



Oxidative damage in metal fragment-embedded Sprague-Dawley rat gastrocnemius muscle

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ABSTRACT

Injuries suffered in armed conflicts often result in wounds with embedded metal fragments. Standard surgical guidance has been to leave fragments in place except under certain circumstances; meaning that individuals may carry these retained fragments for their lifetime. Because of advancements in weapon design and the use of improvised explosive devices, the list of metals that could be found in a wound is extensive. In most cases the toxicological properties of these metals when embedded in the body are not known. To assess the potential damage embedded metals may cause to surrounding tissue, we utilized a rodent model to investigate the effect of a variety of military-relevant metals on markers of oxidative damage. The metals tested included tungsten, nickel, cobalt, iron, copper, aluminum, lead, and depleted uranium. Herein we report our findings on creatine kinase activity, lipid and protein oxidation, total antioxidant capacity, and glutathione levels in gastrocnemius homogenates from Sprague-Dawley rats surgically implanted with metal pellets for periods up to 12 months. Not all embedded metals affected the measured markers equally. However, metal-associated effects were seen at various times for muscle and serum creatinine levels, protein oxidation, total antioxidant capacity, and glutathione levels. No metal-induced effects on lipid peroxidation were observed. Taken together, these data suggest that subtle oxidative damage may be occurring in the muscle surrounding an embedded metal and indicates the need for medical surveillance of those individuals wounded by metal shrapnel.

1. Introduction

Injuries suffered by both combatants and civilians in armed conflicts often result in embedded metal fragments. Because of the risk of morbidity that accompanies extensive surgery, most embedded fragments are left in place except under certain circumstances (Spalding et al., 1991; Maggio et al., 2008; Manring et al., 2009). In addition, there is often an assumption that metals from ballistic injuries are biologically inert. This is not always the case as shown by numerous reports in the literature describing adverse health effects from embedded metals suffered in wartime, albeit many years after the initial injury (Schenck and Kronman, 1977; Knox and Wilkinson, 1981; Symonds et al., 1985; Lindeman et al., 1990; Ligtenstein et al., 1994; Eylon et al., 2005; Bar and Merimsky, 2017). However, advances in armor and weapon design have

now introduced on the battlefield numerous metals and metal mixtures whose toxicological and carcinogenic properties are not well established. This became apparent during the 1990–1991 Persian Gulf War. This conflict saw the first widespread combat use of depleted uranium (DU) munitions. Unfortunately, as a result of “friendly fire” events, several United States military personnel were wounded by DU shrapnel (McDiarmid et al., 2001, 2013, 2017). Due to the unique chemical and radiological properties of DU, there was apprehension over leaving the embedded metal in place for the life of the wounded individual.

Further, because of concern over the potential health and environmental effects of DU munitions, other materials were sought as replacements. From a ballistic standpoint, tungsten-based compositions showed the most promise. However, using a rodent model developed in our Institute to assay for health effects of embedded shrapnel, one of

Abbreviations: AAALAC, Association for Assessment and Accreditation of Laboratory Animal Care; AFRRRI, Armed Forces Radiobiology Research Institute; Al, aluminum; AOPP, advanced oxidative protein products; Co, cobalt; Cu, copper; DoD, Department of Defense; DU, depleted uranium; Fe, iron; GSH, glutathione; IACUC, Institutional Animal Care and Use Committee; Ni, nickel; Pb, lead; Ta, tantalum; TAC, total antioxidant capacity; TBARS, thiobarbituric acid reactive substances; USU, Uniformed Services University; W, tungsten.

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these compositions (tungsten/nickel/cobalt) was found to induce a highly aggressive malignant rhabdomyosarcoma at the metal implantation site (Kalinich et al., 2005; Schuster et al., 2012). Surprisingly, a tungsten/nickel/iron composite did not result in tumor formation (Emond et al., 2015a), despite the presence of nickel, a known carcinogen. These findings further demonstrated our paucity of knowledge regarding the health effects of embedded metal fragments. Because of this, both the United States Department of Defense and Department of Veterans Affairs developed a list of “metals of concern” with respect to embedded fragments (United States Department of Defense, 2007; United States Department of Veterans Affairs, 2017).

We have previously demonstrated that metals selected from this list for surgical implantation into the gastrocnemius muscle of rats to simulate a shrapnel injury were solubilized over time and were eventually detectable in significant levels in the urine of the experimental animals (Vergara et al., 2021). In addition, some of these metals affected expression of proteins involved in signal transduction pathways and also resulted in a significant increase in hydroxynonenal-modified proteins suggesting that some metals can induce long-term oxidative damage in muscle (Hoffman et al., 2022). In this follow-up investigation, we assessed several markers of oxidative damage in the gastrocnemius muscle of metal-implanted Sprague-Dawley rats in an attempt to define the possible mechanisms of these metal-induced effects. This project was part of a multi-center collaborative project with colleagues at the U.S. Department of Veterans Affairs, the University of Maryland School of Medicine, and the University of Kentucky. The overall goal of this combined effort was to study the potential long-term health effects of embedded fragments using a rodent model system alongside a human investigation of military personnel with embedded metal fragment injuries. In this particular study, we examined the levels of several indicators of oxidative damage, including lipid peroxidation (TBARS), protein oxidation (AOPP), glutathione levels, and total antioxidant capacity in homogenates of rat gastrocnemius muscle implanted with metals for up to 12 months. In addition, levels of creatine kinase in both gastrocnemius homogenate and serum were assessed. Creatine kinase is a key enzyme in maintaining energy reserves in skeletal muscle (Wallimann et al., 1992; Watchko et al., 1996). It does so by transferring the gamma phosphate from ATP to creatine to form creatine phosphate in a reversible reaction. Creatinine kinase has also been proposed as a serum indicator of muscle damage (Burch et al., 2016).

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats (*Rattus norvegicus*) were purchased from Envigo (Barrier 208A, Frederick, MD, USA) at approximately 30 days of age and 75–100 g body weight. All animal research conducted under this project was approved prior to initiation by the Armed Forces Radiobiology Research Institute (AFRRI) Institutional Animal Care and Use Committee (IACUC) under protocol 2016–05-006. Further, all procedures were conducted in compliance with guidance found in the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011) in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited research facility. After arriving at the AFRRI vivarium, rats were allowed to acclimate for at least 2 weeks prior to experimentation. Animals were pair-housed throughout the study in plastic microisolator cages with filter tops. Bedding (Teklab Sani-Chips, Envigo) was changed 2–3 times per week. Vivarium rooms were maintained at 21 ± 2 °C with 30–70 % humidity. The rooms were maintained on a 12:12-h light:dark cycle with lights on at 0600. Animals were fed standard rodent chow (Teklad Global Rodent Diet 8604, Envigo) with water available *ad libitum*.

2.2. Study design

Our Institute previously developed a rodent model for studying the health effects of embedded metal fragments, such as those suffered in a shrapnel wound (Castro et al., 1996). Metal loads mimicked what has been previously reported in wounded military veterans (Gaitens et al., 2016; Gaitens et al., 2017). In this study, we used that model system to investigate the effects of eight military-relevant metals including tungsten (W), nickel (Ni), cobalt (Co), iron (Fe), copper (Cu), aluminum (Al), lead (Pb), and depleted uranium (DU). Tantalum (Ta) was used as a control for any changes resulting from the surgical procedure or due to the presence of a foreign material in the muscle. Tantalum has been used for implanted prosthetic devices and is considered inert (Hockley et al., 1990; Johansson et al., 1990; Strecker et al., 1993). Previous studies in our lab and others have shown no differences between naïve and tantalum-implanted rats (Pelmar et al., 1999; Hahn et al., 2002; Kalinich et al., 2005; Schuster et al., 2012). Thus, the total number of rats needed for the study could be reduced and the ARRIVE Guidelines met (du Sert et al., 2020). Animals were randomly assigned to one of the nine metal implantation groups at $n = 8$ per group. The metal groups were conducted in 4 different cohorts: 1-, 3-, 6-, or 12-months post-implantation surgery. A total of 288 rats ($n = 8 \times 9$ metal groups \times 4 cohorts) were used in the study.

2.3. Metal pellets

All metal pellets were obtained from Alfa Aesar (Ward Hill, MA, USA) with the exception of DU which was purchased from Aerojet Ordnance (Jonesboro, TN, USA). The dimensions of all pellets were 1 mm in diameter by 2 mm in length. Due to differences in metal densities, the mass of the individual pellets differed as noted: Ta – 25.57 mg/pellet; W – 30.27 mg/pellet; Ni – 13.99 mg/pellet; Co – 13.97 mg/pellet; Fe – 12.36 mg/pellet; Cu – 14.07 mg/pellet; Al – 4.24 mg/pellet; Pb – 17.82 mg/pellet; DU – 29.99 mg/pellet. Prior to implantation, pellets were cleaned and chemically sterilized as previously described (Emond et al., 2015a, b).

2.4. Pellet implantation procedures

The metal pellets were bilaterally surgically implanted in the gastrocnemius muscle of rats (Hoffman et al., 2021a; Hoffman et al., 2021b). Briefly, animals were anesthetized using isoflurane (Baxter Healthcare, Deerfield, IL, USA). The surgical sites were clipped, swabbed with 70 % 2-propanol, and finally cleansed with betadine (Purdue Pharma LP, Stamford, CT, USA). A prophylactic dose of an analgesic (buprenorphine, 0.05–0.1 mg/kg, s.c., Rickitt and Colman, Hull, UK) was administered prior to surgery to alleviate any potential discomfort involved with the procedure. A small incision approximately 5 mm in length was then made through the skin of each hind leg to expose the gastrocnemius muscle. Each muscle was implanted with two sterile pellets spaced approximately 1.5 mm apart on the lateral side of the muscle by placing the sterile pellet in a 16-gauge needle. Using a specially designed plunger placed inside the needle, the needle was inserted into the gastrocnemius and the plunger depressed, forcing the pellet into the muscle. Tissue adhesive (VetBond; 3 M Corporation, St. Paul, MN, USA) was used to seal the incision. After surgery, rats were closely monitored until ambulatory. The surgery sites were examined daily for two weeks to assess for signs of inflammation, infection, and local metal toxicity, and after that time, weekly, for the duration of the study.

2.5. Euthanasia and tissue collection

Upon reaching their experimental endpoint or when indicated by guidelines approved by the AFRRI IACUC, rats were deeply anesthetized using isoflurane and blood was collected for serum isolation from the

abdominal aorta. Following collection, the rats were humanely euthanized under deep isoflurane by exsanguination and confirmatory pneumothorax as per the guidelines of the American Veterinary Medical Association (AVMA, 2020). After euthanasia, a complete gross pathology examination was conducted and a variety of tissues collected for other investigations associated with this study (Hoffman et al., 2021a; Hoffman et al., 2021b; Hoffman et al., 2022). For this arm of the study, the gastrocnemius was isolated, snap-frozen in liquid nitrogen within a piece of aluminum foil and stored at -80°C until isolation of the tissue surrounding the implanted pellet. In this procedure, the -80°C -stored tissue was placed on a Plexiglas slab chilled over dry ice. The pellet implantation site in the muscle was located and the tissue around the pellet site dissected with a chilled razor blade. The tissue was placed in a chilled 1.5 ml centrifuge tube and processed as described below. In cases where tumor formation impinged on the pellet implantation site, the muscle sample was taken 3–5 mm away from the tumor.

2.6. Serum processing

Blood was collected in serum separator tubes (Becton-Dickinson, Franklin Lakes, NJ, USA), the tubes inverted 5 times, allowed to sit undisturbed for 30 min at room temperature, then centrifuged at $1200 \times g$ for 10 min at room temperature. The serum was aliquoted into 1.5 ml centrifuge tubes and stored at -80°C until analyzed.

2.7. Muscle tissue processing

Tissue samples were suspended in RIPA buffer (Thermo, Waltham, MA, USA) plus Halt Protease and Phosphatase Inhibitor Cocktail (Thermo) and homogenized in a Bullet Blender (Next Advance, Troy, NY, USA) with 1.0 mm zirconium oxide beads (Next Advance) (settings speed 6, 5 min \times 3 runs) and then centrifuged at $1340 \times g$ for 10 min. Total protein from each sample supernatant was measured by Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA), in triplicate, against a BSA standard curve, on a spectrophotometer (BioTek Synergy Model H1M Multimodal Plate Reader with GEN5 Software, BioTek Instruments, Winooski, VT, USA), at 595 nm. Gastrocnemius homogenates were stored at -80°C until analyzed.

2.8. Creatine kinase activity assay

Creatine kinase activity in both gastrocnemius homogenate and serum samples was determined using a colorimetric kit (kit #ab155901, Abcam, Cambridge, MA, USA). The assay measures the ability of creatine kinase in a sample to convert creatine into phosphocreatine and ADP. The "Reaction Mix" provided in the kit converts these compounds to an intermediate which then reduces a colorless probe to a colored end-product that can be determined colorimetrically to indicate NADH production. Briefly, the samples were mixed 1:1 with the prepared kit-provided Reaction Mix in a 96-well plate. The kinetic assay was conducted in the BioTek Synergy Model H1M Multimodal Plate Reader at 37°C in the dark with intermittent shaking. The absorbance of the reaction was read at 450 nm every 2 min for a total of 40 min. Creatine kinase activity was calculated by determining the NADH produced in the sample wells to a standard curve and using this information in the following equation to calculate activity in nmol/min/ml:

$$\text{Creatine kinase activity} = (B/(\Delta T \times V)) \times D.$$

where B is the concentration (nmol) of NADH in the sample as calculated from the standard curve; ΔT is the reaction time (min); V is the original sample volume (ml); and D is the sample dilution factor.

2.9. Thiobarbituric acid reactive substances (TBARS) assay

Lipid peroxidation was monitored using the thiobarbituric acid reactive substances (TBARS) assay (kit #700870, Cayman Chemical, Ann Arbor, MI, USA). The TBARS assay measures the amount of

malondialdehyde (MDA), a naturally occurring product of lipid peroxidation, in a sample by reacting it with thiobarbituric acid (TBA). The MDA-TBA adduct can then be detected fluorometrically. Briefly, 100 μl of sample or standard was placed in a 1.5 ml centrifuge tube, 100 μl of 10 % trichloroacetic acid added followed by 800 μl of "Color Reagent" (TBA in acetic acid/sodium hydroxide, supplied in the kit). The tubes were vortexed, heated at 95°C for 60 min, then placed in an ice bath for 10 min. The tubes were centrifuged ($1600 \times g$ for 10 min) and 200 μl of the resulting supernatant, in duplicate, were removed and placed in a black plate for fluorometric analysis. The BioTek Synergy Model H1M Multimodal Plate Reader was used with an excitation wavelength of 532 nm and an emission wavelength of 585 nm to assess the concentration of the MDA-TBA adduct in both the standards and the samples.

2.10. Advanced oxidative protein products (AOPP) assay

Rat gastrocnemius homogenates were analyzed for advanced oxidative protein products using the OxiSelect AOPP Assay Kit (kit # STA-318, Cell Biolabs, Inc., San Diego, CA, USA). Samples were diluted 1:20 with the assay diluent included in the kit and 200 μl of the prepared samples were added per well to a 96-well plate. Chloramine reaction initiator (provided in the kit) was added to each well and the plate incubated for 5 min at room temperature with shaking at 250 rpm. The reaction was then terminated, and the plate read at 340 nm using the BioTek Synergy Model H1M Multimodal Plate Reader. The AOPP concentrations in the gastrocnemius homogenates were determined by comparing to a standard curve prepared using Chloramine-T.

2.11. Total antioxidant capacity (TAC) assay

The total antioxidant capacity in the gastrocnemius homogenates was assessed using a colorimetric kit (Kit MAK187, Sigma Chemical Co., St. Louis, MO, USA) that determines the ability of small molecule and protein antioxidants to convert Cu^{2+} to Cu^{+} . Binding of the Cu^{+} to a colorimetric probe allows antioxidant capacity to be calculated. Briefly, samples were diluted 1:40 with water and an equal volume of the Cu^{2+} working solution (provided in the kit) was added. After a 90 min room temperature incubation in the dark with shaking (250 rpm), the absorbance of the reaction mixture was determined at 570 nm in a BioTek Synergy Model H1M Multimodal Plate Reader. The antioxidant capacity is given in Trolox equivalents based upon comparison with a standard curve.

2.12. Glutathione (GSH) assay

Glutathione levels in the gastrocnemius homogenates were determined using the DetectX Glutathione Fluorescent Detection Kit (Kit # K006, Arbor Assays, Ann Arbor, MI, USA). Initially, samples were diluted with an equal volume of ice-cold 5 % 5-sulfo-salicylic acid (catalog # S2130, Sigma) and left on ice for 10 min. Samples were centrifuged for 10 min at $21,000 \times g$ after which 30 μl of the resulting supernatant was carefully removed, added to a fresh centrifuge tube and diluted with Assay Buffer provided in the kit. Diluted samples and standards were added to the plate at 50 μl /well followed by 25 μl of ThioStar™ Diluent provided in the kit. The plate was then incubated for 15 min at room temperature after which fluorescence was read using the BioTek Synergy Model H1M Multimodal Plate Reader with excitation at 370 nm and emission at 510 nm. This represents the free (reduced) glutathione concentration. After addition of the kit-provided "Reaction Mixture" (25 μl /well) and a second 15 min room temperature incubation, the plate was again read using the conditions described above. This reading represents the total glutathione concentration. The oxidized (GSSG) glutathione levels were obtained by subtracting the reduced glutathione from the total glutathione results.

2.13. Statistical analysis

Data were analyzed by two-way ANOVA using the variables of time from implant and implanted metal followed by Sidak's multiple comparisons test where each metal implant group within a time from implant is compared back to the corresponding Ta-implanted group value. Analyses were performed using GraphPad Prism Software (version 9.1.2, La Jolla, CA, USA). In all cases P values < 0.05 were considered significant.

3. Results

3.1. Gastrocnemius creatine kinase

Fig. 1 shows creatine kinase activity in the homogenates of gastrocnemius tissue surrounding the implanted metal pellet. Creatine kinase plays a major role in energy production in the cell by catalyzing the transfer of the gamma phosphate from ATP to creatine to form creatine phosphate and, as such, it was of interest to assess metal effects on its activity. For the tantalum control group, activity decreased over time. However, for the various test metals, creatine kinase activity was significantly lower than control at the 1- and 3-month post-implantation time points. Levels generally remained low at the 6- and 12-month points but were not significantly different than control for any of the metal implants.

3.2. Serum creatine kinase

Serum levels of creatine kinase have long been proposed as an indicator of muscle damage. As seen in Fig. 2, there are no statistically significant differences between control and any of the metal implantation groups except for the 12-month post-implantation time point for the Fe, Cu, Al, and Pb cohorts, and both the 6- and 12-month time points for the DU group.

3.3. Lipid peroxidation in gastrocnemius homogenates

In order to determine the type of potential damage the implanted metals may cause; two assessments of cellular damage were conducted.

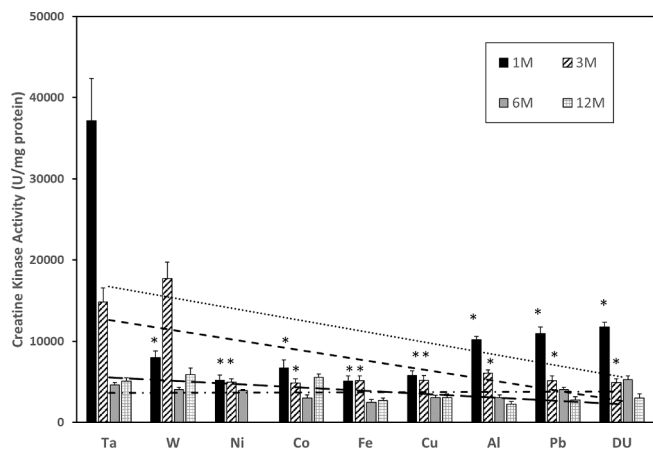


Fig. 1. Creatine kinase activity in metal-implanted rat gastrocnemius muscle. Gastrocnemius homogenates from metal-implanted rats from four different groups: 1 month (M), 3 month (M), 6 month (M), or 12 month (M) post-implantation. Creatine kinase activity expressed as enzyme units per mg protein and represent the mean of 8 individual assessments. Note: 12 month Ni cohort was euthanized at approximately 6 months as a result of tumor formation. Error bars represent the standard error of the mean and an * indicates significant a post-hoc p-value between Ta- and target metal-implanted animals within a post-implantation period. Trendlines are depicted as follows: 1 month (.....); 3 month (-----); 6 month (-.-.-.); 12 month (— — —).

Lipid peroxidation was measured using the TBARS assay which assesses the amount of malondialdehyde, a naturally occurring product of lipid peroxidation, by reacting it with thiobarbituric acid. As seen in Fig. 3, there were no significant differences between control and any of the implanted metal groups. Lipid peroxidation appeared highest at the earliest time point measured (1-month post-implantation) and trended downward as the implantation time increased.

3.4. Protein oxidative damage in gastrocnemius homogenates

Oxidative protein damage was assessed using the advanced oxidative protein product assay which measures the effect of reactive oxygen species on proteins leading to the formation dityrosine residues and protein crosslinkage. Somewhat surprisingly, for all metals except Al, levels of oxidative proteins were significantly lower than control at the 3- and/or 6-month post-implantation time points (Fig. 4). By 12-months post-implantation, all levels were not significantly different from control indicating no time dependency.

3.5. Antioxidant capacity in gastrocnemius homogenates

What role, if any, antioxidant capacity of the implanted muscle tissue played in the manifestation of oxidative damage was investigated by measuring the total antioxidant capacity of the gastrocnemius homogenate. The assay for total antioxidant capacity measures the capability of small molecule and protein antioxidants to convert Cu^{2+} to Cu^+ and is an estimation of their ability to counteract oxidative stress-induced damage in cells and tissues. Fig. 5 shows the total antioxidant capacity, expressed in Trolox equivalents, of gastrocnemius homogenates of the various metal groups. Total antioxidant capacity of the 12-month post-implantation Cu, Al, and Pb groups were significantly higher than control as were the 1- and 6-month post-implantation Ni groups. Only one group exhibited significantly lower total antioxidant capacity versus control and that was the 3-month post-implantation W group; although levels at the later time points were not significantly different than control.

3.6. Glutathione levels in gastrocnemius homogenates

Fig. 6 shows the levels of oxidized glutathione (GSSG, panel A), reduced glutathione (GSH, panel B), and total glutathione (panel C) in gastrocnemius homogenates from metal-implanted rats. Levels of oxidized glutathione were significantly lower in the 3-month post-implantation groups for Co, Fe, Cu, Al, and Pb, but were no different than control at other time points. For reduced glutathione, only Ni at the 3-month time point and Al at the 6-month time point were significantly different from control, with Ni being lower and Al higher. Total glutathione levels in the metal-implanted gastrocnemius homogenates were significantly lower than control in the 3-month post-implantation Ni, Co, and Cu groups.

4. Discussion

Embedded metal fragment injuries are an unfortunate consequence of armed conflict, affecting both combatants and civilians alike. Due to the risk extensive surgery can bring, most embedded fragments are left in place. However, because of advances in weapon design and the use of improvised explosive devices, the list of metals potentially found in embedded fragment wounds are practically endless. In most cases the toxicological properties of these materials, when embedded in the body, are not well understood. This report is part of a larger study investigating the health effects of embedded fragments of military-relevant metals using a rodent model system developed in our Institute (Castro et al., 1996). We previously have shown that metals implanted in the gastrocnemius muscle of Sprague Dawley rats for up to 12 months can solubilize and are excreted in the urine (Hoffman et al., 2021b),

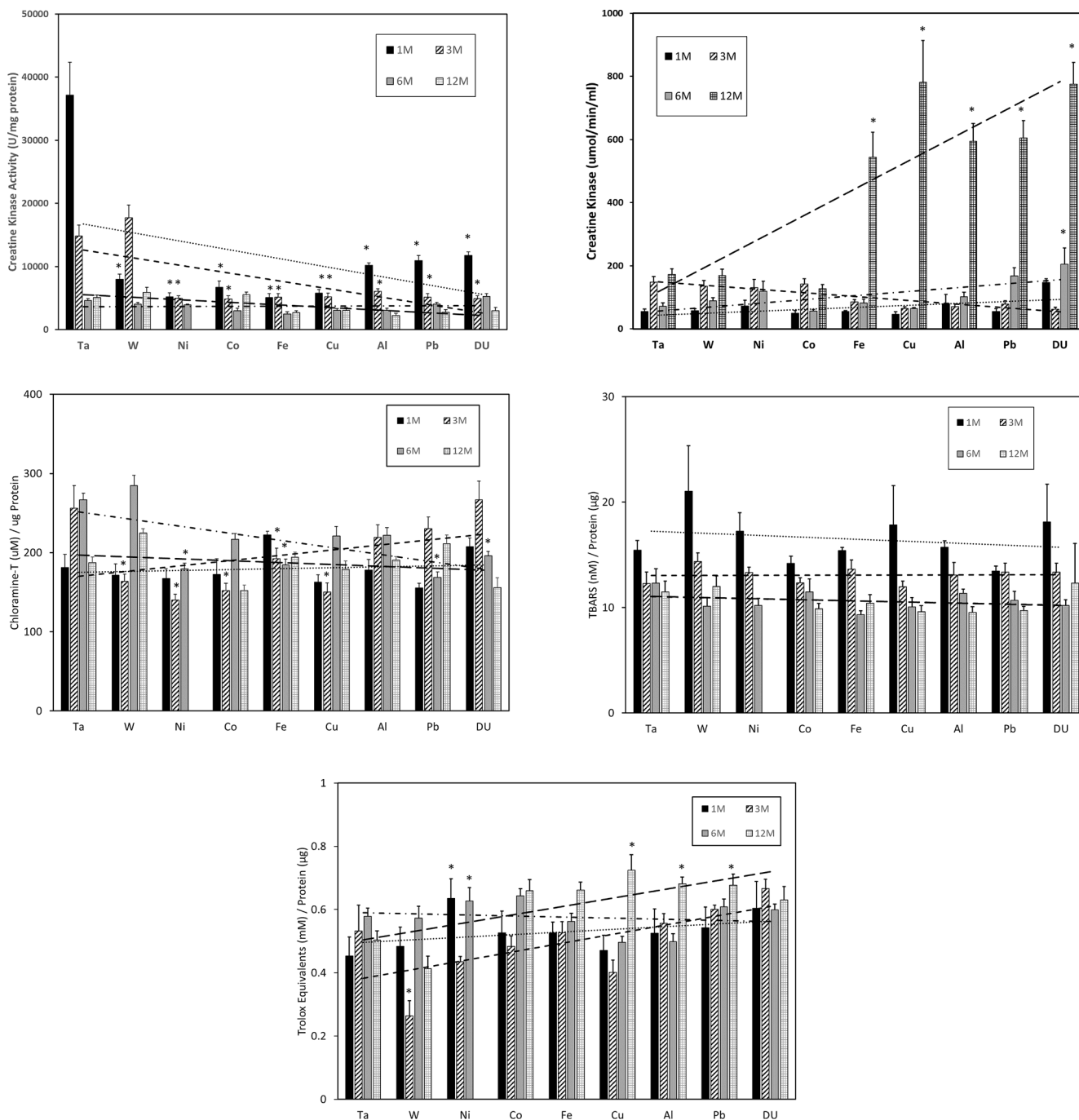


Fig. 2. Creatine kinase activity in serum from metal-implanted rats. Serum from metal-implanted rats from four different groups: 1 month (M), 3 month (M), 6 month (M), or 12 month (M) post-implantation. Creatine kinase activity expressed as $\mu\text{mol}/\text{min}/\text{ml}$ and represent the mean of 8 individual assessments. Note: 12 month Ni cohort was euthanized at approximately 6 months as a result of tumor formation. Error bars represent the standard error of the mean and an * indicates significant a post-hoc p-value between Ta- and target metal-implanted animals within a post-implantation period. Trendlines are depicted as follows: 1 month (.....); 3 month (.....); 6 month (.....); 12 month (.....).

deposited in other tissues (Vergara et al., 2021), and can affect blood-brain barrier markers (Hoffman et al., 2021a). In addition, we have demonstrated that metals can affect protein expression in tissue homogenates isolated from the area surrounding the implanted metal (Hoffman et al., 2022). To expand upon that work and to identify potential damage-response mechanisms, we report here the effect of implanted metals on creatine kinase activity, lipid and protein oxidation, total antioxidant capacity, and glutathione levels in gastrocnemius homogenates obtained from tissue surrounding the implanted metal

pellet in male Sprague Dawley rats. Because of funding limitations only male rats were utilized in this study. Obviously, as more women serve in combat roles their risk of suffering shrapnel wounds will greatly increase. There is a clear need for a study investigating the short- and long-term health effects of embedded metal fragments in females especially with respect to metal distribution patterns and potential fetal effects.

One of the functions of creatine kinase is to maintain intracellular energy levels in skeletal muscle (Wallimann et al., 1992; Watchko et al., 1996). Although there are no reports in the literature on the effect of

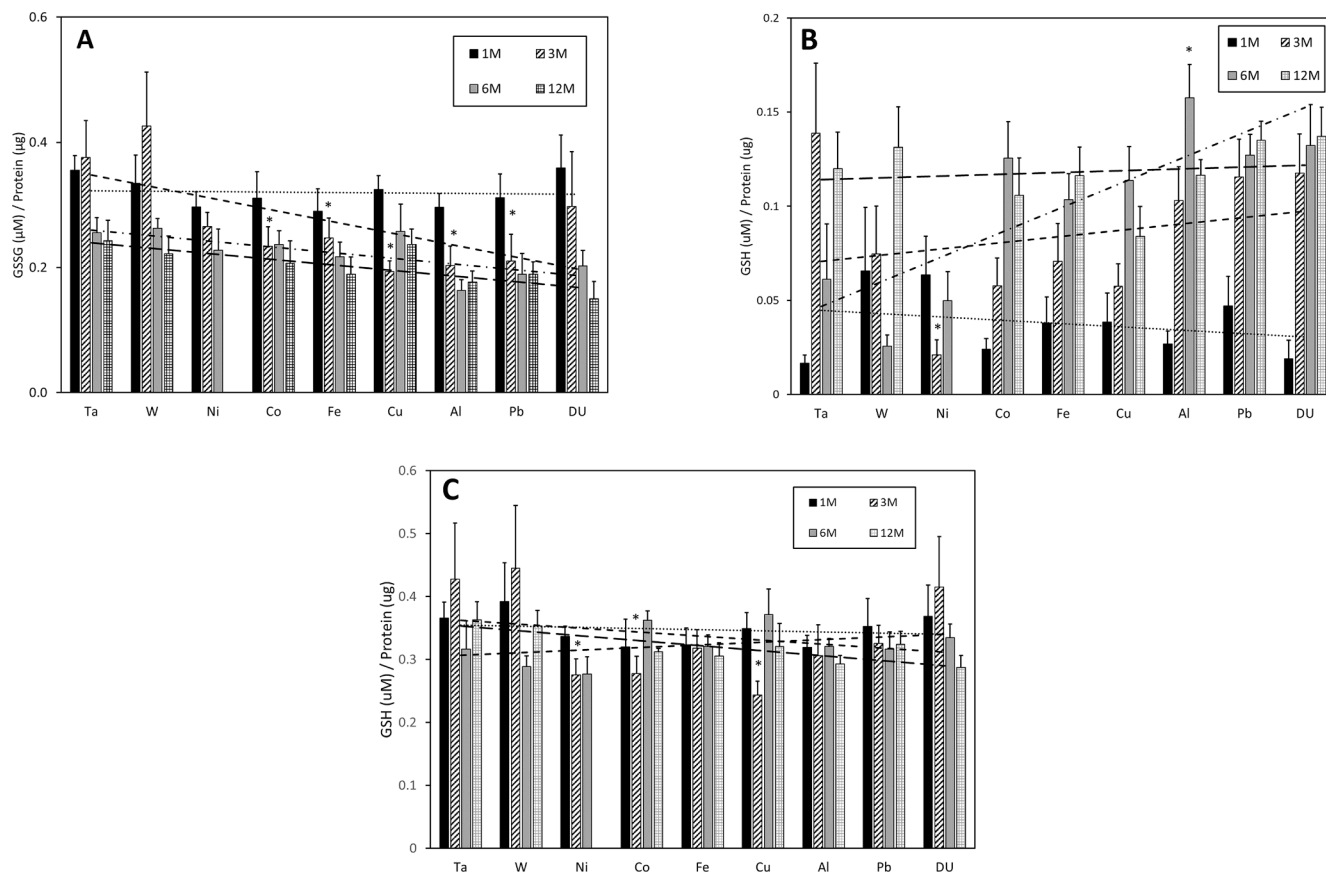


Fig. 2. (continued).

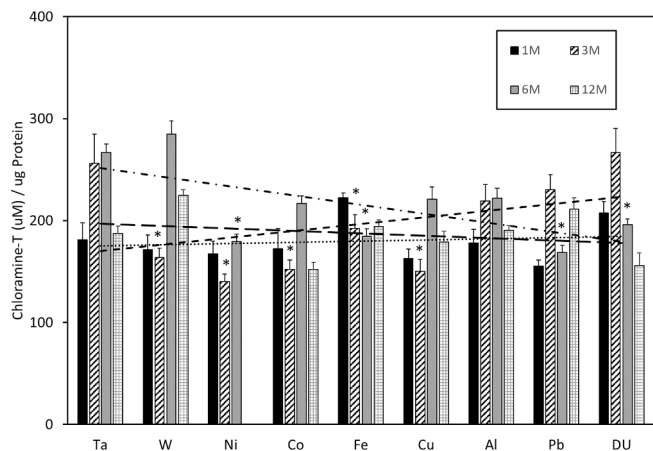


Fig. 3. Lipid peroxidation assessment of metal-implanted rat gastrocnemius muscle. Gastrocnemius homogenates from metal-implanted rats from four different groups: 1 month (M), 3 month (M), 6 month (M), or 12 month (M) post-implantation. Data given as nM of TBARS per μg protein and expressed as the mean of 8 separate determinations. Note: 12 month Ni cohort was euthanized at approximately 6 months as a result of tumor formation. Error bars represent the standard error of the mean and an * indicates significant a post-hoc p-value between Ta- and target metal-implanted animals within a post-implantation period. Trendlines are depicted as follows: 1 month (.....); 3 month (-----); 6 month (-.-.-.); 12 month (- - -).

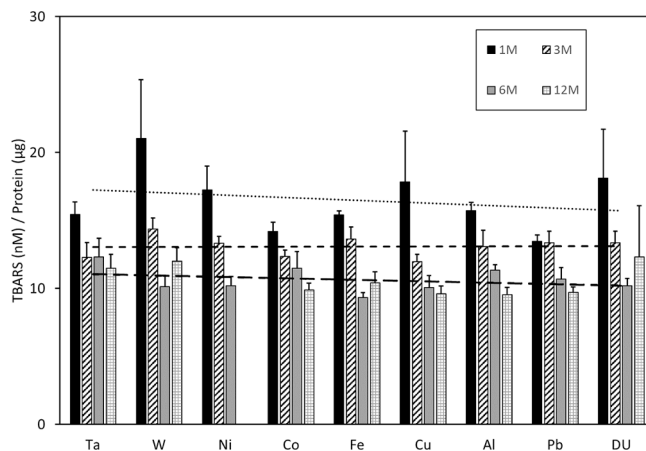


Fig. 4. Advanced oxidation protein products in metal-implanted rat gastrocnemius homogenates. Gastrocnemius homogenates from metal-implanted rats from four different groups: 1 month (M), 3 month (M), 6 month (M), or 12 month (M) post-implantation. Data given as μM of Chloramine-T per μg protein and expressed as the mean of 8 separate determinations. Note: 12 month Ni cohort was euthanized at approximately 6 months as a result of tumor formation. Error bars represent the standard error of the mean and an * indicates significant a post-hoc p-value between Ta- and target metal-implanted animals within a post-implantation period. Trendlines are depicted as follows: 1 month (.....); 3 month (-----); 6 month (-.-.-.); 12 month (- - -).

embedded metals on creatine kinase activity in skeletal muscle, there are indications that environmental metal contamination can affect activity. For example, in a zebrafish model, chronic exposure to copper oxide nanoparticles elevated creatine kinase activity in skeletal muscle (Mani

et al., 2020); while in normal muscle, creatine kinase levels decreased as the animal aged (Nuss et al., 2009). Creatine kinase activity in the gastrocnemius homogenate from tantalum-control rats decreased over time reaching a nadir at 6-months post-implantation. Conversely, with

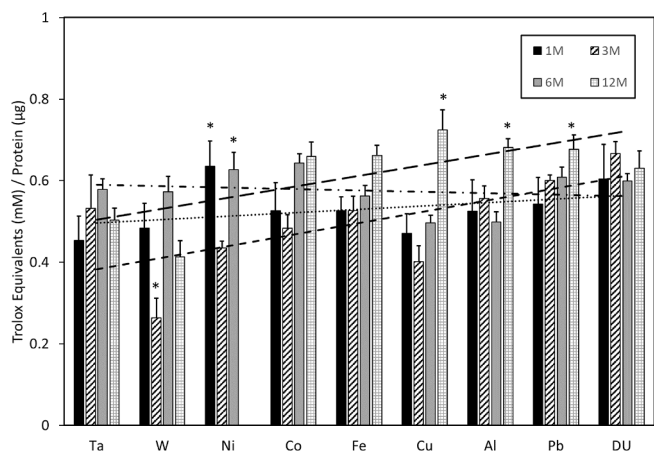


Fig. 5. Total antioxidant capacity in metal-implanted rat gastrocnemius homogenates. Gastrocnemius homogenates from metal-implanted rats from four different groups: 1 month (M), 3 month (M), 6 month (M), or 12 month (M) post-implantation. Data given as mM of Trolox Equivalents per μg protein and expressed as the mean of 8 separate determinations. Note: 12 month Ni cohort was euthanized at approximately 6 months as a result of tumor formation. Error bars represent the standard error of the mean and an * indicates significant a post-hoc p-value between Ta- and target metal-implanted animals within a post-implantation period. Trendlines are depicted as follows: 1 month (.....); 3 month (-----); 6 month (-.-.-.); 12 month (- - - -).

one exception, all 1- and 3-month post-implantation metal groups showed significantly lower creatine kinase levels compared to control. The levels at 6- and 12-months post-implantation remained low but were not significantly different from control. Creatine kinase has been reported to be released to the blood from skeletal muscle injured by either chemical damage or eccentric exercise (Brazeau and Fung, 1990; Sakamoto et al., 1996; Child et al., 1999; Chen et al., 2000; Koch et al., 2014; Baltusnikas et al., 2015; Kawamura et al., 2018). To determine whether the creatine kinase activity decrease observed in metal-implanted gastrocnemius homogenate was the result of an efflux from the muscle to the blood, we measured creatine kinase activity in the serum of the various cohorts. We found no corresponding increase in serum creatine kinase correlating to the decreased gastrocnemius activity in the 1- and 3-month post-implantation metal groups. Interestingly, there were significant increases in serum creatine kinase activity in the 12-month post-implantation Fe, Cu, Al, and Pb cohorts, as well as both the 6- and 12-month post-implantation DU groups. However, it should be noted that except for sarcoma formation in the Ni and Co groups at the later time points, there were no histopathological indications of metal-induced damage in the metal-implanted rat gastrocnemius (Wen et al., 2020). Further, although serum levels of creatine kinase have been shown to be reliable indicators of skeletal muscle damage, other proteins including aspartate transaminase, troponin I, myosin light chain 3, and fatty acid binding protein 3 have been proposed as skeletal muscle injury biomarkers (Goldstein, 2017).

We have previously reported that analysis of gastrocnemius homogenates from metal-implanted rats demonstrated that Fe, Cu, Pb, and DU significantly increased the levels of 4-hydroxynonenal-modified

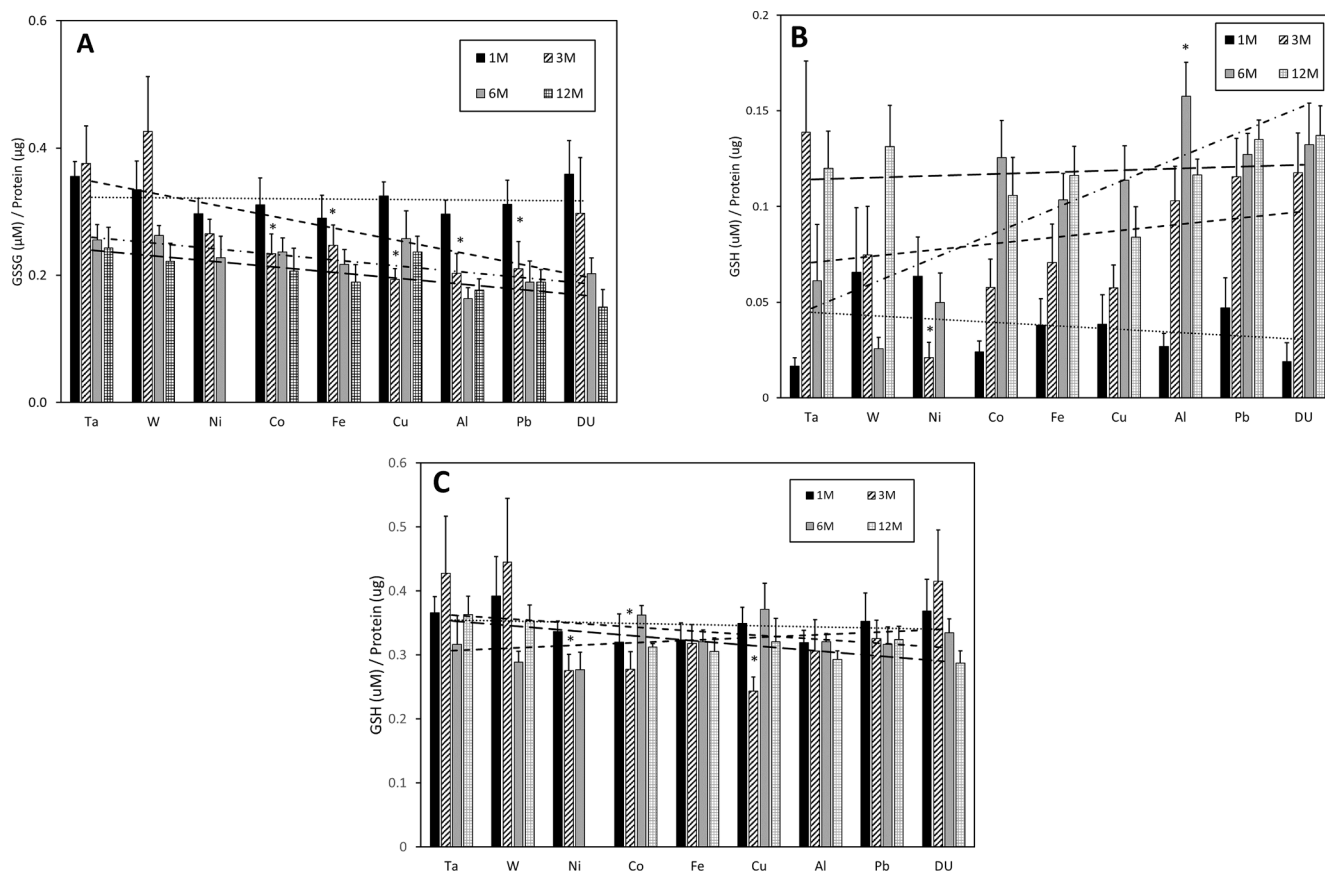


Fig. 6. Oxidized (Panel A), reduced (Panel B), and total glutathione (Panel C) levels in metal-implanted rat gastrocnemius homogenates. Gastrocnemius homogenates from metal-implanted rats from four different groups: 1 month (M), 3 month (M), 6 month (M), or 12 month (M) post-implantation. Data given as μM of glutathione per μg protein and are expressed as the mean of 8 separate determinations. Note: 12 month Ni cohort was euthanized at approximately 6 months as a result of tumor formation. Error bars represent the standard error of the mean and an * indicates significant a post-hoc p-value between Ta- and target metal-implanted animals within a post-implantation period. Trendlines are depicted as follows: 1 month (.....); 3 month (-----); 6 month (-.-.-.); 12 month (- - - -).

proteins at the later experimental time points (Hoffman et al., 2022). 4-Hydroxynonenal results from the peroxidation of ω -6 polyunsaturated fatty acids such as linoleic and arachidonic acids and has been shown to form adducts with biological molecules including lipids and proteins (Zhang and Forman, 2017). To determine if the embedded metals tested would induce more extensive lipid and protein oxidative damage, we assessed lipid peroxidation and protein oxidative damage. None of the metal-implanted groups showed a significant increase in lipid peroxidation in the gastrocnemius homogenates as compared to control. This was somewhat surprising since several of the tested metals, including iron, cobalt, copper, and uranium, are known to induce reactive oxygen species through redox-cycling reactions (Valko et al., 2005, 2016; Matsuda and Nakajima, 2012). In addition, pigeons exposed to excessive lead concentrations by environmental contamination also showed elevated lipid peroxidation levels in muscle (Kurhaluk et al., 2021). However, using an eccentric muscle exercise, neither rats, rabbits, nor humans exhibited elevated lipid peroxidation levels in muscle (Best et al., 1999; Child et al., 1999; Ramos et al., 2013).

Conversely, protein oxidation damage showed significantly lower levels in many of the metal-implantation groups but then only at the 3- and/or 6-month post-implantation time points. No experimental group showed higher protein oxidation damage when compared to control. Again, in lead-contaminated pigeons, significant protein oxidative damage was observed (Kurhaluk et al., 2021) while in exercise-stressed rats, no significant change in muscle protein oxidation was observed (Best et al., 1999; Kawamura et al., 2018).

Considering the lower-than-control protein oxidation levels as well as the lack of a significant rise in lipid peroxidation levels in metal-implanted muscle, we investigated the total antioxidant capacity in the gastrocnemius homogenates to assess whether it may have played a role in our observations. The 3-month post-implantation W cohort exhibited lower total antioxidant capacity than control while most of the other metals and time points assessed were no different than control, although they did tend to trend upward as implantation time increased. Ni at the 1- and 6-month time points and Cu, Al, and Pb at the 12-month post-implantation time point were all significantly higher than control. Whether these increases are the result of the accumulation of small antioxidant molecules or induction of those enzymes involved in the detoxification of reactive oxygen and nitrogen species is not known at this time. It should be noted that using excessive exercise as a stressor, the total antioxidant capacity of measured in rat muscle was found to be no different than control (Ramos et al., 2013; Kawamura et al., 2018). Conversely, total antioxidant capacity in human muscle after excessive exercise was found to increase (Child et al., 1999).

Although an analysis of all components of the cell's antioxidant defense system is well beyond the scope of this study, we did assess glutathione levels in the gastrocnemius homogenates from the various metal-implanted groups. Glutathione is involved in a variety of cellular activities, including radical scavenging, and is considered the most abundant non-enzymatic antioxidant in the cell (Baldelli et al., 2019). In addition, some of the implanted test metals are considered "redox-inactive" and capable of depleting glutathione (Valko et al., 2016). As shown, oxidized glutathione levels were significantly lower than control in the 3-month post-implantation Co, Fe, Cu, Al, and Pb groups. Cobalt, iron, and copper are considered redox-cycling while aluminum and lead are thought to be redox-inactive (Valko et al., 2016). Oxidized glutathione levels also tended to trend lower over time in all groups including control. Conversely, reduced glutathione levels in many of the implanted metal groups tended to trend higher. However, only the 3-month post-implantation Ni group and 6-month post-implantation Al group were significantly lower and higher, respectively, than control. Except for the 3-month post-implantation Ni, Co, and Cu groups, which were significantly lower than control, all other total glutathione levels in the metal-implanted gastrocnemius homogenates were no different than control. Most of the previously published studies on metal effects on glutathione levels have used toxic or near-toxic levels of metals to

demonstrate an effect (Hunaiti and Soud, 2000; Orihuela et al., 2005; Ozcelik and Uzun, 2009; Viehweger et al., 2011; Semprine et al., 2014; Akhtar et al., 2017; Saporito-Magrina et al., 2018). We have previously demonstrated that metal levels in the implanted gastrocnemius homogenates are, for the most part, not significantly different than levels in control homogenate despite the presence of an implanted metal pellet (Hoffman et al., 2022). Blood glutathione levels have been shown to change after eccentric exercise in humans, with GSH decreasing and GSSG increasing (Paschalis et al., 2007). In rabbits, muscle stretch injury results in an increase in both GSH and total glutathione in the injured muscle (Best et al., 1999). In addition, muscle trauma induced during surgery in humans has been shown to decrease GSH levels but not affect GSSG level significantly (Luo et al., 1996). It is not clear whether the presence of the implanted metal pellet or the low level of released metal ions are sufficient enough to significantly affect glutathione levels in the muscle. A limitation on this study was that the levels of antioxidant enzymes in the gastrocnemius homogenates were not assessed. An expanded investigation into the effect of embedded metal on those enzymes involved in antioxidant defense systems (superoxide dismutase, catalase, etc.) as well as the enzymes required for synthesis and maintenance of cellular glutathione levels (glutathione synthetase, glutathione peroxidase, glutathione reductase, etc.) might help further elucidate the potential long-term effects of metal shrapnel injuries.

5. Conclusion

Finally, injuries suffered as a result of armed conflicts often result in wounds with embedded metal fragments. Since standard surgical guidance suggests leaving embedded fragments in place to avoid the risk that extensive surgery often brings, individuals may carry these retained fragments for the remainder of their lives. Because the toxicological effects of many metals when embedded are not fully understood, there is a knowledge gap with respect to proper treatment decisions. As part of our continuing studies on the health effects of embedded metal fragments, in this manuscript we have investigated several indicators of oxidative damage. Not all metals affected all markers at all time points. Muscle creatine kinase activity was significantly decreased in most metal groups at the early experimental time points (1- and 3-months post-implantation). The other markers analyzed appeared most affected at the 3- and 6-month post-implantation time points with the exception of total antioxidant capacity which was greatly enhanced in the 12-month post-implantation Cu, Al, and Pb groups. A lifespan study with embedded metals in our rodent model system using both male and female rats would be informative with respect to assessing whether the presence of embedded metals could induce sarcopenia as the animal reaches old age. Such information would provide the basis for medical treatment decisions for health care providers dealing with shrapnel injuries.

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CRediT authorship contribution statement

John F. Kalinich: Conceptualization, Methodology, Validation, Investigation, Writing – review & editing, Funding acquisition, Software, Formal analysis, Writing – original draft, Visualization, Data curation, Supervision, Project administration, Resources. **Vernieda B. Vergara:** Methodology, Validation, Investigation, Writing – review & editing, Software, Formal analysis, Visualization, Data curation. **Jessica F. Hoffman:** Methodology, Validation, Investigation, Writing – review & editing, Software, Formal analysis, Visualization, Data curation.

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.

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Ethical approval

All procedures involving animals were (a) conducted with maximal possible well-being of the rats, (b) approved by the AFRRRI Institutional Animal Care and Use Committee (IACUC) prior to the start of the study under protocol 2016-05-006, and (c) performed in compliance with the guidelines set forth in the Guide for the Care and Use of Laboratory Animals in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited facility.

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