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Research paper

# The pulmonary inflammatory response to multiwalled carbon nanotubes is influenced by gender and glutathione synthesis

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ABSTRACT

Inhalation of multiwalled carbon nanotubes (MWCNTs) during their manufacture or incorporation into various commercial products may cause lung inflammation, fibrosis, and oxidative stress in exposed workers. Some workers may be more susceptible to these effects because of differences in their ability to synthesize the major antioxidant and immune system modulator glutathione (GSH). Accordingly, in this study we examined the influence of GSH synthesis and gender on MWCNT-induced lung inflammation in C57BL/6 mice. GSH synthesis was impaired through genetic manipulation of *Gclm*, the modifier subunit of glutamate cysteine ligase, the rate-limiting enzyme in GSH synthesis. Twenty-four hours after aspirating 25 μg of MWCNTs, all male mice developed neutrophilia in their lungs, regardless of *Gclm* genotype. However, female mice with moderate (*Gclm* heterozygous) and severe (*Gclm* null) GSH deficiencies developed significantly less neutrophilia. We found no indications of MWCNT-induced oxidative stress as reflected in the GSH content of lung tissue and epithelial lining fluid, 3-nitrotyrosine formation, or altered mRNA or protein expression of several redox-responsive enzymes. Our results indicate that GSH-deficient female mice are rendered uniquely susceptible to an attenuated neutrophil response. If the same effects occur in humans, GSH-deficient women manufacturing MWCNTs may be at greater risk for impaired neutrophil-dependent clearance of MWCNTs from the lung. In contrast, men may have effective neutrophil-dependent clearance, but may be at risk for lung neutrophilia regardless of their GSH levels.


## 1. Introduction

Multiwalled carbon nanotubes (MWCNTs) are concentric cylinders of graphene under 100 nm in diameter which possess useful electronic, optical, and chemical properties [23,25,53]. Following improvements in large-scale manufacturing [34], worldwide carbon nanotube (CNT) manufacturing capacity grew to 4 million kg in 2011 [8,9]. This growing worker population is at risk for inhaling CNTs during manufacturing and handling [8,18,28]. Thus, the National Institute for Occupational Safety and Health recommended an inhalation-based occupational exposure limit of 1 $\mu g/m^3$ [43]. However, this recommendation was limited by a lack of *in vivo* data on sensitive subpopulations [43], which may be at greater risk for MWCNT-induced lung inflammation, fibrosis, and oxidative stress [34,39,48,49,55,63].

CNT-induced oxidative stress has been observed in exposed workers and rodent models. In workers, exposure was associated with elevated breath condensate levels of oxidative stress markers [29], as well as lung dysfunction and suppression of glutathione peroxidase activity [32]. In mice, exposure was associated with depletion of the major cellular tripeptide antioxidant glutathione (GSH). Among C57BL/6 mice exposed via inhalation (5 $mg/m^3$, 5 h/day, 4 days) to single-walled CNTs (SWCNTs) contaminated with 17.7% iron, exposure was associated with approximately a 50% decrease in the lung's total GSH content [55]. Decreases of 40–50% in

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lung total GSH were also observed in juvenile BALB/c mice exposed via inhalation (estimated deposition 5 μg/g/day, 7 days) [51]. Similarly, aspiration of 32 μg MWCNT in C57BL/6 mice was associated with a comparable decrease in total GSH, and a significant increase in the ratio of oxidized (GSSG) to reduced GSH [33].

Conversely, elevated GSH levels may protect against lung damage. Mice treated with the GSH precursor N-acetylcysteine had increased GSH levels and were resistant to the fibrosis and neutrophilia induced by exposure to long MWCNTs contaminated with 4.49% nickel [57]. Similarly, N-acetylcysteine co-treatment ameliorated the SWCNT-induced increase in pro-inflammatory cytokines in murine macrophages [3].

Taken together, these reports indicate that GSH deficiency is a potential consequence of CNT exposure, and that GSH supplementation may protect against CNT-induced lung damage. However, there have been no investigations into how pre-existing GSH deficiency may modulate the lung's pathological response to MWCNTs.

In humans, GSH deficiencies can result from inadequate dietary intake of cysteine or methionine, or from chronic diseases (e.g., chronic bronchitis, idiopathic pulmonary fibrosis) [1,12]. GSH levels can also be affected by polymorphisms in *Gclc* and *Gclm*, which respectively encode the catalytic (GCLC) and modifier (GCLM) subunits for glutamate cysteine ligase (GCL), the rate-limiting enzyme in *de novo* GSH synthesis [11,60]. Functional genetic polymorphisms in *Gclc* and *Gclm* have been reported in 30% and 20% of humans, respectively [41,56].

GSH deficiency can dramatically alter the lung's response to toxicants. Genetic manipulation of *Gclm* in a mouse model of GSH deficiency indicated that *Gclm* heterozygous mice developed significantly greater lung inflammation following diesel exhaust particle exposure [65]. Furthermore, *Gclm* null mice had a significantly impaired inflammatory response to ozone [24] and quantum dots [36]—underscoring GSH's importance in modulating the immune response, as well as oxidative stress [12].

Intriguingly, gender may modulate the effects of GSH deficiency. Following acetaminophen exposure, female *Gclm* null mice had twice the liver damage of wild-type mice; in contrast, male *Gclm* null mice only had 20% more damage than wild-type mice [37]. In humans, gender influences susceptibility to lung diseases: Interstitial lung diseases are approximately 20% more prevalent in men [7,15], while asthma is 34% more prevalent in women [5].

Together, these observations suggest that both gender and GSH deficiency may affect a worker's susceptibility to MWCNTs. Thus, we examined how GSH deficiencies may modulate MWCNT-induced acute lung inflammation in a gender-dependent manner using male and female *Gclm* deficient mice. The information from our study could potentially identify subpopulations of workers who may be more vulnerable to MWCNT-induced lung damage based on their gender and/or GSH status.

## 2. Materials and methods

### 2.1. Reagents

Except where noted, we obtained all reagents from Sigma-Aldrich (St. Louis, MO).

### 2.2. MWCNT characterization

When handling multiwalled carbon nanotubes (MWCNTs) for these experiments, we followed the National Research Council's recommendations on engineering controls and personal protective equipment to reduce the risk of inhalation and dermal exposure to MWCNTs [45].

For these experiments, we used MWCNTs manufactured by Cheap Tubes, Inc., via catalytic chemical vapor deposition. The manufacturer reported that the MWCNTs ranged in length from 500 to 2000 nm, and in outer diameter from 10 to 20 nm. The MWCNTs were supplied to us through our participation in the NIEHS Centers for Nanotechnology Health Implications Research Consortium. We used the MWCNTs as provided, and neither chemically modified nor purified the MWCNTs of non-graphene carbon or residual metal catalysts.

Physicochemical characterization of these MWCNTs has been previously reported. Hamilton *et al*. reported these MWCNTs to have an average length of 1108 nm and an outer diameter of 18 nm, with a surface area of 140.6 $m^2/g$ [17]. The Nanotechnology Characterization Laboratory reported that these MWCNTs have an elemental composition of 94.1% carbon, 4.7% oxygen, 0.8% nickel, and 0.4% iron by weight, and were contaminated with 0.06 EU/mg of bacterial endotoxin [42]. Therefore, our dose of 25 μg MWCNTs/mouse imparted a negligible endotoxin dose of 0.0015 EU/mouse.

To image the MWCNTs, we resuspended the dry powder to 0.5 mg/ml (the same concentration used in mouse exposures) in distilled water, sonicated the solution for 19 s in a 40 kHz Branson 2510DTH bath sonicator (Branson Ultrasonics Corp., Danbury, CT), and vortexed the solution for 1 s. The MWCNT solution was then drop-cast onto a holey carbon transmission electron microscopy grid, and the images captured with a helium ion microscope (ZEISS, Oberkochen, Germany).

To characterize the dispersion state of the MWCNTs in solution, we resuspended them to a concentration of 0.5 mg/ml in either distilled water or dispersion medium dosing vehicle [phosphate buffered saline (PBS)+0.6 mg/ml mouse serum albumin+10 μg/ml 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine surfactant in ethanol (0.1% v/v)] [2]. Following bath sonication and vortexing as described above, we then measured the resuspended sample's hydrodynamic size and zeta potential using dynamic light scattering over 45 min (Malvern Instruments Ltd., Malvern, UK). The relationship between the size of the resuspended particles and their Brownian motion is described by the Stokes-Einstein equation [10]. A particle's zeta potential influences its electrophoretic mobility, as described by the Henry equation and Smoluchowski approximation [22].

### 2.3. Gclm mice

We conducted all animal experiments in accordance with the National Institutes of Health Guide for the Use and Care of Laboratory Animals [44], and with the approval of the University of Washington Institutional Animal Care and Use Committee (UW IACUC Protocol 2384-08). We made all efforts to minimize animal distress and suffering.

For these studies, we used male and female *Gclm* wild-type ($Gclm^{+/+}$), *Gclm* heterozygous ($Gclm^{+/-}$), and *Gclm* null ($Gclm^{-/-}$) mice which had been backcrossed onto a C57BL/6 background [37]. The mice were group housed in a modified specific pathogen free vivarium on a 12-hour light/dark cycle with nesting materials and access to water and chow provided *ad libitum*. At the time of MWCNT exposure, the mice were 3–5 months old with no significant difference in mean age between any of the experimental groups.

### 2.4. Experimental design

To determine if gender and genetic manipulation of GSH levels modulate the lung's pathological response to MWCNTs, we randomly assigned 3- to 5-month-old mice of all *Gclm* genotypes to receive dispersion medium dosing vehicle only, or 25 μg MWCNTs/mouse by oropharyngeal aspiration (n=5–6 mice per