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TISSUE DISTRIBUTION OF TUNGSTEN IN MICE FOLLOWING ORAL EXPOSURE TO SODIUM TUNGSTATE

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14. ABSTRACT

X **A brief (200 words max) factual *summary* of the most significant information.**
The present study describes tissue distribution of tungsten in mice following oral exposure to sodium tungstate. Mice were exposed to sodium tungstate (0, 62.5, 125 and 200mg/kg/d) for 28 days, and then kidney, liver, colon, bone, brain and spleen were harvested for trace element analysis with inductively coupled plasma mass spectrometry. The results showed increasing tungsten levels in all organs, with the highest concentration found in the bones and the lowest concentration found in brain tissue. As part of a complementary study on possible effects on immune functions from tungsten exposure, subgroups of animals were also exposed either to staphylococcal enterotoxin B or lipopolysaccharide. Immune challenge did not have significant effects on tissue distribution, and gender differences were noticed only in spleen (higher concentration of tungsten in female animals). In addition, tungsten levels in this organ were correlated with increased iron levels, something that was not observed for any other organ or either of the other two metals that were analyzed (nickel and cobalt). These findings confirmed most of what has been published on tungsten tissue distribution; they also showed that the brain is relatively protected from oral exposure, and further studies are necessary to clarify the findings on spleen.

15. SUBJECT TERMS
Heavy metal tungsten alloys, Immune challenge, Oral exposure, Sodium tungstate, Tissue distribution, Toxicokinetics, Tungsten

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Abstract

Heavy metal tungsten alloys have replaced lead and depleted uranium in many munitions applications, due to public perception of these elements as environmentally unsafe. Tungsten materials left in the environment from military activities become bioavailable as tungstate, which might lead to population exposure through water and soil contamination. Although tungsten had been considered a relatively inert and toxicologically safe material, recent research findings have raised concerns about possible deleterious effects following acute and chronic exposure to this metal. Thus, in 2002 tungsten was nominated by the Centers for Disease Control and Prevention's National Center for Environmental Health for toxicology and carcinogenesis studies due to the lack of studies on this field. The present study aims to describe tissue distribution of tungsten in mice following oral exposure to sodium tungstate. In this study, laboratory mice were exposed to different oral doses of sodium tungstate (0, 62.5, 125 and 200mg/kg/d) for 28 days, and then six organs were harvested for trace element analysis with high-resolution inductively coupled plasma mass spectrometry (HR-ICP-MS). Kidney, liver, colon, bone, brain and spleen were analyzed by HR-ICP-MS. The results showed increasing tungsten levels in all organs, with the highest concentration found in the bones and the lowest concentration found in brain tissue. As part of a complementary study on possible effects on immune functions from tungsten exposure, subgroups of animals were also exposed either to staphylococcal enterotoxin B or lipopolysaccharide. Immune challenge did not have significant effects on tissue distribution, and gender differences were noticed only in spleen (higher concentration of tungsten in female animals). In addition, tungsten levels in this organ were correlated with increased iron levels, something that was not observed for any other organ or either of the other two metals that were analyzed (nickel and cobalt). These findings confirmed most of what has been published on tungsten tissue distribution; they also showed that the brain is relatively protected from oral exposure, and further studies are necessary to clarify the findings on spleen, focusing on possible immunological effects of tungsten exposure.

Keywords: Heavy metal tungsten alloys, immune challenge, oral exposure, sodium tungstate, tissue distribution, toxicokinetics, tungsten

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Introduction

Tungsten (W), also known as wolfram, is the elemental metal with the highest melting point and tensile strength at high temperatures; for this reason, it is greatly used in industry, from light bulbs to high-performance alloys. It has long been considered an inert material and, due to its resiliency and biocompatibility, W has also become very popular as a constituent of metal alloys in medical implantable devices, like prostheses in orthopedic and maxillofacial surgery,[1] dental implants,[2] intravascular embolization coils[3] and mechanic heart valves.[4] Tungsten has even been studied as a possible pharmacologic agent in the treatment of diabetes, as it has been shown to lower blood glucose both in insulin-deficient[5] and insulin-resistant animal models,[6] without strong evidence of severe toxic effects.[7] Furthermore, heavy metal tungsten alloy (HMTA)-based materials have been recently introduced as replacement for lead (Pb) and depleted uranium (DU) in small caliber ammunition and armor-penetrating munitions, respectively. This is justified by concerns regarding the acute and long term health and environmental effects of exposure to Pb and DU, which have forced the military in many countries to explore the possibility of applying toxicologically safer metals with comparable material characteristics.

The long and broad use of these W-alloys provided them the status of inert and toxicologically safe compounds. However, HMTA have recently been shown to have toxic effects *in vivo* and *in vitro*, a problem brought to attention particularly after chronic tungsten exposure has been investigated as the possible causative factor in a cluster of childhood leukemia in Fallon, Nevada since 1997.[8] Concerns about the toxicity and potential health effects of W-alloys have been corroborated by several *in vitro* studies showing genotoxicity and neoplastic transformation of cell lines exposed to W alloy particles.[9,10] Also, the use of HMTA in munitions applications raises the problem of possible deleterious health effects caused by tissue-embedded W-alloys especially after the discovery that implanted W-alloy pellets induces high-grade rhabdomyosarcoma in rats.[11]

When considering the possible deleterious environmental effects from HMTA used in munitions, the most important exposure route is the oral route. This is because most of the W from these alloys, when left in the environment, will oxidize to tungstate (WO_4^{2-}), a thermodynamically stable molecule under most environmental conditions.[12] In order to better understand the *in vivo* effects of W and W-alloys, the main objective of the present study is to describe W distribution in different organ tissues following oral exposure to sodium tungstate (Na_2WO_4) in laboratory mice. Such approach leads to further understanding of its toxicokinetics properties, revealing novel insights about tungsten effects and distribution in tissues especially after subacute exposure. These objectives are achieved by using state-of-the-art highly sensitive and precise instruments for multiple trace element analysis in biological specimens, such as high-resolution inductively coupled plasma mass spectrometry (HR-ICP-MS). In addition, the possible effects of the immune system activation on tungsten toxicokinetics could also be evaluated, since a subgroup of these animals was challenged with either lipopolysaccharide or staphylococcal enterotoxin B in order to assess the effects of W exposure on immunological parameters (A. Osterburg unpublished results). This was performed because of previous publication showing immunosuppresion after intrasplenic tungsten trioxide (WO_3) injection in rats.[13] Furthermore, iron, nickel and cobalt concentrations were determined for each organ analyzed, as these metals are the most commonly found in HMTA. This approach is justified by the interest in establishing base levels for these elements in tissues, as future studies might be concerned about their possible effects when present in combination with tungsten.

Materials and Methods

Animal procedures

The toxicokinetics of WO_4^{2-} was evaluated in young (6-9 weeks of age) pathogen free C57BL/6 mice kept on low-molybdenum rodent chow (Harlan Teklad, Madison, WI). All animal procedures were conducted under federal guidelines for the care and use of laboratory animals (National Research Council, 1996) and were approved by the University of Cincinnati Institutional Animal Care and Use Committee. They were acclimatized in quarantine for at least one week, and were given access to food and water *ad libitum* throughout the study.

Exposure groups

The cohort of 24 laboratory mice (12 male and 12 female) was divided into four exposure groups (n = 6/group). Three treatment groups were continuously exposed to tungstate for 28 days in drinking water containing sodium tungstate dihydrate ($Na_2WO_4.2H_2O$), and received either 62.5mg/kg/d, 125mg/kg/d or 200mg/kg/d of tungstate. Body weights and water consumption were monitored, and sodium tungstate concentrations in drinking water were adjusted weekly to maintain exposure levels. Negative control animals received deionized water only (*e.g.*, vehicle controls).

Immune challenge

A cohort of animals from each group was immune challenged with either a single intraperitoneal dose of lipopolysaccharide (LPS) (5µg/kg, n = 2/group), staphylococcal enterotoxin B (SEB) (20µg, n = 2/group), administered as a single dose in saline, or saline alone (n = 2/group), 24 hours prior to euthanasia and organ harvest.

Tissue processing and trace element analysis

Following euthanasia, kidneys, liver, colon, bone, brain, and spleen were harvested for tissue processing. Digestion was performed using 70% HNO_3 and 37% H_2O_2 (2:1mL) solution under a pressure and temperature controlled microwave digestion system (MarsXpress, CEM Inc, Mathews, North Carolina, USA), and then reconstituted with 2% HNO_3. After extraction, the samples were analyzed using sector field inductively coupled plasma mass spectrometer (Finnigan Element 2, Thermo Scientific Inc, Bremen, Germany), working under the settings listed on Table 1. Calibration was performed with multi-element standard solutions, with tungsten concentrations ranging from 0.02µg/kg to 20µg/kg and Indium (^{115}In) as an internal standard. Tungsten concentrations in tissues are reported per wet tissue weight.

Statistical analysis

Tungsten toxicokinetics in organ tissues was statistically evaluated for each dose group using the SPSS 14.0 for Windows. Analysis of variance (ANOVA) for mean tissue concentration was carried out to evaluate the data. Dunnett's post-hoc test was used to compare different dose groups, using 0mg/kg/d as the control group. Wilcoxon signed-ranks test was applied for gender and immune challenge comparison. Linear regression was used to assess correlation between tungsten and other trace elements in organ tissues. Differences were considered significant when $p < 0.05$.

Results

All the results were tabulated using SPSS 14.0 for Windows, and are summarized in Table 2. Background W concentrations from non-exposed (*i.e.* control animals) are shown in Table 3. The following analysis of these data was also performed using the same statistical software.

Organ accumulation under different doses

Tungsten tissue concentration, as measured by sector field ICP-MS, was very low for control animals and dramatically increased when animals received increasing daily doses of WO_4^{2-} (Figure 1). This increase was statistically significant for every organ, except for the colon (Table 4).

Total and relative tissue distribution

Among the six organs analyzed, W accumulation was higher in bone tissue, followed by spleen, colon, kidney, liver and brain (Figure 2a). Interestingly, despite inhomogeneous total W accumulation, relative distribution was fairly uniform when expressed as a percentage of the average concentration measured for the group that received 200mg/kg/d (Figure 2b).

Immune challenge effects on organ accumulation

In general, the two different immune challenges did not result in significant difference of tungsten distribution in tissues with increasing dose when compared to controls (Figure 3). This was not true only for LPS, which led to lower tungsten levels for liver and colon tissues (Table 5).

Gender discrepancies on organ accumulation

Regarding genders, tungsten distribution was uniform in all organs except spleen, in which females were found to have higher tungsten concentration when compared to their male matches (Figure 4, Table 6).

Correlation between tungsten and other trace elements

The method used for quantification of trace elements is able to generate data for many different types of elements at a time, and this allowed plotting tungsten concentration results against other metals, like iron, nickel and cobalt. Interestingly, increasing tungsten concentration was correlated to increased iron levels in spleen (Figure 5), something that was not observed for any other organ nor any other element analyzed.

Discussion

The background W concentrations found in the kidney and bone were in the same range as those previously reported in the literature.[8,14,15] None of the publications reviewed in this technical report analyzed brain W concentrations, and measured W concentration either in several intestine segments[14] or in the small intestine[15] instead of colon only. Still, the results found for colon were in accordance to those found for intestine. In addition, the low liver concentrations reported here were higher than the undetected concentrations reported in the literature.[14–16] This discrepancy might be due to the fact that for on these previous studies a quadrupole ICPMS was used (PerkinElmer Elan 6000), an instrument with a lower resolution than the magnetic sector field high-resolution ICPMS used in this investigation.

Regarding the exposure groups, although the boxplot clearly shows an increase in the mean concentration value for every organ, this was not confirmed by statistical analysis for the colon. A possible explanation to this observation is the fact this is not an accumulation organ for W, and the increasing concentration found is solely a reflex of direct deposition to the colonic mucosa due to route of exposure (*i.e.* oral exposure). Nevertheless, literature review shows that the intestine is an organ in which tungsten shows significant concentration after IV administration of 1mg/kg Na_2WO_4, in the same range as observed in the kidney, the organ in which W showed its highest accumulation.[14] This conflicting finding suggests another possible explanation for the statistical insignificance, like the small number of animals in each exposure

group; nevertheless, based on the post-hoc tests, it is still reasonable to state that W concentration in colon are increased.

Another interesting aspect of the tissue distribution after 4 weeks daily exposure to tungstate is the fact that the bone showed the highest W concentration among all tissues analyzed. As a transition metal, W can be found in different oxidation states, something that confers to it the ability to replace other endogenous metals. It has been reported, for instance, that W can replace molybdenum (Mo) in Mo-containing enzymes like xanthine oxidase and sulfite oxidase in kidneys and intestine.[15] Moreover, it has been suggested that WO_4^{2-} can replace phosphate (PO_4^{3-}) in bone, a mimic its property which explains the much higher concentration found in bone tissue when compared to other organs, an observation supported by several publications.[14,15]

On the opposite extreme, it is interesting to notice the very low W levels found in brain tissue, even with increasing daily dose. Despite the fact that there was statistically significant increase in W concentration with increasing exposure, even the highest observations were in the same range as the average baseline concentration for the other organs. This is fairly predictable, as the choroid plexus restrict the diffusion of several hydrophilic molecules through this blood-brain barrier. Interestingly, other W toxicokinetics studies did not describe the tissue distribution in the brain tissue after oral or parenteral exposure to sodium tungstate.

Another remarkable interpretation from these results is the fact that the relative W distribution was fairly constant throughout the six organs, as shown by the Figure 2. This means that those tissues which retained a significant amount of the metal had the same capability regardless of the dose, like the bone; on the other hand, those tissues that retained a very small fraction of the metal behaved the same way for any oral regimen, as seen in the brain.

The fact that the present study used both male and female animals made it possible to compare gender differences regarding tungsten distribution, something that previous publications were not able to do as they used either males only[16] or females only.[14,15] Interestingly, only one of the reviewed publications analyzed spleen W concentration (using a different technique),[17] the only organ in which some difference between genders was found. The finding of higher splenic W concentration in females when compared to their male matches might be the explanation to the decrease in some lymphocyte subpopulations in female animals, a finding from another branch of this same study that will be published elsewhere (A. Osterburg unpublished results). However, the results found for immune challenge differences in liver and colon from animals exposed to LPS when compared to controls are not encouraging, since this difference was not confirmed when comparing LPS against SEB exposure, and SEB did not show any difference when compared to controls. Overall, both immune challenge regimens did not seem to alter W distribution in this study.

Finally, it was surprising to find out that iron and tungsten were fairly well correlated in spleen. As explained in the introduction, the only reason why iron, nickel and cobalt levels were determined in addition to tungsten was the possible interest in future projects dealing with HMTA, since the deleterious health effects reported in literature for these W-alloys might be due to these other elements; pure nickel and cobalt, for instance, are known carcinogenic agents.[18] However, even though the mechanism leading to the correlation shown by Figure 5 is not fully understood, literature review shows that W injection in spleen leads to lymphocytopenia in rats.[13] Such a finding might lead to a further understanding of possible toxic effects from W to the immune system in general, as this increase in iron levels could be a marker of inflammation and tungsten induced toxicity in splenic tissue.

Conclusion

The present document reports data generated and analyzed as part of the study protocol entitled “Acute Effects of Oral Exposure to Sodium Tungstate on Immunological and Behavioral Parameters in Laboratory Mouse (*Mus musculus*)”. In our laboratory, tungsten, nickel, cobalt and iron tissue concentrations were examined using HR/ICPMS, which allowed the further understanding of tissue distribution of tungsten following oral exposure. Thus, those effects observed on immunological and behavioral parameters were studied by other groups, and should be reported elsewhere.

Most of the data reported in this manuscript corroborate other studies employing similar route of exposure in laboratory studies *in vivo*. This is true for the finding that the bone tissue is the main compartment where W is deposited after oral exposure, as well as the liver, colon and kidneys are organs in which W also accumulates in a lower level. Our study suggests that the brain tissue appears to be relatively protected from W exposure, as the concentrations found for that organ were extremely low (in the same range as the base levels for other organs) even after high dose exposure. In addition, the finding of increasing W levels in spleen associated with increasing Fe might be a first step to further understand possible harmful effects of W exposure on the immune system. On the other hand, there were no overall observed effects on W tissue distribution when the animals were immune challenged with either LPS or SEB, when compared to controls. There was also no difference between genders for kidney, liver, colon, bone and brain W distribution; for spleen, however, the higher W concentration found on females, correlated to increasing Fe levels, might be the basis for understanding possible immunological effects observed especially for this gender.

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Figure 1. Increased tungsten levels in all organs as a result of exposure to higher doses.

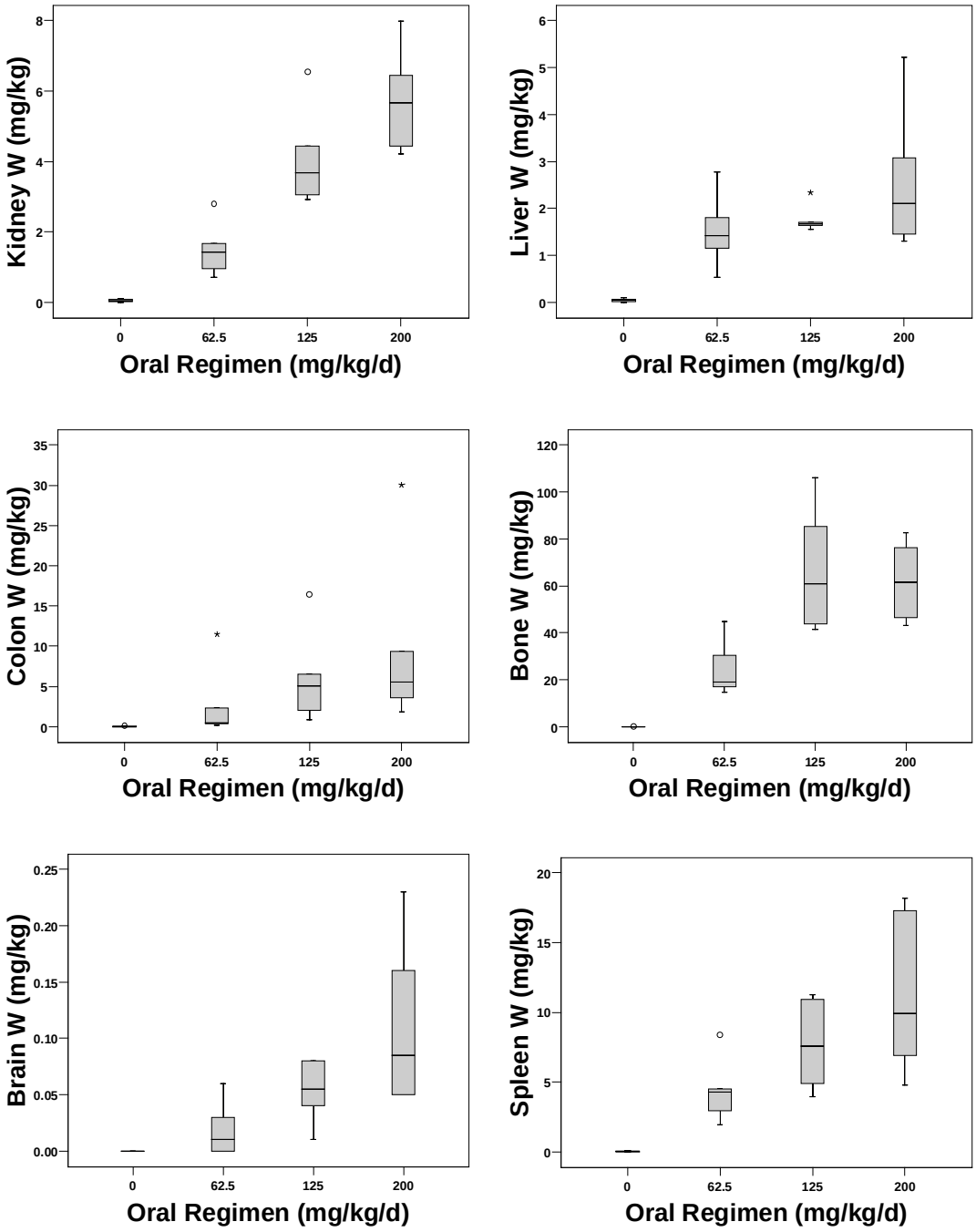

Figure 1. Increased tungsten levels in all organs as a result of exposure to higher doses. Gray bars show median, lower quartile and upper quartile; lines show lowest and highest non-outlier observations; and outliers are shown as circles (mild outliers) or asterisks (extreme outliers). Except for the colon ($p = 0.094$), $p < 0.001$ for all organs.

Figure 2. Analysis of tungsten tissue distribution among six organs.

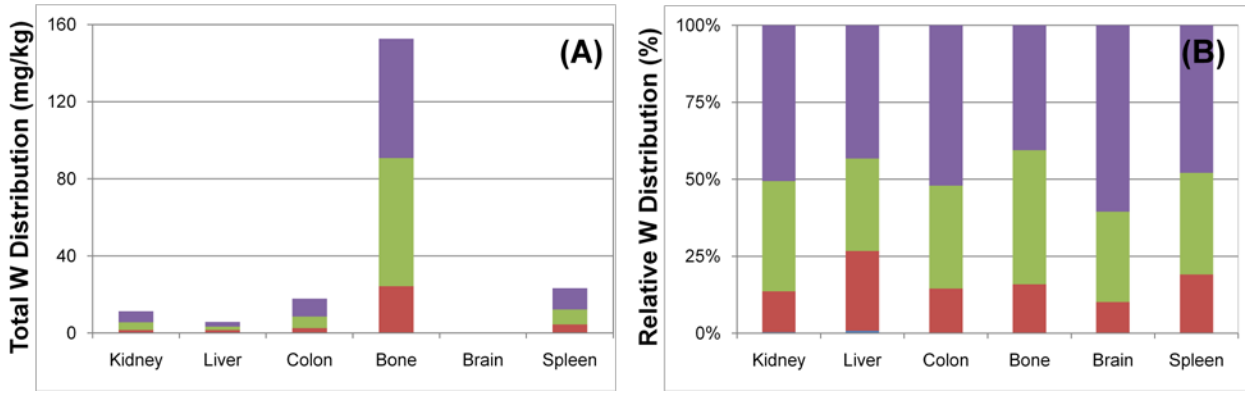

Figure 2. Analysis of tungsten tissue distribution among six organs, with different doses (0mg/kg/d in blue, 62.5mg/kg/d in red, 125mg/kg/d in green and 200mg/kg/d in purple). (A) Tungsten accumulation is not uniform, shown to be highest in bone and lowest in brain tissue. (B) There is no significant difference among the relative distribution ratios.

Figure 3. Effect of immune challenge on tungsten tissue accumulation.

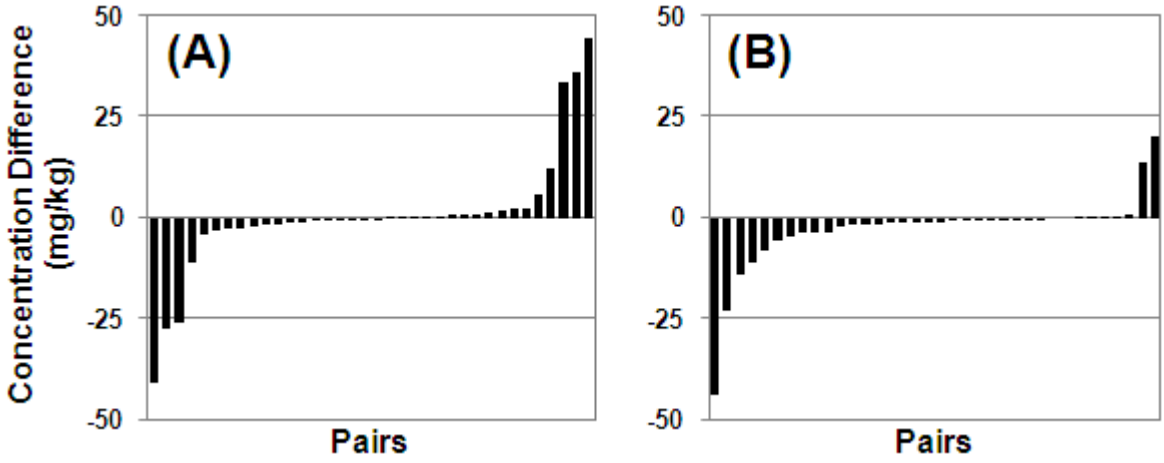

Figure 3. Effect of immune challenge on tungsten tissue accumulation for animals exposed to 62.5, 125 and 200 mg/kg/d. Columns show the net concentration difference between animals immune challenged with either staphylococcal enterotoxin B (SEB) (A) or lipopolysaccharide (LPS) (B) and their respective no challenged match (*i.e.*, same organ, gender, and dose). Negative values mean lower concentration for immune challenged animals, while positive values mean higher concentration for immune challenged animals. (A) Immune challenge with SEB shows no significant difference in tissue distribution. (B) Immune challenge with LPS shows no overall difference, though significant lower tungsten levels were found in liver and colon ($p < 0.01$ and $p < 0.04$, respectively).

Figure 4. Columns show the net concentration difference between males and females.

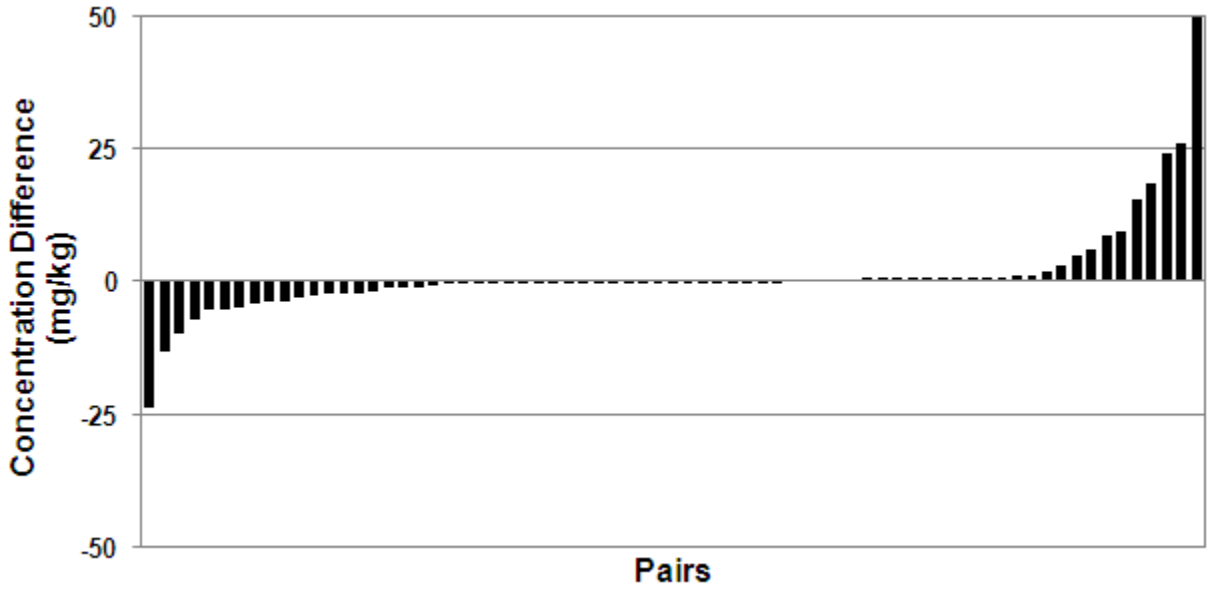

Figure 4. Columns show the net concentration difference between males and females (*i.e.*, same organ, immune challenge and dose). Negative values mean lower concentration for males when compared to their female matches. The symmetry observed expresses the overall lack of difference between males and females, although tungsten levels were significantly lower for males' spleen ($p < 0.005$).

Figure 5. Correlation between iron and tungsten levels in spleen.

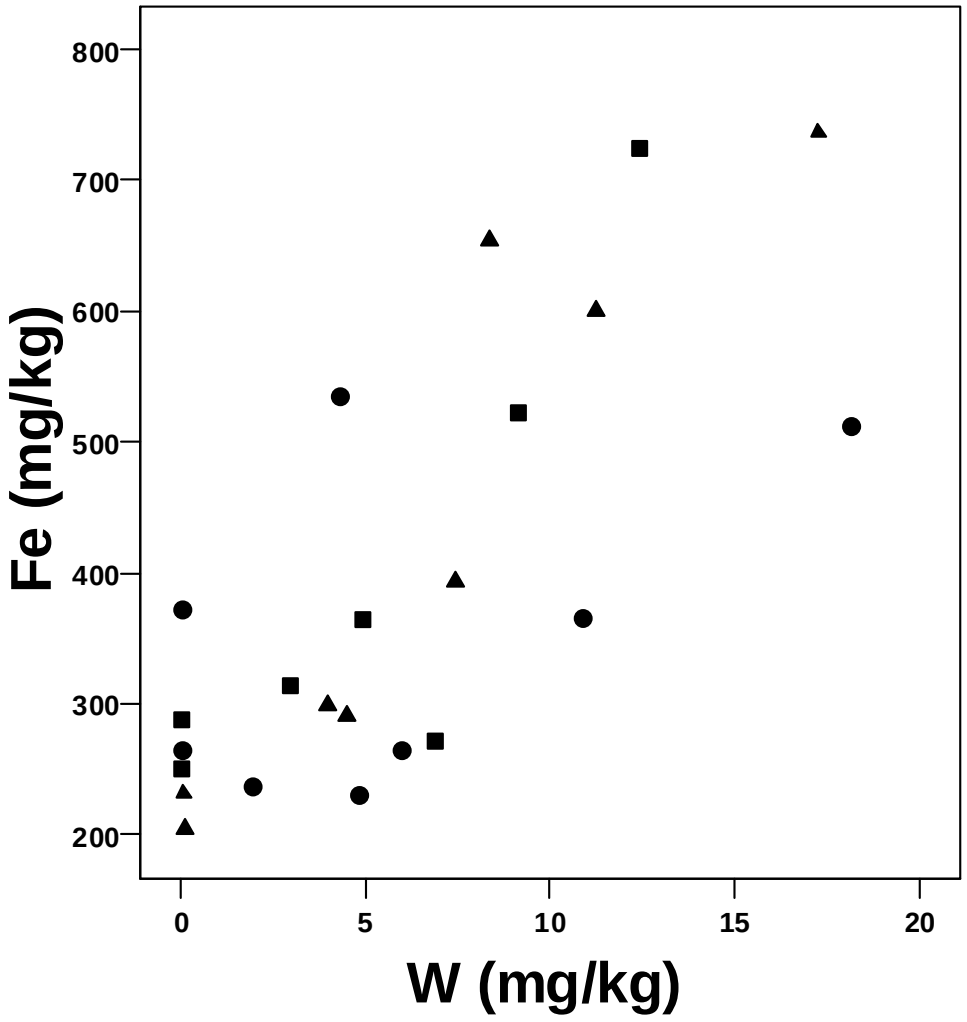

Figure 5. Correlation between iron and tungsten levels in spleen (r = 0.760, p < 0.001). Animals not submitted to any immune challenge are shown as triangles, those submitted to lipopolysaccharide (LPS) are shown as circles, and those submitted to staphylococcal enterotoxin B (SEB) are shown as squares. Such correlation is not seen in any other organ.

Table 1. Operating parameters of the HR-ICP-MS instrument (Thermo Element 2) for measurements of tungsten, iron, nickel and cobalt in tissue samples. Note that the interface cones used were made of platinum instead of nickel, in order to avoid high nickel background levels during measurements.

HR/ICPMS operating parameters	Setting
Plasma gas	Argon
Plasma power	1,150 W
Gas Flows	
Nebulizer gas flow rate	0.97 L min^{-1}
Auxiliary gas flow	0.90 L min^{-1}
Plasma gas flow	16.50 L min^{-1}
Lenses [V]	
Extraction	– 2000
Focus	– 1050
X-deflection	– 9.56
Y-deflection	– 3.02
Interface cones	Platinum

Table 2. Summarized results from ICP/MS performed for the six organs of interest. The tissues were analyzed for tungsten, iron, nickel and cobalt levels. All results are expressed as tissue concentration in mg/kg (wet weight basis). Note the absent spleen results for the animal number 19.

ID	Gender	Organ	Challenge	Dose	Fe	Co	Ni	W
1	F	Kidney	None	0	79.28	0.07	0.00	0.01
2	M	Kidney	None	0	93.37	0.05	0.00	0.02
3	F	Kidney	None	62.5	85.59	0.07	0.00	2.80
4	M	Kidney	None	62.5	81.49	0.00	0.00	1.66
5	F	Kidney	None	125	95.39	0.08	0.00	4.44
6	M	Kidney	None	125	98.79	0.00	0.00	6.54
7	F	Kidney	None	200	91.29	0.05	0.00	6.44
8	M	Kidney	None	200	69.50	0.00	0.00	4.21
9	F	Kidney	SEB	0	69.79	0.11	0.00	0.00
10	M	Kidney	SEB	0	50.56	0.04	0.00	0.10
11	F	Kidney	SEB	62.5	73.76	0.06	0.00	1.21
12	M	Kidney	SEB	62.5	67.27	0.00	0.00	0.72
13	F	Kidney	SEB	125	103.21	0.11	0.05	3.28
14	M	Kidney	SEB	125	71.95	0.00	0.00	4.07
15	F	Kidney	SEB	200	84.28	0.10	0.00	7.99
16	M	Kidney	SEB	200	57.71	0.01	0.00	4.90
17	F	Kidney	LPS	0	68.70	0.14	1.29	0.08
18	M	Kidney	LPS	0	65.70	0.04	0.00	0.04
19	F	Kidney	LPS	62.5	82.27	0.03	2.76	1.64
20	M	Kidney	LPS	62.5	53.54	0.00	0.05	0.96
21	F	Kidney	LPS	125	127.57	0.11	0.00	3.06
22	M	Kidney	LPS	125	66.10	0.00	0.00	2.92
23	F	Kidney	LPS	200	115.86	0.26	0.00	6.44
24	M	Kidney	LPS	200	59.35	0.02	0.00	4.43

Continued

ID	Gender	Organ	Challenge	Dose	Fe	Co	Ni	W
1	F	Liver	None	0	79.27	0.02	0.21	0.09
2	M	Liver	None	0	60.61	0.02	0.14	0.06
3	F	Liver	None	62.5	92.91	0.00	0.00	1.63
4	M	Liver	None	62.5	54.45	0.00	0.24	1.81
5	F	Liver	None	125	76.14	0.00	0.00	1.68
6	M	Liver	None	125	91.57	0.00	0.00	2.34
7	F	Liver	None	200	115.91	0.00	0.06	3.07
8	M	Liver	None	200	74.18	0.00	0.15	2.32
9	F	Liver	SEB	0	82.82	0.03	0.16	0.07
10	M	Liver	SEB	0	53.73	0.00	0.26	0.00
11	F	Liver	SEB	62.5	171.72	0.01	0.00	2.78
12	M	Liver	SEB	62.5	81.20	0.00	0.00	0.54
13	F	Liver	SEB	125	119.09	0.01	0.00	1.70
14	M	Liver	SEB	125	82.23	0.03	0.00	1.65
15	F	Liver	SEB	200	113.73	0.03	0.00	5.22
16	M	Liver	SEB	200	92.70	0.01	0.00	1.45
17	F	Liver	LPS	0	113.57	0.03	0.00	0.03
18	M	Liver	LPS	0	108.98	0.04	0.03	0.02
19	F	Liver	LPS	62.5	171.53	0.04	0.18	1.16
20	M	Liver	LPS	62.5	110.15	0.03	0.00	1.22
21	F	Liver	LPS	125	115.88	0.03	0.00	1.55
22	M	Liver	LPS	125	77.74	0.03	0.00	1.63
23	F	Liver	LPS	200	149.30	0.03	0.00	1.88
24	M	Liver	LPS	200	105.18	0.04	0.00	1.31

Continued

ID	Gender	Organ	Challenge	Dose	Fe	Co	Ni	W
1	F	Colon	None	0	73.58	0.02	0.42	0.00
2	M	Colon	None	0	76.17	0.03	0.45	0.00
3	F	Colon	None	62.5	59.75	0.01	0.00	2.34
4	M	Colon	None	62.5	199.27	0.06	0.72	11.46
5	F	Colon	None	125	37.48	0.00	0.53	5.68
6	M	Colon	None	125	43.34	0.01	0.00	4.38
7	F	Colon	None	200	51.09	0.00	0.00	3.63
8	M	Colon	None	200	45.53	0.00	5.67	30.05
9	F	Colon	SEB	0	69.20	0.02	3.78	0.14
10	M	Colon	SEB	0	42.31	0.02	2.81	0.01
11	F	Colon	SEB	62.5	31.81	0.00	2.45	0.36
12	M	Colon	SEB	62.5	23.18	0.00	1.62	0.22
13	F	Colon	SEB	125	42.49	0.00	2.70	6.55
14	M	Colon	SEB	125	50.71	0.00	1.80	16.42
15	F	Colon	SEB	200	26.51	0.00	1.08	9.37
16	M	Colon	SEB	200	25.64	0.00	2.07	4.03
17	F	Colon	LPS	0	21.05	0.01	1.97	0.04
18	M	Colon	LPS	0	22.77	0.01	2.02	0.00
19	F	Colon	LPS	62.5	38.34	0.00	2.10	0.49
20	M	Colon	LPS	62.5	18.04	0.00	0.00	0.47
21	F	Colon	LPS	125	30.28	0.00	0.00	2.00
22	M	Colon	LPS	125	26.51	0.00	0.00	0.83
23	F	Colon	LPS	200	22.66	0.00	0.01	1.81
24	M	Colon	LPS	200	28.18	0.00	0.25	6.98

Continued

ID	Gender	Organ	Challenge	Dose	Fe	Co	Ni	W
1	F	Bone	None	0	25.85	0.44	9.39	0.07
2	M	Bone	None	0	71.06	0.69	16.83	0.03
3	F	Bone	None	62.5	63.91	0.35	9.27	20.50
4	M	Bone	None	62.5	69.53	0.37	15.57	44.92
5	F	Bone	None	125	140.68	0.00	28.06	85.23
6	M	Bone	None	125	114.23	0.21	20.55	61.44
7	F	Bone	None	200	71.56	0.51	13.74	43.03
8	M	Bone	None	200	86.16	0.51	13.16	46.41
9	F	Bone	SEB	0	64.52	0.74	15.19	0.05
10	M	Bone	SEB	0	30.74	0.69	13.08	0.09
11	F	Bone	SEB	62.5	56.18	0.44	10.62	17.38
12	M	Bone	SEB	62.5	70.14	0.56	14.04	17.12
13	F	Bone	SEB	125	115.74	0.41	9.28	43.95
14	M	Bone	SEB	125	57.73	0.54	10.18	106.15
15	F	Bone	SEB	200	3113.64	0.42	11.43	76.39
16	M	Bone	SEB	200	52.75	0.34	23.03	82.66
17	F	Bone	LPS	0	84.52	0.30	20.86	0.18
18	M	Bone	LPS	0	72.59	0.33	18.44	0.04
19	F	Bone	LPS	62.5	68.39	0.20	6.61	14.74
20	M	Bone	LPS	62.5	78.77	0.33	24.33	30.54
21	F	Bone	LPS	125	63.25	0.34	19.33	41.39
22	M	Bone	LPS	125	95.80	0.32	15.02	60.12
23	F	Bone	LPS	200	80.40	0.34	14.56	62.94
24	M	Bone	LPS	200	179.26	0.34	15.13	60.13

Continued

ID	Gender	Organ	Challenge	Dose	Fe	Co	Ni	W
1	F	Brain	None	0	16.83	0.01	0.66	0.00
2	M	Brain	None	0	13.92	0.01	0.62	0.00
3	F	Brain	None	62.5	18.21	0.01	0.56	0.03
4	M	Brain	None	62.5	10.36	0.01	0.49	0.02
5	F	Brain	None	125	13.26	0.01	0.75	0.05
6	M	Brain	None	125	13.66	0.00	0.59	0.01
7	F	Brain	None	200	12.87	0.00	0.42	0.11
8	M	Brain	None	200	9.06	0.00	0.61	0.05
9	F	Brain	SEB	0	20.81	0.01	0.60	0.00
10	M	Brain	SEB	0	9.77	0.00	0.57	0.00
11	F	Brain	SEB	62.5	7.98	0.00	0.73	0.00
12	M	Brain	SEB	62.5	14.26	0.00	0.45	0.00
13	F	Brain	SEB	125	16.55	0.01	0.46	0.08
14	M	Brain	SEB	125	15.50	0.00	0.64	0.04
15	F	Brain	SEB	200	32.51	0.01	0.51	0.16
16	M	Brain	SEB	200	19.67	0.00	0.00	0.23
17	F	Brain	LPS	0	54.43	0.00	0.00	0.00
18	M	Brain	LPS	0	76.72	0.00	0.00	0.00
19	F	Brain	LPS	62.5	73.23	0.00	0.00	0.00
20	M	Brain	LPS	62.5	19.93	0.00	0.00	0.06
21	F	Brain	LPS	125	110.13	0.00	0.00	0.08
22	M	Brain	LPS	125	15.73	0.01	0.25	0.06
23	F	Brain	LPS	200	46.99	0.00	0.24	0.06
24	M	Brain	LPS	200	34.25	0.00	0.11	0.05

Continued

ID	Gender	Organ	Challenge	Dose	Fe	Co	Ni	W
1	F	Spleen	None	0	231.11	0.13	0.23	0.07
2	M	Spleen	None	0	203.78	0.25	103.88	0.10
3	F	Spleen	None	62.5	654.11	0.06	55.43	8.38
4	M	Spleen	None	62.5	290.25	0.00	19.74	4.51
5	F	Spleen	None	125	599.81	0.03	8.27	11.25
6	M	Spleen	None	125	298.44	0.00	8.17	3.96
7	F	Spleen	None	200	736.38	0.00	7.12	17.28
8	M	Spleen	None	200	392.93	0.00	9.56	7.43
9	F	Spleen	SEB	0	372.53	0.04	7.20	0.00
10	M	Spleen	SEB	0	265.55	0.04	12.06	0.01
11	F	Spleen	SEB	62.5	535.73	0.00	11.00	4.29
12	M	Spleen	SEB	62.5	237.14	0.00	11.64	1.92
13	F	Spleen	SEB	125	366.11	0.00	9.06	10.90
14	M	Spleen	SEB	125	264.42	0.00	5.80	5.99
15	F	Spleen	SEB	200	512.15	0.13	79.33	18.17
16	M	Spleen	SEB	200	230.73	0.06	34.87	4.79
17	F	Spleen	LPS	0	287.93	0.00	0.00	0.03
18	M	Spleen	LPS	0	250.87	0.02	10.40	0.00
20	M	Spleen	LPS	62.5	313.74	0.01	8.97	2.95
21	F	Spleen	LPS	125	521.53	0.01	9.39	9.14
22	M	Spleen	LPS	125	363.99	0.00	1.73	4.92
23	F	Spleen	LPS	200	724.80	0.00	2.13	12.45
24	M	Spleen	LPS	200	271.76	0.00	1.36	6.91

Table 3. Tungsten concentration in tissues from non-exposed and orally exposed groups. Results are shown as an average ± standard deviation, in mg/kg (n = 6 for each group but spleen under 62.5mg/kg/d, for which n = 5).

Organ	0mg/kg/d	62.5mg/kg/d	125mg/kg/d	200mg/kg/d
Kidney	0.04±0.04	1.50±0.74	4.05±1.36	5.74±1.47
Liver	0.05±0.03	1.52±0.76	1.76±0.29	2.54±1.46
Colon	0.03±0.06	2.56±4.43	5.98±5.56	9.31±10.51
Bone	0.08±0.06	24.20±11.57	66.38±25.01	61.93±15.76
Brain	0.00±0.00	0.02±0.02	0.05±0.03	0.11±0.07
Spleen	0.04±0.04	4.41±2.46	7.69±3.15	11.17±5.67

Table 4. One-way ANOVA showing statistical difference (p-values) between different exposure groups for all organs, except for colon. Dunnett's post-hoc test compares each exposure group to the control group (*i.e.*, 0mg/kg/d), providing the p-values found on the table. Even for colon, there was statistical difference between controls and animals exposed to 200mg/kg/d of tungsten.

Organ	ANOVA	62.5mg/kg/d	125mg/kg/d	200mg/kg/d
Kidney	0.000	0.036	0.000	0.000
Liver	0.000	0.008	0.003	0.000
Colon	0.094	0.460	0.137	0.025
Bone	0.000	0.021	0.000	0.000
Brain	0.001	0.420	0.043	0.000
Spleen	0.000	0.066	0.002	0.000

Table 5. Statistical analysis of the immune challenge effects on W concentration. Results were obtained using Wilcoxon signed-ranks test; data were paired by organ, immune challenge and dose. The only significant differences were found for liver and colon tungsten concentration for animals submitted to lipopolysaccharide challenge, which showed lower levels when compared to their respective controls (W+ = 0, W- = 36 and W+ = 1, W- = 27 respectively).

Organ	None x SEB	None x LPS	SEB x LPS
Kidney	$p \leq 0.382$	$p \leq 0.218$	$p \leq 0.382$
Liver	$p \leq 0.742$	$p \leq 0.007$	$p \leq 0.148$
Colon	$p \leq 1.000$	$p \leq 0.031$	$p \leq 0.460$
Bone	$p \leq 0.742$	$p \leq 0.742$	$p \leq 0.148$
Brain	$p \leq 0.218$	$p \leq 0.812$	$p \leq 0.625$
Spleen	$p \leq 0.250$	$p \leq 0.054$	$p \leq 0.382$

Table 6. Statistical analysis of gender discrepancies in W concentration. Results were obtained using Wilcoxon signed-ranks test; data were paired by organ, immune challenge and dose. The only significant difference was found for spleen, which showed higher levels for female animals (W- = 62.50, W+ = 3.50).

Organ	Wilcoxon
Kidney	$p \leq 0.176$
Liver	$p \leq 0.339$
Colon	$p \leq 0.764$
Bone	$p \leq 0.266$
Brain	$p \leq 0.640$
Spleen	$p \leq 0.004$