelation in both treatment groups.

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

To determine if lead from the embedded pellet environment is mobilized into blood and urine with succimer chelation, the pellet lead iso-

topic signature in blood and urine, as a percent of sample lead, was calculated from the measured $^{207}Pb/^{206}Pb$ ratios in blood and urine over the 5-day chelation duration and compared to the isotopic signature of the embedded pellets versus soft tissues (see Supplemental section II, Fig. S3). One day following the last chelation dose, liver and kidney samples were collected from a subset of animals to determine percent pellet lead in the tissue. Within each animal, the liver and kidney percent pellet lead were not significantly different and were therefore averaged to visualize with blood and urine percent pellet lead in Fig. 3A and Supplemental Fig. 3A, G. The relative amount (%) of embedded pellet lead in pre-chelation blood, urine, and tissues for the succimer and vehicle groups was ~35% (Fig. 3A). Mixed model analysis of the percent pellet lead in blood over chelation shows a significant main effect of chelation duration [$F(4,56) = 134$, $p < 0.0001$], no main effect of treatment [$F(1,18.5) = < 0.001$, $p = 1.00$], but a significant interaction of chelation duration x treatment [$F(4,56) = 28.1$, $p < 0.0001$]. This interaction reflects that the percent pellet lead in the blood of the succimer group changed bi-directionally during chelation, in contrast to the unidirectional change of the vehicle group (Fig. 3A). Specifically, following the cessation of ^{206}Pb-enriched drinking water exposure and treatment with succimer, the relative amount of 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 ($p = 0.015$; Fig. 3A). However, over the subsequent 2 – 5 days of chelation, the percent pellet lead in blood significantly increased, such that on the final day of chelation the blood contained 57.7% (± 4.15) pellet lead, a significant increase relative to pre-chelation levels ($p < 0.0001$). In contrast, the percent pellet lead in blood of the vehicle group unidirectionally increased over the entire 5-day course of vehicle treatment to 59.0% (± 4.58), which also was a significant increase relative to pre-chelation levels ($p < 0.0001$) (Fig. 3A). Further, at time of sacrifice the percent pellet lead in blood of the succimer group (66.7% ± 1.48) was significantly higher than the group's average liver/kidney percent pellet lead (51.4% ± 2.85; $p = 0.0006$), while the vehicle group blood and tissue percent pellet lead (53.9% ± 4.16) were not measurably different ($p = 0.428$) (Fig. 3A). Overall, it is noteworthy that from chelation day 1–1-day post-chelation, the percent pellet lead in blood of the succimer group increased > 2-fold more than the vehicle group.

The percent pellet lead in urine over chelation followed similar trends as blood in both treatment groups. There was a significant main effect of chelation duration [$F(3,31.8) = 13.6$, $p < 0.0001$], no main effect of treatment [$F(1,21.3) = 0.000$, $p = 0.994$), but a significant interaction of chelation duration x treatment [$F(3,31.8) = 12.3$, $p < 0.0001$]. Pre-chelation urine contained 38.4% (± 4.00) pellet lead for both succimer and vehicle groups (Fig. 3A). In the succimer group, the percent pellet lead signature in urine after 1 day of chelation (16.5% ± 2.49) was significantly lower than the pre-chelation urine levels noted above ($p = 0.0006$; Fig. 3A) and was also significantly lower than the percent pellet lead in blood at the same time point ($p = 0.025$) (Fig. 3A). By 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 at 53.5% (± 5.31) pellet lead following the final dose of chelation; a significant increase relative to pre-chelation levels ($p = 0.002$; Fig. 3A).

Overall, it is noteworthy that the succimer group blood and urine percent pellet lead decreased over the initial stage (1 day) of chelation, then increased thereafter with an apparent positive slope of change towards the embedded pellet isotopic signature that appears to steepen with continued chelation (Fig. 3A). In contrast, the vehicle group blood and urine unidirectionally increased then asymptoted over the final days of chelation.

The percent pellet lead in post-chelation tissue (average of liver and kidney) for the succimer and vehicle groups increased significantly relative to the estimated pre-chelation tissue values in the chelated animals (p's < 0.0001), and the measured baseline animals sacrificed prior to chelation ($p = 0.006$ and 0.002, respectively), but were not significantly different between treatment groups post-chelation ($p > 0.95$; Fig. 3A-C). Specifically, baseline animals sacrificed prior to the start of chelation contained 33.7% (± 4.46) pellet lead in the liver, which significantly increased to 50.5% (± 2.93) and 52.6% (± 4.21) in the succimer and vehicle groups, respectively ($p = 0.044$ and 0.018) post-chelation; there was no significant difference between succimer and vehicle groups post-chelation ($p = 0.996$, Fig. 3B). Similarly, the percent pellet lead in kidney also significantly increased in the succimer (60.4% ± 3.03, $p = 0.001$), and trended to increase in the vehicle group (50.1% ± 4.24, $p = 0.060$), compared to kidney levels in the measured baseline animals (33.1% ± 4.81). The percent pellet lead in kidney also was not significantly different between succimer and vehicle groups post-chelation ($p = 0.452$, Fig. 3C). Finally, the percent pellet lead in the muscle tissue surrounding the lead pellets (99.7% ± 0.10) essentially matched the pellets and was not different between treatment groups ($p = 0.780$) (Fig. 3D).

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

To determine if lead from the pellet/pellet environment is mobilized over the post-chelation rebound period, lead concentrations were measured in blood and urine samples from a subset of animals (n = 8/treatment) 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 comparable at 17.6 ng/mL (± 2.24) and 16.7 ng/mL (± 1.36), respectively (Fig. 4A). Immediately following 5 days chelation, blood lead concentrations in the succimer group were significantly lower than vehicle (2.99 ± 0.61 ng/mL and 11.5 ± 0.87 ng/mL, respectively, $p < 0.0001$) (Fig. 4A).

Over the 3-week post-chelation rebound period, 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 [$F(3,41) = 67.8$, $p < 0.0001$] on blood lead concentrations. One week after the end of chelation the succimer group exhibited a significant rebound in blood lead concentrations to 7.23 ng/mL (± 0.77) ($p < 0.0001$ vs blood lead levels immediately following chelation) (Fig. 4A). In contrast, blood lead levels in the vehicle group continued to decline to 8.82 ng/mL (± 0.73) over the same 1-week post-chelation rebound period ($p = 0.0002$ vs levels immediately following chelation) (Fig. 4A). As a result, blood lead levels between the succimer and vehicle groups were no longer measurably different beyond 1-week post-chelation and beyond ($p > 0.77$, Fig. 4A). In contrast, 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) but did not measurably change thereafter (Fig. 4A). Further, urine lead levels were not measurably different between treatment groups at any time point post-chelation (p's > 0.9) (Fig. 4A).

Following the 3-week post-chelation 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 one day following the final dose of chelation (p's > 0.5, Fig. 2B). Similarly, the succimer group kidney lead levels trended to increase to 324 ng/g (± 43.4) post-rebound, whereas the vehicle group trended to decrease to 337 ng/g (± 38.3), relative to one day post chelation (p's = 0.084 and 0.070, respectively, Fig. 2C). After the 3-week rebound period, liver and kidney lead levels were not significantly different between succimer and vehicle groups (p's > 0.99; Fig. 2B, C).

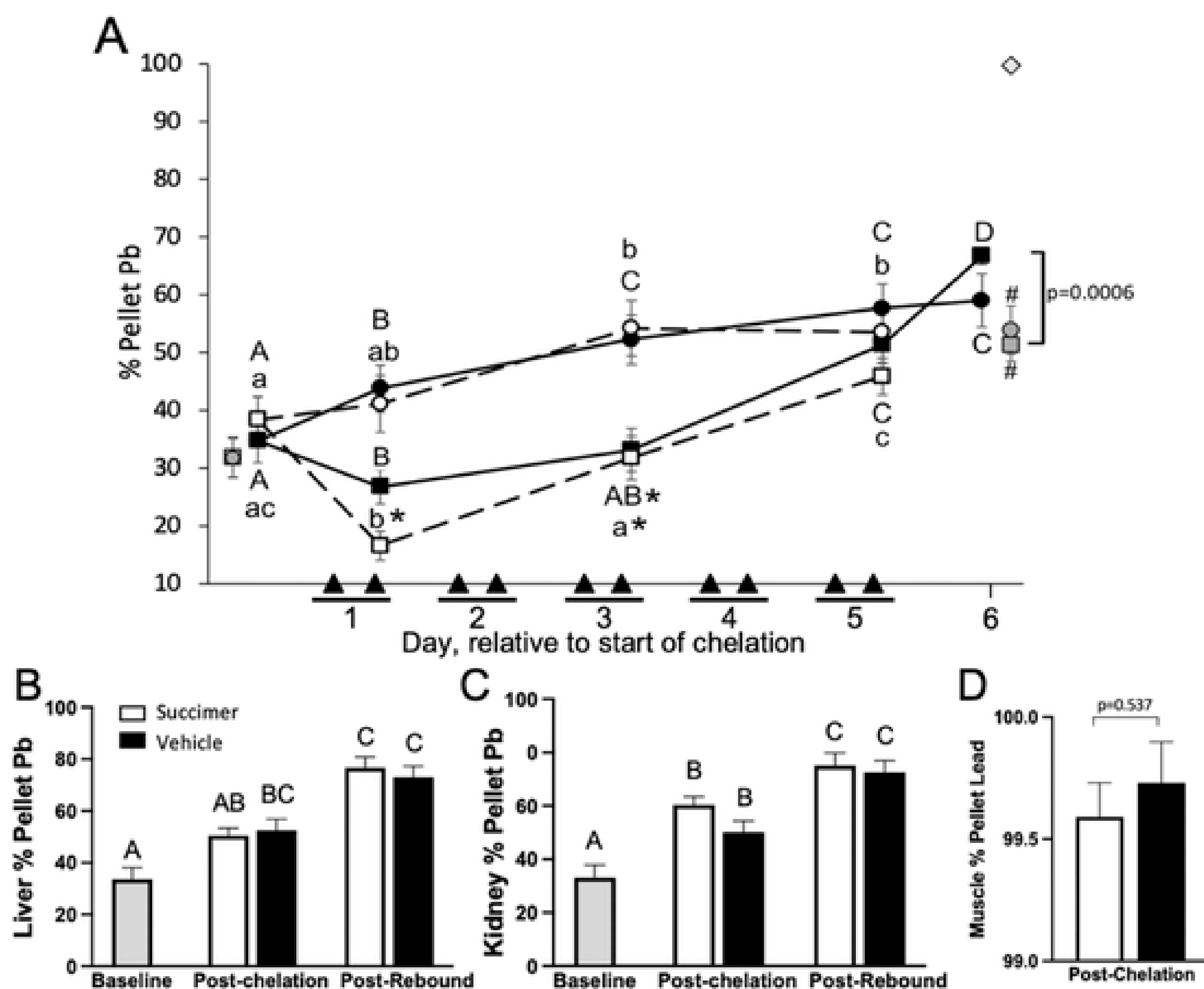

Fig. 3. Percent pellet lead in blood, urine, and tissue increase with ongoing succimer chelation. (A) Normalized percent pellet lead in blood (filled symbols, solid lines) and urine (open symbols, dashed lines) over chelation (succimer = squares, vehicle = circles). The timing of each oral succimer or vehicle dose is indicated on the x-axis. Diamond denotes derived percent pellet from the $^{207}Pb/^{206}Pb$ of the implanted lead pellet. Estimated pre- and measured post-chelation percent pellet lead in the tissue are displayed as grey squares (succimer) and circles (vehicle). Data are mean ± SE (n = 8/group for blood and tissue, 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 ($p < 0.05$). # represents statistical difference between percent pellet in measured post-chelation tissue (average of liver and kidney) compared to estimated pre-chelation tissue within each treatment group ($p < 0.05$). **(B, C)** Measured percent pellet lead in liver and kidney of baseline group animals pre-chelation, 1 day after the final succimer dose (post-chelation), and 3 weeks after the final succimer dose (post-rebound). Bars with different superscripts are statistically different ($p < 0.05$) based on Tukey's multiple comparisons test. **D)** Measured percent pellet lead in the muscle tissue surrounding the embedded lead pellets of animals sacrificed post-chelation. Data are mean ± SE (n = 8/group). Statistical differences noted based on Wilcoxon test.

3.2.4. *Pellet lead levels increase in blood, urine, and tissues of the succimer and vehicle groups over the post-chelation rebound period*

The percent pellet lead in blood, urine, and liver/kidney tissue increased in both succimer and vehicle groups over the first week post-chelation, but not thereafter (Fig. 4B). This is evidenced by mixed model analysis of the percent pellet lead in blood over the post-chelation rebound period, which shows a significant main effect of time [$F(3,40) = 75.3$, $p < 0.0001$], no main effect of treatment [$F(1,14.8) = 1.96$, $p = 0.182$], and a significant interaction of time x treatment [$F(3,40) = 3.11$, $p = 0.037$]. Specifically, 1 day after the end of chelation, blood of the succimer group contained 66.4% (± 3.96) pellet lead, which significantly increased to 73.8% (± 4.61) 1 week later ($p < 0.0001$; Fig. 4B). A similar significant increase in percent pellet lead in the blood was observed in the vehicle group (i.e., increasing from 57.3% ± 4.78 post-chelation to 68.4% ± 4.11 1 week later, $p < 0.0001$) (Fig. 4B). However, at 1-week post-chelation and thereafter the percent pellet lead in blood did not measurably change in either treatment group and the treatment groups did not differ (p's > 0.34) (Fig. 4B).

Urine percent pellet lead followed a similar trend as blood in both treatment groups, with mixed model analysis showing a significant main effect of time [$F(3,32.3) = 29.9$, $p < 0.0001$], no effect of treatment [$F(1,15.9) = 1.04$, $p = 0.323$], and a significant interaction of time x treatment [$F(3,32.3) = 3.12$, $p = 0.040$]. In the succimer group, percent pellet lead in urine increased from 65.8% (± 5.01) post-chelation to 70.3% (± 4.78) 1 week later ($p = 0.226$), while in the vehicle group, it increased from 57.0% (± 4.02) pellet lead post-chelation to 72.6% (±4.45) pellet lead 1 week later ($p < 0.0001$) (Fig. 4B). Over the subsequent weeks of the rebound period the succimer and vehicle blood and urine percent pellet lead were not significantly different (p's > 0.95) (Fig. 4B).

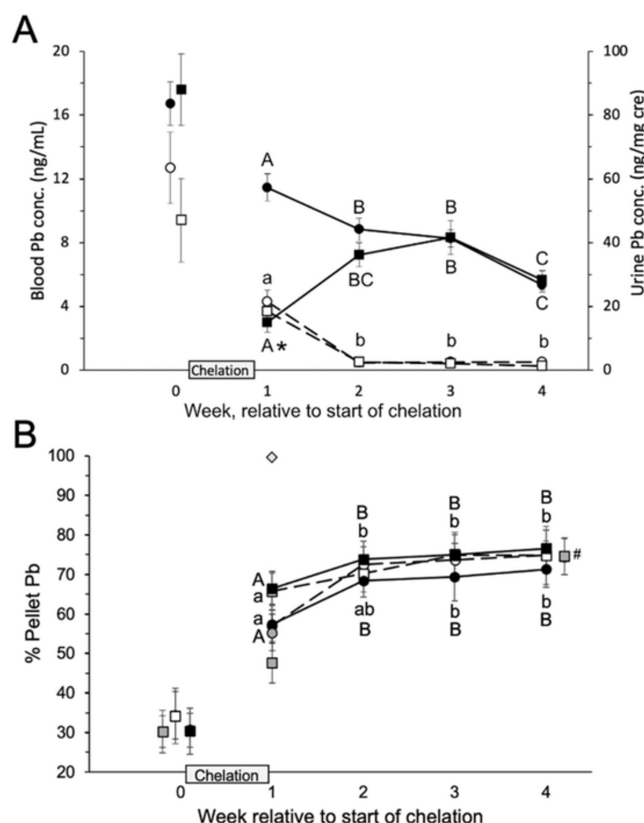

Fig. 4. : Blood lead concentration and percent pellet lead increase over the rebound period. (A, B) Blood (filled symbols, solid lines) and urine (open symbols, dashed lines) lead levels (A) and percent pellet lead (B) over 3 weeks following 5 days of treatment with succimer (squares) or vehicle (circles). Diamond denotes derived percent pellet from the $^{207}Pb/^{206}Pb$ of the implanted lead pellet. Estimated pre-chelation, estimated post-chelation, and measured post-rebound percent pellet lead in the tissue are displayed as grey squares (succimer) and circles (vehicle). The timing of each oral succimer or vehicle dose is indicated on the x-axis. Data are mean ± SE (n = 7–8/group for blood and tissue, n = 6–8/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 ($p < 0.05$). # indicates statistical difference between measured percent pellet in post-rebound tissue (average of liver and kidney) compared to estimated post-chelation tissue ($p < 0.05$).

The percent pellet lead in post-rebound tissue (average of liver and kidney) for the succimer and vehicle groups increased significantly relative to the estimated post-chelation tissue values in the rebound animals (p's < 0.0001), and the measured post-chelation animals sacrificed prior to chelation (p = 0.002 and 0.021, respectively), but were not significantly different between treatment groups post-rebound (p = 0.984; Figs. 3B-C, 4A). Specifically, relative to post-chelation, the percent pellet lead in liver significantly increased from ~51% and ~53 to 76.6% (± 4.20) and 73.0% (± 4.29) in the succimer and vehicle groups, respectively (p = 0.001 and 0.009), although there was no difference between treatment groups (p = 0.970, Fig. 3B). Similarly, the percent pellet lead in kidney of the succimer group increased (non-significantly) from ~60% to 72.6% (± 4.40%, p = 0.133), while levels in vehicle group increased from ~50% to 75.1% (± 4.77%, p = 0.006), from the post-chelation animals. Kidney percent pellet lead was not significantly different at post-rebound between groups (p = 0.993, Fig. 3C).

3.2.5. Embedded pellets elicited an immune/inflammatory response and fibrosis encapsulation of the pellets

To determine whether the embedded lead pellets generated an inflammatory response leading to fibrosis encapsulation, and whether the degree of encapsulation was associated with the relative amount of pellet lead over chelation, histological analyses of muscle tissue from the post-chelation animals (n = 8/treatment, ~3 weeks since the pellets were implanted) and a subset of the post-rebound animals (n = 3, ~6 weeks since the pellets were implanted) was conducted and scored by a board-certified veterinary pathologist (Supplemental Table 1). Findings in the post-chelation animals show that minimal to mild fibrosis was present in all cases and that the fibrosis encapsulated the implanted pellet. There was minimal to mild inflammation in some rats (9 out of 16), and the composition of inflammatory cells was histiocytic (i.e., macrophage). In some cases, macrophages contained brown variably refractile material, possibly indicating phagocytosis of lead particles. There was no relationship between any histopathological score (i.e., fibrosis, inflammation, muscle atrophy) and the percent pellet lead in blood or urine on the days (day 5 or 6) following the final dose of chelation in either treatment group (or pooled groups) post-chelation, based on ANOVA (p's > 0.18). Similar findings were evident in the subset of post-rebound animals (see Supplemental section III, Fig. S4).

4. Discussion

In light of the large number of retained metal fragment injuries in civilian and military populations, the risk of lead poisoning from retained lead bullet and other metal fragments is a potentially significant, though under-recognized public health threat(Gaitens et al., 2017, 2020; Weiss, Tomasallo et al., 2017; Oda et al., 2021; Kershner et al., 2022). Moreover, while guidelines for treating lead and other metal poisoning from environmental sources of exposure are well-established, there are no well-accepted guidelines for the medical management of asymptomatic patients with moderate elevations in blood lead levels from retained fragments, and it is not known whether treatment options such as chelation therapy offer long term benefit(Kane, Kasper and Kalinich, 2009; Riehl et al., 2013; Nickel et al., 2018; Gaitens et al., 2020). Our results show that treatment with succimer chelation significantly reduces blood lead and increases urine lead concentrations during chelation when compared with cessation of lead exposure alone, consistent with past studies investigating the efficacy of succimer chelation therapy to reduce body lead burdens(Friedheim et al., 1978; Graziano et al., 1992; Smith et al., 2000; Rogan et al., 2001; Bradberry, Sheehan and Vale, 2009; Smith and Strupp, 2013). Notably, the most marked decreases in blood lead and increases in urine lead occurred over the initial stage (day 1) of chelation (Fig. 2A). Following this (i.e., chelation days 2–5), blood lead levels continued to decline only slightly, while urine lead levels remained significantly elevated in the succimer group, suggesting a significant amount of body lead continued to be chelated (Fig. 2A).

Here, labelling tissue lead with a stable lead isotopic signature that was measurably different from the embedded lead pellets allowed us to distinguish the internal source of chelated lead in blood and urine. Our findings indicate that during the initial stage (day 1) of chelation, lead is mobilized from blood/soft tissues and not from the embedded pellets, but over the subsequent days of continued chelation significant amounts of pellet lead are mobilized (Fig. 3A). Evidence for this is that during the initial day 1 of chelation, in which the greatest reductions in blood lead and increases in urine lead occur, the percent pellet lead isotopic signature in blood and urine decrease to below their pre-chelation levels and move towards the $^{207}Pb/^{206}Pb$ isotopic signature of the ^{206}Pb-enriched drinking water pre-chelation exposure source. This evidences mobilization of recently acquired labile lead from soft tissues into blood and urine over the initial first day of chelation treatment. This interpretation is further supported by the significantly lower percent pellet lead

in urine (16.5% ± 2.49) relative to blood (26.8% ± 2.92) of the succimer (but not vehicle) group after the first day of chelation, consistent with urine lead reflecting the most soluble/labile fraction of chelatable lead. In comparison, whole blood is known to contain both a small fraction of soluble chelated lead (in plasma) and a majority fraction of lead complexed within the cellular fraction of blood (e.g., erythrocytes) that is less available for renal filtration into urine(Smith, 2000; Smith et al., 2000).

Over the subsequent chelation days (i.e., days 2 – 5), the percent pellet lead significantly increases with each day of chelation, with the percent pellet lead in blood of the succimer group increasing > 2-fold more over this period than the vehicle group (Fig. 3A). Considering that 1) the readily chelatable lead in sub-compartments of blood/tissue extracellular fluid may have become relatively depleted after the initial day of chelation(Smith et al., 2000),[48], and 2) lead concentrations in the pellet/tissue environment remained extremely high (~96,000 ng Pb/g post-chelation), these findings suggest that lead from the pellet environment was mobilized down its concentration gradient into compartments of lower concentrations, such as the continuously chelated extracellular fluid and blood compartment (Figs. 2D, 3D). Finally, the fact that lead concentrations in the liver and kidney decrease with chelation, while the percent pellet lead isotopic signature increases in those tissues, likely reflects the initial removal of labile lead from those tissues over the first day of chelation, and the redistribution of lead from the pellet environment (with a pellet lead isotopic signature) into blood and tissues over the subsequent days of chelation (Figs. 2A-C, 3A-C).

Notably, while blood lead concentrations of the succimer group were significantly lower than the vehicle following chelation treatment, they rebounded over the first post-chelation week, such that they were no longer measurably different from the vehicle group, and blood levels in both groups continued to decline by the third post-chelation week (Fig. 4A). In contrast, liver and kidney lead concentrations did not measurably change over the 3-week post-chelation period (Fig. 2B, C). However, the percent pellet lead in blood and urine continued to increase in both treatment groups over the first post-chelation week, but not thereafter (Fig. 4B). Similarly, the percent pellet lead in liver and kidney significantly increased in both treatment groups over the 3-week post-chelation period (Fig. 3B, C). Collectively, these findings indicate that succimer chelation did not produce prolonged (>1 week) reductions in blood and tissue lead concentrations relative to the vehicle treatment, and that post-chelation, lead from the embedded pellet environment continues to be remobilized into blood and tissues.

Clinical studies have reported mixed outcomes in patients with retained fragments and elevated blood lead levels who underwent chelation treatment, with some reporting short term reductions in blood lead levels, while others reported more complex longer term outcomes that made it difficult to evaluate chelation efficacy(Cyrus et al., 2011; Weiss, Lee et al., 2017b; Yen and Yen, 2021). For example, in one case series, a patient with retained lead fragments from gunshot reported elevated blood lead levels (>23 μg/dL) following surgical debridement of only superficial shotgun pellets(Cyrus et al., 2011). After the subject's blood lead levels transiently increased over time (>35 μg/dL), and symptoms of nausea, myalgias, and fatigue emerged, succimer chelation treatment was administered. While blood lead levels modestly declined over the ensuing 6 months (to 16–18 μg/dL), symptoms persisted, which prompted additional lead fragment removal surgeries, followed by continuing succimer treatment; the patient's blood lead levels remained elevated at ~15 μg/dL, with persistent symptoms and ongoing chelation (Cyrus et al., 2011).

A number of studies have used stable isotopic tracer methods to evaluate mobilization of endogenous lead stores into blood, including mobilization of lead from the skeleton, and chelation of lead from different body lead stores (Smith and Flegal, 1992; Smith et al., 1992, 2000; Smith et al., 1996; Gulson et al., 1997; Seaton et al., 1999; Gwiazda and Smith, 2000; Cremin et al., 2001; Gwiazda et al., 2005). Several studies have used stable lead isotope tracer approaches to demonstrate that embedded fragments constitute a significant endogenous source of lead exposure, and to distinguish endogenous exposure from embedded fragments from exogenous (outside the body) exposure sources. For example, in a human case study of elevated blood lead levels from fragments embedded in soft tissue, stable lead isotope methodologies were used to show that the elevated blood lead concentrations possessed a lead isotopic signature matching the embedded lead fragments, proving that the fragments were the source of the elevated blood lead levels(Weiss et al., 2017a). In a canine model study of embedded intra- and extra-articular lead fragments, implantation of ^{208}Pb or ^{206}Pb isotopically-enriched lead fragments into the knee joint or muscle were used to show that significantly more lead is mobilized from intra-articular lead fragments, leading to greater increases in blood lead levels than extra-articular soft tissue-embedded fragments(Manton and Thal, 1986). Collectively, these studies demonstrate that stable lead isotopic methodologies are ideally suited to differentiate endogenous sources of lead exposure.

Our in vitro 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 (Fig. 1). 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 et al., 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 ($Ka = 1 \times 10^{17.4}\ M^{-1}$), the reduced thiols of DMSA would readily give up their protons upon coordinating with lead in the solid metal(Harris et al., 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.

This study has a number of limitations. First, while findings from our in vitro experiment show that direct mobilization of lead from a solid pellet may be possible in a biological system depending on the chemical properties of the chelating agent, different outcomes may arise in vivo since the aECF buffer solution used in our in vitro model lacks many components of a biological system, including bioligands of both DMSA and $CaNa_2EDTA$ that have been documented in in vivo studies(Aposhian, 1983; Aposhian et al., 1989; Rivera et al., 1989; Maiorino et al., 1993). Second, there are several limitations of the animal model study that may qualify extrapolation of the findings to humans. For example, our experimental rodent model represents sub-chronic exposure to pellet lead (i.e., 2 weeks prior to starting chelation) with a sub-chronic (5 day) chelation regimen. In contrast, patients that have elevated lead levels from retained fragments often do not present to clinicians until months to years after first retaining the metal fragments. Further, while symptomatic lead intoxication is the main indication for chelation in human subjects, our animal model did not experience blood lead levels high enough to produce symptomatic toxicity or warrant chelation following clinical guidelines. However, while reductions in blood and tissue lead levels from succimer chelation may not be clinically translatable, the patterns of blood and urine lead concentrations during and after chelation are entirely consistent with the substantial body of experimental and clinical evidence in the literature and do not diminish the novel findings of pellet lead mobilization evidenced using the stable lead isotopic tracer methods presented here.

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5. Conclusions

We show that embedded lead fragments in soft tissue pose a significant source of lead to blood and other tissues. While oral succimer chelation leads to a transient reduction of tissue and blood lead levels, mobilization of embedded pellet lead post-chelation diminishes the efficacy of chelation. Given that embedded lead fragments represent a significant source of endogenous lead, these findings suggest that succimer chelation may not be efficacious for reducing longer-term risk of lead poisoning from embedded lead fragments.

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Authorship Contribution Statement

D.R.S., M.A.M., J.F.K., and J.M.G. conceived and designed research. S.M.T., T.J., and S.M.H. performed experiments. D.R.S. and T.J. development ICP-MS methodology. S.M.T., T.J., and D.R.S. analyzed and interpreted results of experiments. S.M.T. prepared figures. S.M.T. and D.R.S. drafted manuscript. S.M.T., D.R.S., M.A.M., J.M.K., and J.M.G. edited and revised manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Disclaimer

The contents do not represent the views of the U.S. Department of Veterans Affairs or the United States Government.

Appendix A. - Supporting Information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.etap.2023.104283.

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