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Research article

Tissue distribution patterns of solubilized metals from internalized tungsten alloy in the F344 rat

Vernieda B. Vergara[1], Christy A. Emond[1,2] and John F. Kalinich[1,*]

[1] Internal Contamination and Metal Toxicity Program, Armed Forces Radiobiology Research Institute, Uniformed Services University, 8901 Wisconsin Avenue, Bethesda, Maryland 20889-5603, USA

[2] Current address: Department of Veterans Affairs, Manchester Veterans Affairs Medical Center, 718 Smyth Road, Manchester, New Hampshire 03104, USA

* **Correspondence**: Email: john.kalinich@usuhs.edu; Tel: +1-301-295-9242; Fax: +1-301-295-1731.

Abstract: Because of its unique physical and chemical properties, tungsten has been increasingly utilized in a variety of civilian and military applications. This expanded use also raises the risk of human exposure through internalization by various routes. In most cases the toxicological and carcinogenic properties of these tungsten-based compounds are not known nor are the dissolution biokinetics and ultimate fate of the associated metals. Using a laboratory rodent model system designed to assess the health effects of embedded metals, and a tungsten alloy comprised of tungsten (91.1%), nickel (6.0%), and cobalt (2.9%), we investigated the tissue distribution patterns of the metals over a six month period. Despite its perceived insolubility, tungsten rapidly solubilized from the implanted metal fragments, as did nickel and cobalt. All three metals distributed systemically over time with extremely elevated levels of all three metals found in kidney, liver, and spleen. Unexpectedly, tungsten was found to cross the blood-brain and blood-testis barriers and localize in those tissues. These results, along with recent reports suggesting that tungsten is a tumor promoter, raises serious concerns as to the long-term health effects of exposure to tungsten and tungsten-based compounds.

Keywords: tungsten alloy (WA); tungsten (W); nickel (Ni); cobalt (Co); wound; contamination; rat; inductively coupled-plasma mass spectrometry (ICP-MS)

1. Introduction

Tungsten, Swedish for "heavy stone", was first isolated in the mid-18th century. Its physical properties, including its high density and melting temperature, make it uniquely suited for a variety of applications. Tungsten is found in air, soil, and water in varying concentrations. In soil, it is found as a mineral mixture, primarily as scheelite or wolframite [1]. Tungsten can then be released into the air or groundwater as a result of the weathering and dissolution of soil. Environmental levels of tungsten are usually very low, except in areas containing natural deposits or tungsten mines [2]. As a result, uptake of tungsten is generally very low, with occupational exposures being the most likely route of internalization in humans.

Because of its unique physical characteristics, tungsten is used in a variety of applications including light bulb filaments, thermocouples, counterweights, and radiation shields. Since pure tungsten is extremely brittle, it is often combined with other elements to obtain the specific characteristics desired. For example, tungsten carbide (WC) and tungsten carbide-cobalt (WC/Co) are used in cutting blades and drill bits. As a result of the potential risk of lead toxicity, tungsten-based materials have also been used as replacements for lead in small-caliber ammunition. The United States Fish and Wildlife Service banned the use of lead shot for waterfowl hunting starting in 1991 and recommended the use of formulations that would be nontoxic if ingested by wildlife [3]. Many of these new formulations contain varying amounts of tungsten in combination with other metals such as copper, tin, bismuth, nickel, and iron. Toxicity assessments have thus far shown no adverse health effects of these formulations [4-9].

Tungsten is also used in a variety of military applications, most notably as a proposed replacement for depleted uranium in kinetic-energy armor penetrating munitions [10]. In these formulations, tungsten is usually combined with two or more transition metals such as iron, cobalt, nickel, or copper. However, the formulations and manufacturing processes for civilian and military tungsten-based munitions differ greatly which leads to varying metal bioavailablity and health effect outcomes when these materials are internalized. For example, while internalized tungsten-based munitions designed for hunting waterfowl showed no adverse health effects, military-grade munition material comprised of tungsten (91.1%), nickel (6.0%), and cobalt (2.9%) induced malignant rhabdomyosarcomas when implanted into the leg muscle of laboratory rodents [11-13]. Conversely, no tumors were observed when a military-grade material composed of tungsten (91%), nickel (7%), and iron (2%) was implanted [12,13].

For many years, tungsten was thought to be insoluble and relatively inert; however, that has not proven to be the case. As described above, some tungsten-based materials (W/Ni/Co) are carcinogenic while others (W/Ni/Fe) induce no adverse health effects. Studies with laboratory rodents demonstrated, in both cases, after implantation, both materials began to solubilize resulting in the release of the component metals of the material, including tungsten [12,13]. Additionally, studies investigating the environmental fate of munition-grade tungsten material in soil demonstrated that, over time, tungsten would eventually solubilize and could migrate into the groundwater [14-16]. Of greater concern is that the solubilization of the tungsten resulted in a more acidic soil pH enhancing the solubility and migration ability of other metal contaminants [17-19]. Increasing use of tungsten-based materials in both civilian and military applications will increase the risk of exposure to materials whose health effects are not entirely known. Exposure can occur by a variety of routes including inhalation, ingestion, and wound contamination. Our laboratory has been at the forefront investigating the health effects of internalized military-relevant metals. We report here our results on

the tissue distribution patterns of metals solubilized from an implanted military-grade tungsten alloy composed of tungsten, nickel, and cobalt.

2. Materials and methods

2.1. Test samples

The metals used in this experiment were in the form of pellets, cylindrical in shape with a diameter of 1 mm and a length of 2 mm. Tungsten alloy (WA) pellets were produced by Aerojet Ordnance Tennessee (Jonesborough, TN USA) and consisted of 91.1% tungsten, 6.0% nickel, and 2.9% cobalt. Tantalum (Ta) pellets (99.95% Ta, Alfa Aesar, Ward Hill, MA USA) were used as a control. Before implantation surgery, all pellets were cleaned and chemically sterilized as previously described [13].

2.2. Rodents

F344 rats (male, 6 weeks of age, Harlan, Frederick, MD USA) were maintained in an Association for Assessment and Accreditation of Laboratory Animal Care-International (AAALAC)-accredited facility in accordance with the Guide for the Care and Use of Laboratory Animals [20]. All procedures were approved by the Armed Forces Radiobiology Research Institute (AFRRI) Animal Care and Use Committee prior to initiation. Upon arrival, animals were screened for common rodent pathogens. Rodents were paired-housed in plastic microisolator cages with hardwood chips for bedding and fed a certified NTP-2000 (Quality Lab Products, Elkridge, MD USA) diet [21]. Water was provided *ad libitum*. Animals were on a 12-hr light/dark cycle with no twilight.

2.3. Metal implantation

All rats were implanted at 9 weeks of age with a total of 20 pellets, evenly split between each hind leg. Experimental groups (10 rats per group) included a non-surgical control (no metal), Ta (control, 20 Ta pellets), low-dose WA (4 WA pellets and 16 Ta pellets), and high-dose WA (20 WA pellets). Tantalum was used as a negative implantation control because it is considered inert and has been used in human prostheses [22-24]. For pellet implantation, anesthesia was induced by continuous administration of isoflurane using an open circuit system with a scavenger/recapture system. All surgery was done using aseptic techniques. After the surgical sites were clipped and cleansed with betadine, an incision was made through the skin to expose the gastrocnemius muscle. Pellets were implanted in the muscle; spread approximately 1.5 mm apart on the lateral side of each leg. The incision was closed with sutures and surgical adhesive. Rats were closely monitored after surgery until they were ambulatory. A prophylactic dose of analgesic (buprenorphine hydrochloride, Reckitt and Colman, Hull, UK) was administered preoperatively and then as needed postoperatively. At 1, 3, and 6 months post-implantation or when moribund, rats were humanely euthanized by exsanguination under deep isoflurane-induced anesthesia and a variety of tissues collected.

2.4. Sample preparation

Prior to metal analysis, tissue samples were first dried in a muffle furnace (Fisher Scientific, Pittsburgh, PA USA) at 100 ºC for 24 h. The temperature of the furnace was then ramped to 350 ºC, at 5 ºC/min, and the samples held there for 24 h. The furnace temperature was then ramped to 600 ºC, at 5 ºC/min, and the samples held there for 48 h. After cooling, the samples were wet-ashed with 5 ml of 70% nitric acid (Optima Ultrapure Grade, Fisher Scientific) and 200 µL of 30% hydrogen peroxide (Semiconductor Grade, Sigma-Aldrich Chemical Co., St. Louis, MO USA) then heated to just below boiling until completely evaporated. The samples were then dry-ashed for 12 h at 600 ºC in a muffle furnace prior to another cycle of wet-ashing with nitric acid and hydrogen peroxide. After the second run of wet-ashing, the resulting white residue was dissolved in 2% nitric acid and analyzed.

2.5. Metal analysis

Inductively coupled plasma-mass spectrometry was used for determination of metal content. An X Series 2 Inductively Coupled Plasma-Mass Spectrometer (ThermoElectron North America, LLC., Madison, WI USA) equipped with a Cetac ASX520 Autosampler (Cetac Technologies, Omaha, NE USA) was utilized. The plasma gas was high-purity (99.997%) liquid argon. The appropriate metal standards obtained from SPEX CertiPrep (Metuchen, NJ USA) in 2% HNO_3 were used for instrument calibration. Metal concentration levels were obtained by reference to the slope of the calibration curve (counts per second/ng per liter) as well as an internal standard [13,25]. Calibration was done with a 7-point standard external calibration curve with tungsten, nickel, and cobalt standard concentrations ranging from 0.05 to 50 ppb and was done after every ten experimental samples. The internal standard utilized was indium (^{115}In). The limits of detection and limits of quantitation, respectively, are as follows: tungsten (0.12 ppb/0.15 ppb); nickel (0.17 ppb/0.21 ppb); cobalt (0.03 ppb/0.06 ppb).

2.6. Statistical analysis

Data were analyzed by a one-way ANOVA to assess the effect of the embedded pellets on tissue metal levels. Experimental groups were considered statistically different from the non-implanted control group if $P < 0.05$.

3. Results

Inductively coupled plasma-mass spectrometry (ICP-MS) was used to analyze metal levels in the collected tissues. The instrument parameters and operating conditions are given in Table 1.

Samples of muscle tissue from the gastrocnemius, distal to the site of pellet implantation, were analyzed for cobalt, nickel, and tungsten content. As seen in Table 2, low levels of cobalt and nickel were found in both the non-surgical control group as well as the tantalum (metal load) control group. Tungsten levels were below the limit of detection in these groups.

Table 1. ICP-MS operating conditions and parameters.

Instrument parameters	
Nebulizer type	Concentric
Spray chamber	Conical, with impact bead
Sampler cone	Platinum, 1 mm orifice diameter
Skimmer cone	Platinum, 0.7 mm orifice diameter
Sample uptake rate	1.0 ml/min
Sample read delay	60 sec
Plasma conditions	
RF power	1400 W
Plasma argon gas flow	13.0 L/min
Auxiliary argon gas flow	0.80 L/min
Nebulizer gas flow	0.94 L/min
Mass spectrometer settings	
Scanning mode	Peak Jump
Sweeps	100
Dwell time	600 µs
Channels/mass	1
Acquisition time	18 sec
Number of readings/replicate	5
Number of replicates	2

Table 2. Muscle metal levels in the 1-, 3-, and 6-month experimental groups.

1 Month Groups			
	Cobalt	Nickel	Tungsten
Non-surgical Control	0.82 ± 0.04	9.91 ± 1.65	BD
Tantalum Control	0.73 ± 0.02	4.24 ± 0.41 *	BD
WA-low dose	7.32 ± 0.90 *	13.76 ± 1.39	2.57 ± 0.46 *
WA-high dose	19.64 ± 1.35 *	25.09 ± 1.06 *	5.94 ± 0.42 *
3 Month Groups			
	Cobalt	Nickel	Tungsten
Non-surgical Control	0.52 ± 0.04	2.54 ± 0.11	BD
Tantalum Control	0.62 ± 0.05	6.22 ± 0.81*	BD
WA-low dose	5.40 ± 0.41 *	8.24 ± 1.58 *	1.86 ± 0.22 *
WA-high dose	14.51 ± 0.84 *	14.49 ± 1.32 *	8.39 ± 1.68 *
6 Month Groups			
	Cobalt	Nickel	Tungsten
Non-surgical Control	0.49 ± 0.03	4.79 ± 0.95	BD
Tantalum Control	0.46 ± 0.02	2.20 ± 0.22 *	BD
WA-low dose	5.33 ± 0.49 *	4.72 ± 1.29 *	3.12 ± 1.01 *
WA-high dose	14.75 ± 1.25 *	8.10 ± 0.66 *	35.77 ± 9.02 *

Data represent mean and standard error of the mean of 10 independent measurements expressed as ng metal per g tissue. * indicates a result statistically different than the non-surgical control at $P < 0.05$ using one-way ANOVA. BD—below the limit of detection.

Conversely, muscle samples from both the low-dose tungsten alloy (WA) group (4 pellets of WA + 16 pellets of Ta) and the high-dose tungsten alloy group (20 pellets of WA) showed significantly elevated levels of all three metals over time. This indicates that the internalized pellets are capable of being solubilized *in vivo* with low levels of the released metals capable of diffusing through the adjacent tissue.

Metal levels in liver samples from the experimental groups showed similar results (Table 3), although far higher levels of tungsten were found in the livers of the WA groups.

Table 3. Liver metal levels in the 1-, 3-, and 6-month experimental groups.

1 Month Groups			
	Cobalt	Nickel	Tungsten
Non-surgical Control	4.43 ± 0.15	4.01 ± 0.59	BD
Tantalum Control	3.92 ± 0.21	1.64 ± 0.15 *	BD
WA-low dose	48.53 ± 3.84 *	6.95 ± 0.98 *	6.31 ± 0.65 *
WA-high dose	139.03 ± 8.59 *	13.02 ± 0.78 *	31.82 ± 1.82 *
3 Month Groups			
	Cobalt	Nickel	Tungsten
Non-surgical Control	1.80 ± 0.13	3.17 ± 0.35	BD
Tantalum Control	1.54 ± 0.13	8.04 ± 2.20	BD
WA-low dose	43.44 ± 2.37 *	4.49 ± 0.20 *	6.66 ± 0.76 *
WA-high dose	121.75 ± 4.98 *	15.70 ± 2.89 *	39.92 ± 4.70 *
6 Month Groups			
	Cobalt	Nickel	Tungsten
Non-surgical Control	2.28 ± 0.14	2.98 ± 0.41	BD
Tantalum Control	4.63 ± 1.29	6.20 ± 0.38 *	2.47 ± 0.60 *
WA-low dose	72.66 ± 10.49 *	7.21 ± 0.76 *	62.29 ± 6.41 *
WA-high dose	231.22 ± 21.30 *	16.32 ± 1.42 *	306.44 ± 60.75 *

Data represent mean and standard error of the mean of 10 independent measurements expressed as ng metal per g tissue. * indicates a result statistically different than the non-surgical control at $P < 0.05$ using one-way ANOVA. BD—below the limit of detection.

Again, accumulation of all three metals comprising the WA was highest in the tissue from the WA-high dose animals, with tungsten showing both time- and dose-dependency. There was a small amount of tungsten found in the livers of the tantalum control group, but only at the 6-month time point, probably due to accumulation of diet-associated tungsten. Spleen metal levels are presented in Table 4. As seen with other tissues, both WA-implanted groups accumulated significantly higher levels of all three metals than the non-surgical and tantalum control groups. As before, metal accumulation was greatest for the WA-high dose group. Surprisingly, measureable levels of tungsten were detected in both the non-surgical and tantalum control groups at all three experimental time points.

Table 4. Spleen metal levels in the 1-, 3-, and 6-month experimental groups.

1 Month Groups			
	Cobalt	Nickel	Tungsten
Non-surgical Control	2.39 ± 0.12	58.25 ± 7.90	1.28 ± 0.41
Tantalum Control	3.33 ± 0.09 *	63.99 ± 4.23	3.74 ± 0.34 *
WA-low dose	22.24 ± 1.49 *	68.31 ± 3.02	67.59 ± 5.60 *
WA-high dose	48.00 ± 2.24 *	115.22 ± 7.18 *	243.65 ± 8.99 *
3 Month Groups			
	Cobalt	Nickel	Tungsten
Non-surgical Control	1.96 ± 0.19	17.77 ± 4.64	1.48 ± 0.31
Tantalum Control	2.18 ± 0.14	19.64 ± 1.47	3.21 ± 0.24 *
WA-low dose	24.97 ± 1.40 *	22.76 ± 2.10	112.06 ± 5.04 *
WA-high dose	70.30 ± 3.76 *	35.25 ± 3.21 *	363.04 ± 21.67 *
6 Month Groups			
	Cobalt	Nickel	Tungsten
Non-surgical Control	2.20 ± 0.22	36.58 ± 5.81	4.48 ± 1.27
Tantalum Control	2.06 ± 0.27	27.23 ± 8.75	7.64 ± 0.85
WA-low dose	23.11 ± 0.91*	64.38 ± 3.72 *	227.59 ± 22.84 *
WA-high dose	70.38 ± 4.26 *	153.61 ± 10.48 *	696.66 ± 27.40 *

Data represent mean and standard error of the mean of 10 independent measurements expressed as ng metal per g tissue. * indicates a result statistically different than the non-surgical control at $P < 0.05$ using one-way ANOVA. BD—below the limit of detection.

By far, the greatest accumulation of metals solubilized from embedded WA pellets was found in the kidney (Table 5). Again, significantly elevated levels of cobalt, nickel, and tungsten were found in both WA groups compared to both non-surgical and tantalum control groups. Tungsten accumulation in the kidneys of the WA groups occurred in a dose- and time-dependent manner. Conversely, although all three of the assayed metals were found at detectable levels in the kidneys of non-surgical and tantalum control animals, these levels were low and showed no correlation with the experimental time course.

Table 5. Kidney metal levels in the 1-, 3-, and 6-month experimental groups.

1 Month Groups			
	Cobalt	Nickel	Tungsten
Non-surgical Control	36.29 ± 0.55	7.15 ±1.77	3.58 ± 0.84
Tantalum Control	32.97 ± 0.88	16.31 ± 1.65 *	1.30 ± 0.07
WA-low dose	162.87 ± 12.72 *	104.18 ± 4.44 *	41.47 ± 1.87 *
WA-high dose	321.17 ± 10.78 *	264.75 ± 9.16 *	258.80 ± 7.94 *
3 Month Groups			
	Cobalt	Nickel	Tungsten
Non-surgical Control	36.59 ± 0.31	5.33 ± 0.94	2.50 ± 0.45
Tantalum Control	33.84 ± 0.46	9.77 ± 1.81	2.53 ± 0.10
WA-low dose	151.53 ± 5.61 *	78.72 ± 4.10 *	87.91 ± 10.77 *
WA-high dose	368.70 ± 19.03 *	263.62 ± 16.43 *	656.27 ± 102.04 *

6 Month Groups			
	Cobalt	Nickel	Tungsten
Non-surgical Control	29.63 ± 0.61	5.00 ± 1.35	1.96 ± 0.38
Tantalum Control	47.11 ± 2.91 *	4.60 ± 0.93	8.54 ± 2.28
WA-low dose	217.52 ± 43.21 *	162.33 ± 30.02 *	1023.27 ± 183.58 *
WA-high dose	385.26 ± 17.90 *	280.54 ± 12.36 *	2157.74 ± 15.63 *

Data represent mean and standard error of the mean of 10 independent measurements expressed as ng metal per g tissue. * indicates a result statistically different than the non-surgical control at $P < 0.05$ using one-way ANOVA. BD – below the limit of detection.

Two organs with robust blood-tissue barriers are the testes (Sertoli cell barrier) and the brain (blood-brain barrier). These semi-permeable barriers are designed to tightly regulate the passage of molecules from the blood to the protected tissue. To that end, metal levels in the testes (Table 6) and brain (Table 7) of non-surgical and tantalum control rats are extremely low in the case of cobalt and nickel, and below the limit of detection for tungsten. However, for both the low- and high-dose tungsten alloy groups, significantly elevated levels of all three metals were found in both tissues at all experimental time points. Further, although dose-dependent differences were apparent between the WA groups, the time-dependency observed for these groups showed either a biphasic or reverse reciprocal response.

Table 6. Testes metal levels in the 1-, 3-, and 6-month experimental groups.

1 Month Groups			
	Cobalt	Nickel	Tungsten
Non-surgical Control	0.59 ± 0.03	0.73 ± 0.23	BD
Tantalum Control	0.58 ± 0.02	0.20 ± 0.03	BD
WA-low dose	3.81 ± 0.36 *	5.50 ± 0.57 *	4.46 ± 0.29 *
WA-high dose	17.02 ± 0.81 *	13.17 ± 0.40 *	18.99 ± 0.86 *
3 Month Groups			
	Cobalt	Nickel	Tungsten
Non-surgical Control	0.57 ± 0.02	0.60 ± 0.12	BD
Tantalum Control	0.60 ± 0.01	2.42 ± 0.37 *	BD
WA-low dose	3.51 ± 0.40 *	4.13 ± 0.14 *	3.70 ± 0.34 *
WA-high dose	8.72 ± 0.52 *	8.77 ± 0.48 *	13.51 ± 1.12 *
6 Month Groups			
	Cobalt	Nickel	Tungsten
Non-surgical Control	0.66 ± 0.04	1.77 ± 0.30	BD
Tantalum Control	0.71 ± 0.03	1.03 ± 0.13	BD
WA-low dose	3.38 ± 0.26 *	4.23 ± 0.45 *	11.79 ± 1.10 *
WA-high dose	15.76 ± 1.88 *	13.97 ± 1.30 *	54.44 ± 9.43 *

Data represent mean and standard error of the mean of 10 independent measurements expressed as ng metal per g tissue. * indicates a result statistically different than the non-surgical control at $P < 0.05$ using one-way ANOVA. BD—below the limit of detection.

Table 7. Brain metal levels in the 1-, 3-, and 6-month experimental groups.

1 Month Groups			
	Cobalt	Nickel	Tungsten
Non-surgical Control	0.90 ± 0.07	3.66 ± 1.21	BD
Tantalum Control	0.73 ± 0.02	1.83 ± 0.15	BD
WA-low dose	3.53 ± 0.26 *	9.66 ± 0.61 *	0.79 ± 0.06 *
WA-high dose	12.74 ± 0.43 *	19.47 ± 0.94 *	2.96 ± 0.27 *
3 Month Groups			
	Cobalt	Nickel	Tungsten
Non-surgical Control	0.64 ± 0.02	3.17 ± 0.57	BD
Tantalum Control	0.75 ± 0.03	7.49 ± 1.82 *	BD
WA-low dose	3.98 ± 0.30 *	6.75 ± 0.77 *	0.82 ± 0.15 *
WA-high dose	11.56 ± 0.58 *	12.44 ± 1.12 *	2.01 ± 0.11 *
6 Month Groups			
	Cobalt	Nickel	Tungsten
Non-surgical Control	0.73 ± 0.04	2.79 ± 0.42	BD
Tantalum Control	0.71 ± 0.03	1.40 ± 0.32 *	BD
WA-low dose	4.45 ± 0.39 *	4.14 ± 0.93 *	1.06 ± 0.23 *
WA-high dose	9.34 ± 0.51 *	36.53 ± 2.38 *	2.28 ± 0.25 *

Data represent mean and standard error of the mean of 10 independent measurements expressed as ng metal per g tissue. * indicates a result statistically different than the non-surgical control at $P < 0.05$ using one-way ANOVA. BD—below the limit of detection.

4. Discussion

For decades, tungsten was considered both insoluble and inert. However, a greater appreciation of the unique chemical and physical properties of tungsten led to the development of compounds which combine tungsten with other elements, usually transition metals. The components (and percentages) comprising these mixtures can be adjusted to produce a material with the desired strength and chemical properties. However, the expanding role of these tungsten-based materials in civilian and military applications has greatly increased the chance of human exposure to these compounds. In most cases, little is known as to the toxicological properties and long-term health risks of these unique mixtures.

The health effects of exposure to environmental sources of tungsten also came under scrutiny following the appearance of cancer clusters in the Fallon, Nevada and Sierra Vista, Arizona areas. Both these regions have elevated levels of tungsten, as well as other heavy metals, in soil and water [26]. An investigation by the United States Center for Disease Control discounted tungsten exposure as a direct cause of the observed cancers [27]; however, adverse synergistic interactions between tungsten and the other heavy metal contaminants found, including cobalt, uranium, and antimony, as well as arsenic, were not ruled out.

For the past decade, our laboratory has investigated the health effects of embedded fragments of military-relevant metals and metal mixtures. The tungsten alloy used in this study was originally proposed to replace depleted uranium in armor-penetrating munitions. However, using a laboratory rat model to simulate a shrapnel injury and assess the health effects of embedded fragments of this

material, we demonstrated the tungsten/nickel/cobalt alloy induced highly aggressive malignant rhabdomyosarcomas at the pellet implantation site [11]. Further, despite the assumed insolubility of tungsten, we also found that all three component metals of the alloy solubilized *in vivo* and could be easily detected in serum and urine [28]. A second study in a laboratory mouse model also showed that embedded tungsten/nickel/cobalt alloy could induce rhabdomyosarcomas, but these tumors were not as aggressive as those found in the rat [13]. As seen in the rat model, the tungsten/nickel/cobalt alloy also solubilized in the mouse model. Surprisingly, a tungsten/nickel/iron alloy showed no carcinogenic properties in either the rat or mouse model [12,13]. An investigation designed to determine if a single metal was responsible for the tumor induction seen with the tungsten/nickel/cobalt alloy suggested that no one metal was responsible and that a synergistic effect was the most likely scenario [25]. Similar findings were also reported for an *in vitro* cellular transformation model where transformation of cells to a tumorigenic phenotype occurred to a far greater extent with the tungsten/nickel/cobalt alloy than with the metals individually [29,30]. Additionally, when added, the tumorigenic potential of the individual metals was far less than the tumorigenic potential of the alloy. This suggests that a synergistic effect between the various metal components may be responsible for the adverse health effects observed.

Metal analysis showed that tungsten, nickel, and cobalt were all solubilized from the implanted pellets. In addition, as described previously, very small amounts of tungsten, nickel, and cobalt (5, 182, and 96 ng/gm, respectively) are present in rodent chow [31] with negligible levels found in water. The three metals from the WA-implanted rodents distributed systemically. In most cases, but not all, the amount of tissue-associated metal increased as implantation time increased. Kidney showed the highest metal levels which is not surprising since all three metals have been shown to be excreted in the urine [28]. There were also low levels of tungsten, nickel, and cobalt in kidney tissue from both control groups, presumably derived from food intake.

While tungsten has been shown to translocate to the spleen [32], the spleen represented the second highest tissue with respect to tungsten concentration with the high-dose WA group 150-fold higher than control at the 6-month experimental time point. It also represented the only other control tissue with detectable tungsten levels. Elevated metal levels in the spleen could potentiate adverse immune system effects, as will be discussed shortly. Significantly elevated levels of cobalt and nickel were also found in the spleens of both WA groups. Liver was the only other tissue analyzed that contained extremely elevated levels of all three metals; however, no tungsten was detected in the livers from the control animals. Low, yet statistically significant, levels of all three metals were found in the remaining WA-group tissues analyzed. For muscle, it appears that as metals from the implanted pellet solubilize, they can diffuse through the muscle tissue to a small extent. Unexpectedly, we found that significant amounts of tungsten, nickel, and cobalt could cross the blood-brain and blood-testis (Sertoli cell) barriers in both WA groups. Although very low levels of cobalt and nickel were detected, no tungsten was found in the brain or testes from non-surgical or tantalum control rats. It is not known at this time whether localization of tungsten to these normally protected tissues is a result of disruption of the blood-tissue barriers or the hijacking of a normal transport mechanism designed to maintain metal homeostasis.

The results from this study suggest metals and metal mixtures internalized as a result of embedded fragments or wound contamination can solubilize and deposit in a variety of tissues, thus providing a long-term depot for potentially toxic metals. Although nickel and cobalt have been shown to solubilize from internalized solid fragments [11,33], tungsten was generally thought to be

insoluble. The presence of tungsten in kidney and spleen of control animals was not unexpected since tungsten is present in rat chow and biokinetic studies have shown that these organs are depot sites [32]. What was unexpected was the extremely high metal levels that accumulated in these tissues in the WA-implanted animals. Schuster et al. suggested that the metals solubilized from the embedded WA pellets come from the matrix that binds the tungsten grains [12]. The fact that embedded tungsten/nickel/cobalt pellets lose only approximately 5% of their total mass after 6-months of implantation supports this theory [11]. Earlier studies from our laboratory also demonstrated that embedded fragments of a tungsten alloy comprised of tungsten/nickel/cobalt induced rhabdomyosarcomas at the implantation sites in a rat model [11]. Another finding from that study was that the alloy also induced splenic and hematological changes indicative of polycythemia. While cobalt has been used to experimentally induce polycythemia in rats [34,35], the concentrations required are far greater than the total amount of cobalt in the WA pellets. Implantation of nickel or cobalt fragments in laboratory rats also did not result in the induction of polycythemia [11,33] nor have there been any reports in the literature of induction by tungsten administration alone. This again suggests a possible synergistic effect between the metals. Clearly, more work is needed to assess the potential health effects of internalized metal mixtures. Not only do the mixtures themselves need to be tested, but also each component metal, at the concentration found in the mixture, should be assessed. Unfortunately, funding is not always available for these extensive studies.

Recently, there have been reports in the literature suggesting that tungsten exposure, through ingestion or inhalation, can have profound effects on the immune system. Ingestion or inhalation of tungsten, followed by exposure to Respiratory Syncytial Virus, resulted in severe splenomegaly in C57BL/6J mice [36]. Further, mice exposed to tungsten in drinking water exhibited altered B-cell development and DNA damage suggesting that tungsten could act as a tumor promoter [37]. Recently, it was reported that breast cancer patients undergoing intraoperative radiotherapy using a device with a tungsten shield were left with small tungsten particulates in the breast tissue [38,39]. These tungsten particles solubilized resulting in elevated levels of tungsten in urine and blood. Further, using a rodent model of breast cancer, it was found that while tungsten exposure did not directly induce carcinogenesis, it affected the tumor microenvironment allowing for increased metastasis [38]. The findings from this study showing tungsten is capable of crossing both the blood-brain and blood-testis barriers in rodents with internalized tungsten-based material raises concern as to potential neurological effects resulting from long-term exposure to elevated levels of tungsten. The exact mechanism of tungsten-induced health effects is not known. Although several studies have investigated the effect of tungsten and tungsten-based materials on cellular toxicity, oxidative stress, and gene expression [40-43], many questions remain unanswered.

While this is the first report on tissue distribution patterns of solubilized metals from embedded tungsten alloy in the rat, an earlier study using the same alloy in a mouse model showed similar tissue distribution patterns of the solubilized tungsten [13]. In that investigation, tungsten levels increased in a time-dependent manner in kidney, liver, spleen, and testes over a 12-month period. It also appears that the biokinetic and tissue deposition patterns of tungsten are consistent, regardless of the route of internalization, as results from this study correlated with those using ingestion and inhalation as routes of administration [44-47]. After acute oral administration of sodium tungstate in mice, tungsten was found in a variety of tissues including kidney, liver, spleen, and brain [44]. Results from a chronic oral administration of sodium tungstate to pregnant rats showed that tungsten deposited systemically, was able to cross the placenta and deposit in the pup's tissues, and was also

found in the mother's milk [45]. Studies using one-time inhalation exposures to tungsten in both rats and mice again showed that tungsten deposited systemically in the body [46,47]. As the use of tungsten in civilian and military applications increases, the risk of human exposure through ingestion, inhalation, and wound contamination also rises. This concern has led the National Toxicology Program of the U.S. Department of Health and Human Services to add tungsten to their list of compounds to be assessed for adverse health effects [48] and the U.S. Environmental Protection Agency to consider tungsten as an "emerging contaminant of concern" [49]. This increased focus on the health effects of tungsten should help address any potential risk involved with exposure.

5. Conclusion

Human exposure to tungsten and tungsten-based compounds is an increasing concern due to its expanded use in civilian and military applications. In this study we have shown that, using a laboratory rat model system, a tungsten alloy embedded in the gastrocnemius muscle rapidly solubilized. All three metals comprising the alloy were distributed systemically over time. Unexpectedly, tungsten was found to cross the blood-brain and blood-testis barriers and deposit in those tissues. The tissue distribution patterns raise concern over the potential long-term health effects of tungsten internalization.

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Conflict of interest

All authors declare no conflicts of interest in this paper.

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