Tungsten toxicity on kidney tubular epithelial cells induces renal inflammation and M1-macrophage polarization

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Abstract

Tungsten is widely used in medical, industrial, and military applications. The environmental exposure to tungsten has increased over the past several years and few studies have addressed its potential toxicity. In this study, we evaluated the effects of chronic oral tungsten exposure (100 ppm) on renal inflammation in mice. We found that 30- or 90-day tungsten exposure led to the accumulation of LAMP1-positive lysosomes in renal tubular epithelial cells. In addition, the kidneys of mice exposed to tungsten showed interstitial infiltration of leukocytes, myeloid cells, and macrophages together with increased levels of proinflammatory cytokines and p50/p65-NFkB subunits. In proximal tubule epithelial cells (HK-2) in vitro, tungsten induced a similar inflammatory status characterized by increased mRNA levels of CSF1, IL34, CXCL2 and CXCL10 and NFkB activation. Moreover, tungsten exposure slowed HK-2 cell proliferation and enhanced reactive oxygen species generation. Conditioned media from HK-2 cells treated with tungsten induced an M1-proinflammatory polarization of RAW macrophages as evidenced by increased levels of iNOS and interleukin-6 and decreased levels of the M2-antiinflammatory marker CD206. These effects were not observed when RAW cells were exposed to conditioned media from HK-2 cells treated with tungsten and supplemented with the antioxidant N-acetylcysteine (NAC). Similarly, direct tungsten exposure induced M1-proinflammatory polarization of RAW cells that was prevented by NAC co-treatment. Altogether, our data suggest that prolonged tungsten exposure leads to oxidative injury in the kidney ultimately leading to chronic renal inflammation characterized by a proinflammatory status in kidney tubular epithelial cells and immune cell infiltration.

Introduction

Exposure to pollutants including metals has been recognized as a risk factor for chronic kidney disease (CKD) development (Orr & Bridges, 2017; Tsai et al., 2021) Tungsten (W, atomic number 74) is a naturally occurring element possessing exceptional industrial features such as having the highest melting point, high density, flexibility, tensile strength, and conductivity. Therefore, it is widely used in the manufacturing of electronics, implanted medical devices, tools, munitions and other industrial and military applications (Bolt & Mann, 2016; Lemus & Venezia, 2015; Yu et al., 2023) Owing to its broad use, occupational and environmental exposure to tungsten is increasing and it has been detected in soil, ground sources and potable water (Du et al., 2022; Keith et al., 2007; Shi et al., 2022) In the clinical setting, it has been reported that patients exposed to treatments based on tungsten display high levels of tungsten in the blood and urine (Domingo, 2002; Lalak & Moussa, 2002; Tajima, 2001, 2003) Notably, it appears that tungsten can bioaccumulate as evidenced in a cohort of breast cancer patients with a tungsten-based shield during intraoperative radiotherapy in which tungsten was detectable in their urine following several months postsurgery (Bolt et al., 2015) Emerging evidence shows that tungsten may lead to toxic effects in organs such as the lungs, kidneys, bones, and intervertebral discs; however, more research is needed to fully understand the potential health risks of chronic tungsten exposure (Grant et al., 2022; Grant et al., 2021; Miller et al., 2021)
Once tungsten enters the body, it is excreted mainly through the kidneys and gut, but it also accumulates in bone, spleen, colon, liver, brain, intervertebral disc, and the kidneys. (Grant et al., 2021; Guandalini et al., 2011) The kidneys are particularly susceptible since they are not only a site of bioaccumulation but are also responsible for tungsten excretion. Kidneys are particularly susceptible since tungsten induces mitochondrial dysfunction and oxidative injury in renal mitochondria. (Cheraghi et al., 2019) Only a few studies have evaluated the potential kidney toxic effects of chronic tungsten exposure. In the rat, daily gavage of tungsten for 90 days produced mild to severe basophilia in renal cortical tubules at doses of 125 and 200 mg/kg/day. (McCain et al., 2015) Moreover, exposure of rats to 500 ppm of tungsten for 28 days lead to increased reactive oxygen species (ROS) generation and lipid peroxidation. (Sachdeva et al., 2022) We recently showed that chronic oral exposure to tungsten in mice for 1 to 3 months induced fibrotic tissue accumulation in the kidneys accompanied by increased levels of myofibroblast markers, extracellular matrix and matricellular proteins. (Grant et al., 2022) In addition, we observed vacuole formation in renal tubular epithelial cells following tungsten exposure; these alterations were associated with a reduction in the glomerular filtration rate, indicating that tungsten nephrotoxicity may predispose to CKD. (Grant et al., 2022) The notion that tungsten exposure contributes to CKD development is supported by one study in the population from San Luis Valley in Colorado, in which it was found that higher urinary tungsten concentrations were associated with decreased time to CKD, independently of diabetes or hypertension. Moreover, doubling tungsten concentrations in the urine were associated with a 27–31% higher risk of developing CKD within 5 years. (Fox et al., 2021)

Some studies have shown that tungsten can induce hepatic and pulmonary damage mainly by producing oxidative injury and increasing the levels of inflammatory cytokines and macrophage activation. (Armstead & Li, 2016; Mao et al., 2021; Roedel et al., 2012) To further explore the nephrotoxic effects of chronic tungsten exposure, in this study we investigated the effect of low-dose orally administered tungsten on renal inflammatory changes in mice and whether oxidative stress is the main mechanism leading to these alterations.

**Material And Methods**

**Animal Model**

We used male C57BL/6J mice purchased from The Jackson Laboratory (USA). The mice were housed within the Maisonneuve-Rosemont Hospital Research Center animal facility and fed Harlan Teklad rodent diet (#2018 Envigo, Canada) and water ad libitum. All experiments were conducted according to the Canadian Council on Animal Care guidelines for the care and use of laboratory animals and under the supervision and approval of our local animal care committee, Comité de protection des animaux du Centre Intégré Universitaire de Santé et de Services Sociaux (CIUSSS) de l’Est-de-l’île-de-Montréal (Approved Protocol No. 2018 – 1261), in compliance with the Animal Research: Reporting of in vivo Experiments (ARRIVE) Guidelines. At 5 weeks of age, twenty-four mice were divided into two treatment groups: 1) **Control (CTL) group** receiving acidified water [12 mL 5 N hydrochloric acid (HCl) in 20 L water] or 2) **Sodium tungsten (NaW) group** consisting of mice receiving 100 ppm of tungsten in their drinking
water for 1- or 3-months as previously reported (Grant et al., 2022) (n = 6 per treatment group/timepoint). Sodium tungstate dihydrate (Na$_2$WO$_4$·2H$_2$O; Sigma-Aldrich) was dissolved in acidified water (1.79 g Na$_2$WO$_4$·2H$_2$O:1 g W) and was replaced every 2 or 3 days to limit conversion to polytungstates. (Kelly et al., 2013) Following 1 or 3 months of treatment the mice were euthanized under isoflurane anesthesia and the kidneys were collected. A portion of the kidney was further processed for histological analysis and the other portions were flash-frozen in liquid nitrogen for quantitative polymerase chain reaction (qPCR) and Western blot analyses.

**Immunofluorescence**

The kidneys were fixed in 10% formaldehyde, dehydrated, and embedded in paraffin. The tissue was sectioned (5 mM) and was subjected to antigen retrieval in citrate solution at pH 6. The sections were blocked with anti-donkey serum 5% and labelled with anti-LAMP1 (1:200; Cell signaling), anti-CD45-FITC (1:200; BioLegend), anti-CD11b-FITC (1:200; BioLegend), and anti-F4/80-AF647 (1:100; BioLegend). For LAMP1, the slides were subsequently exposed to donkey anti-rabbit AF647-conjugated (1:400 Jackson ImmunoResearch Laboratories). Fluoroshield with DAPI (Millipore-Sigma) was used for nuclear staining and mounting. Slides were imaged using a Zeiss AxioObserver.Z1 inverted microscope coupled to an X-Cite 120LED Boost High-Power LED illumination system. Images for quantitative analysis were captured with a 20X objective and the number of positive cells was determined as the average of positive cells in at least 8 fields per kidney section.

**Cell Culture**

The human proximal tubule cell line HK-2 and the RAW 264.7 murine macrophage cell line were obtained from American Type Culture Collection (ATCC, USA) and maintained in a humidified atmosphere of 5% CO$_2$ at 37ºC with Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12) supplemented with 10% fetal bovine serum (FBS) (Gibco/Life Technologies, USA) or DMEM supplemented with 10% FBS, respectively. For tungsten treatment, HK-2 or RAW cells were seeded in 6-well plates and allowed to adhere for 24 hours. After the 24 hours adhesion time, the media was replaced with DMEM containing tungsten to a final concentration of 100 ppm for 48 to 72 hours depending on the assay. Tungsten was dissolved in acidified water and then diluted in DMEM before supplementation. Vehicle was used in control wells. For the experiments with N-acetylcycteine (NAC) addition, NAC was dissolved in water and added at a 1 mM concentration. For conditioned media experiments, HK-2 cells were treated for 72 hours as described above, next fresh DMEM media was added to allow conditioned media enrichment for an additional 48 hours. The media was then collected and added to RAW cells seeded in 6-well plates for a 48-hour incubation.

**Protein extracts preparation and western blotting**

Total protein extracts were prepared from kidney samples or cells lysed by RIPA buffer (Pierce) containing a cocktail of protease and phosphatase inhibitors (Roche). The extracts were sonicated and centrifuged at 13,000 rpm for 10 min at 4ºC, the supernatant was collected, and the proteins were quantified using the Pierce BCA protein assay (Thermo Scientific). Following the quantification, the proteins were denatured
for 10 min at 95°C in Laemmlli buffer. A total of 20–40 µg of proteins from each condition were loaded and migrated on 8 to 10% SDS-PAGE acrylamide gels and transferred on 0,22 µm PVDF membranes (BioRad). The membranes were blocked in 5% non-fat milk TBS-Tween (TBS-T) for 90 min and incubated overnight at 4°C with the following primary antibodies: anti-LAMP1 (99437S, Cell Signaling, 1:1000), anti-p65 (sc-372, Santa Cruz, 1:2000), anti-p50 (sc-7178, Santa Cruz, 1:2000), anti-phospho-Ser536-p65 (sc-136548, Santa Cruz 1:5000), anti-CD206/Mannose receptor (24595S, Cell Signaling, 1:1000), anti-NOS2/iNOS (13120S, Cell Signaling, 1:1000), anti-Arginase 1 (93668S, Cell Signaling, 1:1000), anti-interleukin-6 (sc-57315, Santa Cruz, 1:1000), anti-GAPDH (9485, Abcam, 1:4000), anti-SOD1, anti-Theoredoxin, anti-smooth muscle Actin (ab179843, Abcam, 1:250), anti-Catalase (ab76110, Abcam, 1:1000). After primary antibodies incubation, membranes were washed during 30 min in TBS-T and incubated with HRP- conjugated secondary antibodies: anti-mouse (Sc-516102, Santa Cruz, 1:2000) or anti-Rabbit (Sc-2357, Santa Cruz, 1:2000) during 90 min at room temperature. The membranes were washed for 60 min and exposed to enhanced chemiluminescence solution (ECL, BioRad) using ImageQuant LAS 4000.

**Quantitative real time-PCR**

The total RNA was extracted from frozen tissue or fresh cells using TRizol reagent (Invitrogen). The RNA obtained from the aqueous fraction was then purified using the RNeasy Mini kit (Qiagen) according to the manufacturer's protocol. Next, we synthesized cDNA using 1 µg of total RNA and the SuperScript VILO cDNA Synthesis kit with DNase (Invitrogen). The transcript levels were analyzed by quantitative real-time PCR (SYBR green fluorescence detection) in an ABI 7500 Real-Time PCR System (Applied Biosystems) using the primer pairs listed in Supplementary Table 1 and SsoAdvanced Universal SYBR Green Supermix (BioRad). All samples were measured with technical triplicates and normalized against 18S and GAPDH as endogenous controls. The relative quantification of all gene expressions was performed using the comparative 2^{−DDCT} method. (Livak & Schmittgen, 2001)

**Hydrogen Peroxide Detection Assay**

HK-2 cells were seeded in 6-well plates on glass coverslips coated with Fibronectin [0.5 µg/mL]. Cells were cultured in HK-2 medium supplemented with 100 ppm tungsten for nine days. Media was changed every three days. Detection of cellular hydrogen peroxide was performed using a Hydrogen Peroxide Assay Kit following the manufacturer’s guidelines (ab138874, Abcam). Cells were imaged on a Zeiss LSM800 confocal microscope with Airyscan (Carl Zeiss, Oberkochen, Germany) using an excitation 490 nm and emission 525 nm filter set.

**Cell proliferation assays**

RAW cells were seeded in triplicate on a 96-well plate and allowed to adhere for 6 hours. Next, control, tungsten or conditioned media were added. Cell confluence was monitored using the Incucyte Live Cell SX5 system (Sartorius) as specified in the manufacturer’s instructions. The plate was photographed every 2 hours for 96 hours. The data was analyzed using the Incucyte software.
Alamar blue assay was used to determine the effects of tungsten on HK-2 cell proliferation/viability. HK-2 cells (5 x 10^5) were seeded in 6-well plates and cultured in 2 mL medium supplemented with the indicated amounts of tungsten (0, 5, 15, 50, 100 and 200 ppm) for up to 9 days. Every three days including day 0, pellets were incubated with 2 mL medium containing 10% resazurin (alamarBlue reagent, ThermoFisher Scientific, Waltham, Massachusetts, USA, Cat. #DAL1025) for three hours. Supernatant (100 µL) was added to a black 96-well plate and quantified for reductions in resazurin by measuring changes in its fluorescence (Ex 540 nm/Em 590 nm) using a spectrophotometer. Results were normalized to Day 0 within each treatment group. All experiments were performed in triplicate. Pellets were rinsed in PBS prior to the addition of fresh medium supplemented with the indicated concentrations of tungsten.

**Statistical Analysis**

The results are presented as means ± standard error. For multiple group comparisons, the statistical significance was calculated by ANOVA followed by Dunnett's multiple-comparison post hoc test. To compare between two groups, a two-tailed Student’s t-test was performed. We considered p values < 0.05 as statistically significant. Statistical analysis and graphical representation were performed using GraphPad Prism v9.1.0. 5.

**Results**

**Chronic tungsten exposure induced lysosome accumulation in tubular epithelial cells**

We previously showed that daily treatment with water containing sodium tungsten (100 ppm) over a period of 1 or 3 months induced markers of renal fibrosis and tubular cell vacuolization in mice.(Grant et al., 2022) To further characterize vacuole/granule formation in tubular epithelial cells, we stained kidney sections from mice exposed to tungsten over a 1- or 3-month period against LAMP1, a marker of lysosome formation that has been reported to be involved in toxicant-induced proximal tubule nephropathy.(Vervaet et al., 2020) In control mice, we observed a diffuse cytoplasmic localization of LAMP1 that switched to a granular localization in mice exposed to tungsten at 1 or 3 months of treatment (Figs. 1A and 1B). Thus, chronic tungsten exposure in mice induced LAMP1-positive lysosome accumulation in renal tubular epithelial cells.

**Chronic tungsten exposure stimulated renal inflammation**

Since tungsten exposure induced a toxic effect on tubular epithelial cells (Fig. 1), we hypothesized that this toxic effect could trigger an inflammatory response in the kidney. Therefore, we next evaluated the effect of chronic tungsten ingestion on the renal recruitment of inflammatory cells. Following 1 month of daily tungsten exposure, we observed a significant increase in the infiltration of leukocytes (CD45⁺), myeloid cells (CD11b⁺) and macrophages (F4/80⁺) to the kidney interstitial space as shown in the representative immunofluorescence images (Fig. 2A) and the quantification of positively stained cells by high power field (Fig. 2B). A similar pattern of increased infiltration was found for CD45⁺ and CD11b⁺
positive cells in mice receiving tungsten for 3 months, whereas macrophage accumulation was unchanged at this time-point as compared to controls (Figs. 3A and 3B).

It has been described that injured tubular epithelial cells develop a proinflammatory status characterized by increased NFkB activity and proinflammatory cytokine production. (Kirita et al., 2020) In mice exposed to tungsten in their drinking water for 3 months, we observed that the enhanced infiltration of inflammatory cells was associated with increased levels of the p65- and p50-NFkB subunits, without changes following a 1-month treatment (Figs. 4A and B). In addition, we detected increased renal mRNA levels of inflammatory cytokines known to enable communication between injured epithelial cells and leukocytes (Kirita et al., 2020), including Csf1, Il34 and Ccl7 at month 1 (Fig. 4C) and Il34, Ccl7 and Cxcl2 following 3-month exposure to tungsten (Fig. 4D).

Tungsten treatment induces proinflammatory changes and oxidative stress in kidney tubular epithelial cells

To confirm if the inflammatory changes induced by tungsten in the kidney are triggered by an inflammatory status in tubular epithelial cells, we used a proximal tubule cell line (HK-2 cells) exposed to tungsten 100 ppm in vitro. Following 72-hour exposure of HK-2 cells to tungsten, we observed an increase in the phosphorylation of p65-NFkB and in the protein levels of p50-NFkB (Fig. 5A). Moreover, incubation of HK-2 cells with tungsten for 72 hours promoted an increase in the mRNA levels of CSF1, IL34, CXCL2 and CXCL10 (Fig. 5B); similar proinflammatory cytokines that were upregulated in the kidney tissue of mice chronically exposed to tungsten (Figs. 4C and D). This inflammatory state was accompanied by an oxidative environment induced by tungsten as evidenced by an increase in the reactive oxygen species (ROS) generation as detected by a fluorescent probe for hydrogen peroxide / H2O2 following 9 days of tungsten exposure (Fig. 6A). Moreover, the antioxidant enzyme catalase was significantly diminished with 6 days of tungsten treatment at 100 and 200 ppm, without altering the levels of other antioxidant enzymes such as SOD1 or thioredoxin (Fig. 6B). In addition, 100 and 200 ppm of tungsten slowed HK-2 cell growth from day 3 to 9 following tungsten exposure, as compared to control cells (Fig. 6C). These data suggest that tungsten induces a toxic effect on tubular epithelial cells that leads to increased ROS generation, reduced proliferation and triggers an inflammatory response.

Conditioned media from epithelial cells or direct tungsten exposure modulated macrophage polarization

To evaluate if the release of inflammatory mediators by epithelial cells exposed to tungsten would influence inflammatory cell phenotype, we treated RAW macrophages with conditioned media (CM) from HK-2 cells exposed to tungsten. Following 48 hours of treatment with the conditioned media from tungsten treated HK-2 cells, the RAW cells showed a reduction in the levels of the M2-antiinflammatory macrophage polarization marker CD206 and an increase in the levels of the M1-proinflammatory polarization markers iNOS and IL-6 (Figs. 7A). Of note, these effects were partially prevented in RAW cells that were treated with CM from HK-2 cells under co-treatment with tungsten and N-acetylcysteine (NAC), as an antioxidant to prevent the ROS generation induced by tungsten (Figs. 7A). This data suggests that
tungsten induces oxidative stress in HK-2 cells that leads to the production of proinflammatory mediators that modify macrophage phenotype.

Finally, to investigate if tungsten would have a direct effect on inflammatory cell activation once they are recruited to the kidney, we also evaluated the influence of direct tungsten treatment on RAW macrophages polarization and if this effect could be prevented by NAC co-treatment. Tungsten treatment of RAW cells for 48 hours induced a reduction in the levels of the M2-marker CD206 with a concomitant upregulation of the M1-markers iNOS and IL-6 levels. These changes were prevented by NAC treatment (Figs. 7B). Despite the marked changes in macrophage activation, RAW cell proliferation was unaffected by either conditioned media from tungsten treated HK-2 cells or direct tungsten treatment (Sup. Figure 1).

**Discussion**

Chronic kidney disease is a health problem that affects around 850 million people worldwide.(Jager et al., 2019) Environmental exposure to pollutants including metals has the potential of increasing the susceptibility to develop kidney disease and/or accelerate its progression.(Orr & Bridges, 2017; Tsai et al., 2021) In a recent study, we showed that chronic oral tungsten exposure for 90 days led to functional and structural renal damage in the mice as evidenced by a reduction in the glomerular filtration rate and by the presence of tubular vacuoles and fibrotic tissue, accompanied by increased expression of matricellular proteins and the myofibroblast marker αSMA.(Grant et al., 2022) Here, we further expanded on the nephrotoxic effects of chronic tungsten exposure by showing that tungsten promotes the upregulation of inflammatory cytokines and infiltration of inflammatory cells to the kidney, mechanisms that might contribute to chronic renal injury and fibrosis. Indeed, persistent inflammation is a well-known risk factor for CKD progression(Mihai et al., 2018; Yilmaz et al., 2007), and tungsten has been shown to induce inflammatory responses in other organs. Mice exposed to tungsten (15ppm) for 4 weeks in the drinking water showed tungsten accumulation in lumbar intervertebral disk and increased levels of the inflammatory cytokines TNF-α and interleukin-1b.(Grant et al., 2021) Female mice exposed to inhaled tungsten particles (1.7 mg/m³ < 1 mm) had increased lung levels of IL-1β and CXCL1 accompanied by infiltration of neutrophils and macrophages to the lungs.(Miller et al., 2021) Our data shows that the kidney is also a target for inflammatory effects following chronic tungsten exposure as shown by increased infiltration of leukocytes, myeloid cells, and macrophages. Moreover, we found increased expression of inflammatory cytokines that have been shown to be released by injured epithelial cells to facilitate leukocyte chemotaxis(Kirita et al., 2020), such as Csf1, Il34, Ccl7 and Cxcl2. This inflammatory signature was also upregulated in human proximal tubule epithelial cells that were treated with tungsten, suggesting that prolonged tungsten exposure induces an inflammatory state in epithelial cells that leads to the release of inflammatory mediators and inflammatory cell recruitment in the kidney (Fig. 8). In agreement with our findings, it has been reported that in primary cultures of human renal proximal epithelial cells that were isolated from diabetic and non-diabetic individuals, tungsten stimulated the secretion of pro-inflammatory cytokines such as IL-6, IL-8 and MCP-1.(Bertinat et al., 2017)
Immune cell infiltration to the kidney has an essential role in the regulation of injury and repair mechanisms. (Lee et al., 2017; McWilliam et al., 2021) Depending on the microenvironment and stimulus received by macrophages, they can polarize to a “classically” M1-proinflammatory or an “alternative” M2-antiinflammatory phenotype that plays dual roles in kidney injury. (Lee et al., 2011) Macrophages expressing M1 markers such as CD80, CD64 or iNOS are pro-inflammatory and produce cytokines including IL-6, TNF-α, and IL-1β, whereas macrophages that express M2 markers such as CD206 or arginase-1 are essentially anti-inflammatory. (Liu et al., 2014; Meng et al., 2015) Several intermediate and dynamic populations have also been described. During kidney damage, enhanced M1 macrophage accumulation amplifies the initial injury and leads to further damage and fibrosis. (Jo et al., 2006; Ko et al., 2008) Here, we showed that tungsten exposure stimulated the infiltration of macrophages to the kidney but also modulated macrophage polarization to an M1-proinflammatory phenotype characterized by the upregulation of iNOS and IL-6 and the downregulation of CD206. In agreement with our findings, THP-1 macrophages exposed to nanoparticles composed of a tungsten carbide-cobalt mix displayed an increase in the proinflammatory cytokines IL-1β and IL-12, while stimulating the M1 phenotype as determined by higher levels of CD40 following nanoparticle exposure. (Armstead & Li, 2016) Similarly, intratracheally instillation of powder mixtures consisting mostly of tungsten (> 90%), promoted lung inflammation and upregulation of genes associated with oxidative and metabolic stress responses in the rat. Moreover, this effect was linked to macrophage activation, ROS generation and neutrophilia. (Roedel et al., 2012)

In addition to inflammation, tungsten might induce tissue injury by increasing ROS generation. We found that in HK-2 cells, tungsten treatment increased H₂O₂ production and reduced the levels of the antioxidant enzyme catalase. Moreover, the addition of NAC, a potent antioxidant (Ezerina et al., 2018), prevented the effects of HK-2 cell-conditioned media on promoting M1 polarization of RAW macrophages. These observations suggest that the oxidative stress induced by tungsten in proximal tubular epithelial cells leads to a proinflammatory state that signals immune cell recruitment to the kidney and macrophage activation. This is in agreement with observations suggesting that kidney mitochondria are susceptible to tungsten-induced alterations leading to oxidative injury (Cheraghi et al., 2019) as evidenced in rats in which exposure to 500 ppm of tungsten-induced renal (ROS) generation and lipid peroxidation. (Sachdeva et al., 2022) The pro-oxidant effects of tungsten have also been evaluated in other organs. In Wistar rats, sodium tungsten (100 ppm) administration for 3 months induced oxidative stress as determined by increased levels of oxidized glutathione and thiobarbituric acid reactive species in the liver and spleen, while these effects were prevented by NAC co-administration. (Sachdeva & Flora, 2014) Moreover, this study found reduced blood activity of antioxidant enzymes including d-aminolevulinic acid dehydratase and catalase accompanied by increased blood ROS detection, effects prevented by NAC. (Sachdeva & Flora, 2014) In the liver, tungsten trioxide nanoparticles caused hepatic structural and functional alterations, an effect that was mediated by increased oxidative stress and prevented by melatonin pre-treatment as an antioxidant. (Mao et al., 2021) Similar to our observation of increased ROS in HK-2 cells following tungsten exposure, it has been documented that tungsten also induces increased ROS generation in HEK-293 (kidney) and HepG2 (liver) cell lines. (Sachdeva & Maret, 2021)
Altogether, our data demonstrate that chronic oral exposure to sodium tungsten (100 ppm) for 30 or 90 days induces epithelial cell ROS generation and renal inflammation characterized by immune cell infiltration and by the upregulation of inflammatory cytokines. In addition, RAW macrophages were polarized towards an M1 proinflammatory phenotype when directly exposed to tungsten or when treated with conditioned media from proximal tubule cells treated with sodium tungsten, effects that were prevented by an antioxidant treatment.

**Declarations**

**Ethical Approval**

*In vivo* experiments were carried out according to the Canadian Council on Animal Care guidelines for the use of laboratory animals, under the supervision and approval of our local animal care committee (Comité de protection des animaux du Centre Intégré Universitaire de Santé et de Services Sociaux (CIUSSS) de l'Est-de-l'île-de-Montréal) with the approved protocol number 2018-1261, in compliance with the Animal Research: Reporting of in vivo Experiments (ARRIVE) Guidelines.

**Authors’ contributions**

Study design and conduct: C.G., and J.B.C., Data collection: J.B.C., N.H., M.G., and S.C. Data analysis and interpretation: C.G., J.B.C., M.G., P.G., and V.P. Drafting manuscript: J.B.C. and M.P.G. Revising manuscript content and approving the final version of manuscript: All authors reviewed the manuscript.

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**Availability of data and materials**

Not applicable.

**DISCLOSURE**

The authors have no competing interests to declare.

**References**


**Figures**

**Figure 1**

**Tungsten induces LAMP1⁺ lysosomes accumulation.** Immunofluorescence of kidney tissue from mice exposed to sodium tungsten 100 mg/L (NaW) in their drinking water or control mice (Ctl) for A) 1 month or B) 3 months, showing LAMP1 (green) and DAPI (blue). Examples of LAMP1⁺ granules accumulation are indicated in white arrows. Images are representative of n = 4-6 mice per group. Scale bar: 100 mM. Western-blot analysis was performed to evaluate the kidney levels of LAMP1 following tungsten exposure.
for A) 1 month or B) 3 months. The upper insets display representative blot images and the lower graph the densitometric analysis with normalization to GAPDH as a loading control (n=6).

**Figure 2**

**Tungsten exposure for 1 month increases the infiltration of CD45⁺, CD11b⁺ and F4/80⁺ cells.** A) Immunofluorescence images of kidneys from mice receiving sodium tungsten 100 mg/L (NaW) in the drinking water for 1 month showing CD45⁺ - leukocytes (green), CD11b⁺ -myeloid cells, (green) or F4/80⁺ - macrophages (green) and DAPI (blue). Images are representative of n = 4-6 mice per group. Scale bar: 100 mM. B) Quantification of positive cells for the respective staining counted in high power field (HPF) images from at least 8 fields per mice. *p<0.05, **p<0.01.
Figure 3

**Figure 3**

**Tungsten exposure for 3 months increases the infiltration of CD45^+ and CD11b^+ cells.**  
A) Immunofluorescence images of kidneys from mice treated with sodium tungsten 100 mg/L (NaW) in the drinking water for 3 months showing CD45^-leukocytes (green), CD11b^-myeloid cells, (green) or F4/80^-macrophages (green) and DAPI (blue). Images are representative of n = 4-6 mice per group. Scale bar: 100 mM. B) Quantification of positive cells for the respective staining counted in high power field (HPF) images from at least 8 fields per mice. *p<0.05.
Figure 4

**Chronic tungsten exposure promotes renal inflammation.** Western-blot analysis were performed to evaluate the kidney levels of the NFkB subunits p65 and p50 following sodium tungsten (NaW) exposure for A) 1 month or B) 3 months. Representative blot images with their respective densitometric analysis are shown. GAPDH was used as the loading control. In addition, quantitative real-time PCR was performed to determine the renal mRNA levels of \( Csf1, \, Il34, \, Ccl7 \) and \( Cxcl2 \) at C) month 1 or D) month 3 of NaW treatment. The mRNA levels were normalized against \( GAPDH \). *\( p<0.05 \), **\( p<0.01 \) and ***\( p<0.001 \)
**Figure 5**

Tungsten induces an inflammatory state in human proximal tubule HK-2 cells. HK-2 cells were treated with sodium tungsten 100 mg/mL (NaW) or water (Ctl) for 72-hours. A) Western-blot analysis to quantify the levels of the NFkB subunits p65 and p50 and p65 phosphorylation (Ser-536) following NaW exposure. Representative blot images and their respective densitometric analysis are shown. GAPDH was used as the loading control. B) Quantitative real-time PCR was performed to determine the effect of 72-hours NaW treatment on the mRNA levels of CSF1, IL34, CCL7, CXCL2 and CXCL10 in HK-2 cells. The mRNA levels were normalized against 18S. *p<0.05, **p<0.01 and ***p<0.001
Figure 6

**Tungsten promotes oxidative stress in human proximal tubule HK-2 cells.** A) HK-2 cells were treated with sodium tungsten 100 mg/mL (NaW) or without (0 ppm, Ctl) for 9-days and detected for intracellular hydrogen peroxide accumulation. B) Western-blot analyses to determine the levels of superoxide dismutase 1 (SOD1), thioredoxin and catalase. b-actin or GAPDH were used as loading controls. C) Cell growth was measured for up to 9 days following 0-200 ppm tungsten exposure. ANOVA; posthoc Dunnett’s multiple comparison test; *, p < 0.05; ***, p < 0.001; ****, p < 0.0001; ##, p<0.01 vs. 0 ppm, ####, p<0.0001 vs. 0 ppm. n = 3 or 4.

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Figure 7

*Image not available with this version*
RAW Macrophage proinflammatory phenotype induced by tungsten and conditioned media from tungsten-treated HK-2 cells. A) RAW cells were treated for 48 hours with conditioned media collected from HK-2 cells treated with water (Ctl), sodium tungsten 100 mg/mL (NaW) or NaW 100 mg/mL plus n-acetylcysteine 1 mM (NaW + NAC). B) RAW cells were treated for 48 hours with water (Ctl), sodium tungsten 100 mg/mL (NaW) or NaW 100 mg/mL plus n-acetylcysteine 1 mM (NaW + NAC). For A) and B) the cell lysates were analyzed by western blot to evaluate the levels of Mannose receptor (CD206), inducible nitric oxide synthase (iNOS), Arginase-1 (Arg1) or interleukin 6 (IL-6). Representative blot images and their respective densitometric analysis are shown. GAPDH was used as the loading control. *p<0.05 and **p<0.01.

**Schematic representation of tungsten effects on renal inflammation.** Chronic tungsten ingestion leads to oxidative injury in tubular epithelial cells which creates a proinflammatory status characterized by increased production of proinflammatory cytokines that mediate the attraction of leukocytes, myeloid cells, and macrophages to the kidney. In addition, tungsten has direct effects on macrophages by promoting its polarization towards a proinflammatory phenotype. These effects are attenuated by an antioxidant treatment with N-acetylcysteine.

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.
• SupplementaryTable1.docx
• Supple.Figure1.pdf