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Pulmonary toxicity after exposure to military-relevant heavy metal tungsten alloy particles ☆

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ABSTRACT

Significant controversy over the environmental and public health impact of depleted uranium use in the Gulf War and the war in the Balkans has prompted the investigation and use of other materials including heavy metal tungsten alloys (HMTAs) as nontoxic alternatives. Interest in the health effects of HMTAs has peaked since the recent discovery that rats intramuscularly implanted with pellets containing 91.1% tungsten/6% nickel/2.9% cobalt rapidly developed aggressive metastatic tumors at the implantation site. Very little is known, however, regarding the cellular and molecular mechanisms associated with the effects of inhalation exposure to HMTAs despite the recognized risk of this route of exposure to military personnel. In the current study military-relevant metal powder mixtures consisting of 92% tungsten/5% nickel/3% cobalt (WNiCo) and 92% tungsten/5% nickel/3% iron (WNiFe), pure metals, or vehicle (saline) were instilled intratracheally in rats. Pulmonary toxicity was assessed by cytologic analysis, lactate dehydrogenase activity, albumin content, and inflammatory cytokine levels in bronchoalveolar lavage fluid 24 h after instillation. The expression of 84 stress and toxicity-related genes was profiled in lung tissue and bronchoalveolar lavage cells using real-time quantitative PCR arrays, and *in vitro* assays were performed to measure the oxidative burst response and phagocytosis by lung macrophages. Results from this study determined that exposure to WNiCo and WNiFe induces pulmonary inflammation and altered expression of genes associated with oxidative and metabolic stress and toxicity. Inhalation exposure to both HMTAs likely causes lung injury by inducing macrophage activation, neutrophilia, and the generation of toxic oxygen radicals.

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Introduction

As the sophistication of enemy armed forces has increased, the United States and its allies have pursued more advanced armor and munitions. Although depleted uranium (DU) is considered very effective as an anti-armor ballistic material, significant controversy over the environmental and public health impact of its use in the Gulf War and the war in the Balkans has prompted the investigation and use of other materials including heavy metal tungsten alloys (HMTAs) as nontoxic alternatives (Miller and McClain, 2007; Monleau et al., 2006; van der Voet et al., 2007).

HMTAs are a category of tungsten-based substances containing 90 to 98% by weight tungsten (W) in combination with nickel (Ni), iron (Fe), copper, and/or cobalt (Co) (van der Voet et al., 2007). Due to their impressive ballistic properties (high density, strength, and stiffness) and a belief that tungsten and its alloys were relatively inert (based on studies investigating pure tungsten or tungsten carbide), HMTAs have been the leading candidates to replace DU and lead in military munitions (Gold et al., 2007; van der Voet et al., 2007). Discovery of the superior mechanical properties obtained from the W–Ni–Co and W–Ni–Fe alloy systems has led to their recent use and development for fragmentation warheads and kinetic energy penetrators for defeating heavy armor (Gold et al., 2007; van der Voet et al., 2007). Kinetic energy penetrators are armor-piercing weapons which do not contain explosives but use kinetic energy to penetrate the target; therefore they are made from the densest metals available. Because of the manufacturing process and the high melting temperature of W, HMTAs are actually a heterogeneous

Abbreviations: BAL, bronchoalveolar lavage; Cdkn1a, cyclin-dependent kinase inhibitor 1A; CINC, cytokine-induced neutrophil chemoattractant; CYP2A3, cytochrome P450, family 2, subfamily A, polypeptide 3; DCF, 2′,7′-dichlorodihydrofluorescein; DCFH-DA, 2′,7′-dichlorodihydrofluorescin diacetate; DU, depleted uranium; HMTA, heavy metal tungsten alloy; IL, interleukin; LDH, lactate dehydrogenase; Nos2/iNOS, nitric oxide synthase 2, inducible; ROS, reactive oxygen species; RNS, reactive nitrogen species; TNF-α, tumor necrosis factor alpha; WNiCo, reconstituted mixture of tungsten (W), nickel (Ni), and cobalt (Co); WNiFe, reconstituted mixture of tungsten (W), nickel (Ni), and iron (Fe).

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mixture of pure W particles (tungsten phase) embedded in a matrix that is a true alloy (binder phase) made up of a small amount of dissolved W and alloying elements such as Ni, Co, and Fe (Harris et al., 2011; Kalinich et al., 2008; Machado et al., 2010, 2011). The binder phase corrodes more rapidly than the tungsten phase, releasing the alloying elements into solution (Kalinich et al., 2008; Ogundipe et al., 2006).

Interest in the health effects of HMTAs has peaked since the recent discovery that rats intramuscularly implanted with pellets containing 91.1% W, 6% Ni, and 2.9% Co rapidly developed aggressive metastatic tumors at the implantation site (Kalinich et al., 2005). This unanticipated finding raised extreme concern over the potential health effects of HMTA-based munitions, and, as a result, long-term exposure from internalized retained HMTA fragments has been a major focus of attention by the military medical community (Kalinich et al., 2008; Kane et al., 2009; van der Voet et al., 2007). The *in vivo* carcinogenic potential of HMTAs containing W, Ni, and Co is supported by *in vitro* studies which demonstrated that exposure to military-relevant mixtures of W, Ni, and Co (WNiCo) induced malignant transformation, generation of reactive oxygen species (ROS), oxidative DNA damage, and expression of several stress genes in various cultured cell types, suggesting a synergistic effect that exceeded the effects of the metals individually (Harris et al., 2011; Miller et al., 2002, 2004). Although military-relevant mixtures of W, Ni, and Fe (WNiFe) also induced genotoxic effects and induced cell transformation *in vitro* (Miller et al., 2001), other studies found that WNiFe was less toxic than WNiCo and did not induce tumors in rats (Harris et al., 2011; Kalinich et al., 2005; Roszell et al., 2008).

In addition to risks associated with HMTAs after internalization as retained fragments, military personnel may be at risk of respiratory tract injury after acute exposure to aerosolized HMTA particles generated at high temperature when tungsten alloy munitions strike hard targets (Gold et al., 2007; Machado et al., 2010, 2011). The Capstone DU Aerosol Characterization and Risk Assessment Study was conducted initially to assess inhalation exposure to DU aerosols and risk to soldiers in combat vehicles and to first responders at the time of perforation by a DU penetrator (Parkhurst and Guilmette, 2009). The predicted median inhalation intakes of DU from the generated aerosols ranged from a low of 10 mg for a 1-minute exposure in a ventilated Abrams tank with DU armor to a high of 710 mg for a 5-minute exposure in an unventilated Abrams tank with DU armor. Gold et al. (2007) conducted a quantitative analysis of aerosols generated inside of an armored vehicle perforated by a kinetic energy penetrator containing W, Ni, and Co. They showed that respirable particles (<10 μm) were generated at doses twice the concentration listed as immediately dangerous to life or health by the National Institute for Occupational Safety and Health. More recently, Machado et al. (2010, 2011) characterized the size distribution and composition of particulates created by W–Ni–Co and W–Ni–Fe penetrator rods perforating a series of steel target plates and determined the relative fractions of Co, Ni, W, and Fe. Particulates collected on individual filters were discovered to be highly toxic to human lung epithelial cells, suggesting severe human toxicity potential for inhaled ballistic aerosolized metals (Machado et al., 2010, 2011).

Despite these concerns, pulmonary toxicity caused by HMTA-based metallic particles has never been investigated *in vivo*, and very little is known concerning the molecular mechanisms leading to the pathological effects associated with inhalation exposure to HMTAs. In the present study we wished to determine whether *in vivo* exposure to military-relevant reconstituted HMTA mixtures of W, Ni, and Co (WNiCo) or W, Ni, and Fe (WNiFe) induces pulmonary inflammation and altered expression of genes associated with oxidative and metabolic stress and toxicity in the rat. We also wished to determine the effects of *in vitro* exposure on two parameters of lung macrophage function, the oxidative burst response and phagocytosis.

Material and methods

Metal powders. The weight percentage composition of HMTAs containing W/Ni/Co or W/Ni/Fe currently used in military munitions is approximately 91–93% W, 3–5% Ni, and either 2–4% Co or 2–4% Fe (Miller et al., 2001; van der Voet et al., 2007). Because HMTAs used by the military are not commercially available, we used a mixture of these metals, in the same percentages used by the military, to model the particles of the alloy similar to the methods of Miller et al. (2001). Metals were obtained from Alfa Aesar (Ward Hill, MA) and included tungsten powder (W; Alfa Aesar 10400, 99.9% purity, median particle size 1–5 μm), nickel powder (Ni; Alfa Aesar 10256, 99.9% purity, median particle size 3–7 μm), cobalt powder (Co; Alfa Aesar 10455, 99.8% purity, median particle size 1.6 μm), and iron powder (Fe; Alfa Aesar 00170, 99.9% purity, median particle size <10 μm). The alloys tested in this study (WNiCo and WNiFe) consisted of a pure mixture of insoluble W (92%), Ni (5%), and either Co (3%) or Fe (3%) particles made in the laboratory. Dose–response experiments were conducted by altering the amounts of each metal powder, based upon its percentage of 100% of the total amount of powders. For example, 1 mg WNiCo was composed of 0.92 mg W, 0.05 mg Ni, and 0.03 mg Co, while 2 mg WNiCo consisted of 1.84 mg W, 0.1 mg Ni, and 0.06 mg Co. Thus the total amount (weight) of the metal powder mixture was varied while the ratio of the component metals was held constant. Effects of individual metals were also examined according to their percentages in the WNiCo and WNiFe mixtures. For *in vivo* studies, insoluble metal particles were suspended in sterile saline and agitated immediately prior to intratracheal instillation. For *in vitro* studies, particles were suspended in acetone and vortexed immediately before being dispensed into cell cultures.

Animals. Adult male Sprague–Dawley rats (9 weeks of age, approximately 300 g) were obtained from Taconic (Germantown, NY) and housed in polycarbonate cages in HEPA-filtered laminar airflow racks with wood chip bedding under controlled lighting conditions (12 h light/12 h dark). The rats were allowed free access to standard laboratory diet and tap water. The study protocol was approved by the Institutional Animal Care and Use Committee at Tripler Army Medical Center. Investigators complied with the policies as prescribed in the USDA Animal Welfare Act and the National Research Council's "Guide for the Care and Use of Laboratory Animals." Facilities are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Each experiment or assay was performed using 3–6 different rats per treatment group.

Intratracheal instillation. Rats were lightly anesthetized by an intraperitoneal (ip) injection of sodium pentobarbital (20 mg/kg) and/or isoflurane (2–5%). Animals were intratracheally instilled with WNiCo or WNiFe (1, 2, and 4 mg/100 g body weight) or individual metals in an instillation volume of 1 ml/kg body weight sterile saline suspension using a 16-gauge catheter (1.7×50 mm, B. Braun Medical, Inc., Bethlehem, PA) according to the method of Brain et al. (1976). Rats in the vehicle control group were instilled with 1 ml/kg body weight sterile saline. Doses chosen for this study were adapted from those used in *in vivo* studies of hard metal alloys consisting of tungsten carbide and Co particles and fell within the range of doses that induced acute pulmonary toxicity (De Boeck et al., 2003; Huaux et al., 1995; Lison and Lauwerys, 1994). Because this study focused on the effects of acute exposure, all rats were allowed to recover for 24 h before subsequent experiments were performed.

Bronchoalveolar lavage (BAL) and cell differentials for measurement of pulmonary inflammation. Twenty-four hours after intratracheal instillation, rats were euthanized with sodium pentobarbital (150 mg/kg, ip). A 16 gauge catheter was inserted into the trachea through a cut