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Particle and Fibre Toxicology

LETTER TO THE EDITOR Open Access

Comment on Shvedova et al. (2016), "gender differences in murine pulmonary responses elicited by cellulose nanocrystals"

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Abstract

A recent publication in "Particle and Fibre Toxicology" reported on the gender differences in pulmonary toxicity from oro-pharyngeal aspiration of a high dose of cellulose nanocrystals. The study is timely given the growing interest in diverse commercial applications of cellulose nanomaterials, and the need for studies addressing pulmonary toxicity. The results from this study are interesting and can be strengthened with a discussion of how differences in the weights of female and male C57BL/6 mice was accounted for. Without such a discussion, the observed differences could be partially explained by the lower body weights of females, resulting in higher doses than males when standardized to body weight or lung volume. Further, few conclusions can be drawn about the pulmonary toxicity of cellulose nanocrystals given the study design: examination of a single high dose of cellulose nanocrystals, administered as a bolus, without positive or negative controls or low dose comparisons, and at an unphysiological and high dose rate. Simulating the bolus type delivery by inhalation would require a highly unrealistic exposure concentration in the g/m^3 range of extremely short duration. A discussion of these limitations is missing in the paper; further speculative comparisons of cellulose nanocrystals toxicity to asbestos and carbon nanotubes in the abstract are both unwarranted and can be misleading, these materials were neither mentioned in the manuscript, nor evaluated in the study.

Keywords: Cellulose nanocrystals, Cellulose nanomaterials, Inhalation, Gender differences, Pulmonary toxicity

Commentary

In their recent publication, "Gender differences in murine pulmonary responses elicited by cellulose nanocrystals", Shvedova et al., [1] exposed C57BL/6 mice by pharyngeal aspiration to suspensions of cellulose nanocrystals (CNCs) (40 μg/mouse/day; cumulative dose of 240 μg/mouse). The authors employed a variety of biochemical, cellular, histopathological and physiological measures to compare responses observed in the lungs of male and female mice. As strong advocates for proactive approaches to assessing the safety of nanomaterials, we would be most interested in these findings, however the study design limits the ability to relate the results to effects from CNC exposure under realistic conditions.

The authors state that the "primary goal of this study was to determine whether gender affects pulmonary function, global mRNA expression, and cytokine/chemokine inflammatory responses in the lung of C57BL/6 mice". The findings of observed gender differences, with females showing a higher pulmonary toxicity is interesting, and as the authors point out, such gender differences in respiratory diseases have been reported in previous studies. However, the observed "gender differences…" would be strengthened with a discussion of how differences in the weights of female and male C57BL/6 mice was accounted for. At 7–8 weeks, when Shvedova et al. began their acute exposures, female C57BL/6 mice have an average weight of 18 g while male mice have an average weight of 23 g [2], more than a 20 % difference, which might explain the greater responses in female mice because of the higher dose per unit BW in females. The authors state the

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C57BL/6 mice used in their study weighed 20.0 ± 1.9 g, but a discussion of the weight distributions of males and females used in their study was not provided, it certainly would strengthen the interpretation of results. The observed gender differences might simply be explained by lower body weights of females, resulting in higher doses than males when standardized with body weight.

As mentioned, the results of the study are difficult to interpret in terms of human health impact, given its overall design. Specifically, the exposure method (pharyngeal aspiration of bolus doses) and examination of effects from a single concentration (240 μg/mouse cumulative exposure), equivalent to a very high deposited dose in humans, requires closer examination. Shvedova et al. estimated that the CNC dose administered to mice is equivalent to a human worker exposed to the Occupational Safety & Health Administration (OSHA) limit for 42 days. However, it is scientifically not justifiable in terms of effects to equate a deposited bolus dose (exposure duration is a fraction of a minute) with the same dose achieved after an exposure for many days in the lung. A more realistic comparison of the dose in mice to the dose deposited in workers' lungs has to consider the following: Key is that there is a difference in effects and underlying mechanisms induced by very high *vs*. very low dose rates [3]. Effects induced by high bolus-type delivery can be used for hazard identification, provided that dose–response data are established to determine a slope [4]. Unfortunately, though, results from such studies cannot be used for risk characterization (establishing limit values).

The principle of our approach for mouse-human extrapolation modeling involves the following steps: We used the Multiple-Path Particle Dosimetry (MPPD) model (Version 3.04) to determine the deposited fraction inhaled by a 20 g mouse of an aerosol with mass median aerodynamic diameter (MMAD) of 0.6 μm and geometric standard deviation (GSD) of 2.0 and aerosol density of 1. Respiratory parameters (tidal volume and breathing frequency) were allometrically adjusted to body weight which is essential when running the MPPD model. The model derived deposition fraction in the alveolar region of the mouse lung gave a value of 5.1 %.

As a next step, an estimate of the deposited dose in humans over an 8 h (hr) workplace exposure was performed, at a concentration of 5 mg/m^3, which is the OSHA occupational limit for respirable cellulose dust. The deposition fraction in the alveolar region of the human lung, using the MPPD model with the MMAD and GSD given above, turned out to be 8.1 %. The 8-h deposited dose in the alveolar region under light physical exercise breathing conditions was calculated as 3,985 μg/day at 5 mg/m^3 exposure concentration. This is equivalent to 6.3 ng/cm^2 of the alveolar surface area (634,620 cm^2 at functional residual capacity [FRC]) in the human lung. The equivalent deposited dose in the mouse by inhalation would then be 3.3 μg/mouse for a one day (8 h) exposure (mouse alveolar surface area at FRC of 526 cm^2 x 6.3 ng/cm^2). This is 12 times less than the 40 μg/mouse delivered in the study. In addition, as mentioned above, the impact of an 8-h inhalation exposure *vs*. a less than a minute bolus delivery has to be considered.

Finally, in order to determine a mouse equivalent inhalation exposure concentration that results in the same deposited lung dose as human workers deposit when exposed to the OSHA limit of 5 mg/m^3 for 8 h, we used the following correlation:

Deposited Dose = Minute Ventilation x Expos. Concentration x Depos. Fraction x Expos. Duration

(The deposited dose over 8 h is 3.3 μg/mouse; the MPPD derived deposition fraction is 0.051 [see above]; body weight allometrically adjusted tidal volume and breathing frequency for a 20 g mouse are 0.148 ml and 252/min, respectively; and exposure duration is 8 h). Rearranging and solving the above equation for Exposure Concentration gives a value of 3.6 mg/m^3, which is in a similar range as the OSHA exposure limit for workers.

However to simulate the bolus type delivery used in the mouse study, the dose of 3.3 μg/mouse would have to be inhaled in one minute - rather than 8 h - at an exposure concentration of 1.73 g/m^3. Nobody would claim this Exposure Concentration to be realistic; and yet, that is exactly the equivalent to bolus-type dosing in terms of the dose delivered to the respiratory tract. Unfortunately, this has been done - and continues to be done, and accepted without question - in numerous other studies. (Additional issues of unequal distribution between aspiration and inhalation are not considered here).

Conclusions from this derivation of a human/mouse equivalent dose in the alveolar region are: (*i*) Shvedova et al. exceeded the estimated human daily deposited dose—at the Permissible Exposure Limit allowable by OSHA - by a factor of 12 when dosing the mice. (*ii*) simulating the bolus type delivery with inhalation would require a highly unrealistic exposure concentration in the g/m^3 range of extremely short duration; (*iii*) effects and induction of underlying mechanisms are due to the high, unrealistic and unphysiological exposure conditions which have to be interpreted with great caution [5]. Part of any study must be a critical assessment of the relevance of administered doses in animal (and in vitro) experimental studies in order to avoid erroneous conclusions. For example, the reported significant gender differences in this study may be simply a result of differences in male/female body size if doses have not been adjusted; or are they due to the study design of only one very high dose? Again, determining the slope of a dose–response relationship would be essential to answer these and other questions. We encourage further

discussion of the importance of dosing, which would include more details of our dosimetric calculations.

In order to comment on the possible human toxicity of CNCs, the authors should have investigated several doses in order to demonstrate response related to dose of CNC exposure. This is especially important given that high-dose effects in in vivo studies are inherently difficult to interpret. It is well documented that dose-dependent transitions in the principle mechanism of toxicity occur at high exposures [5]; for example, high doses - amplified by very high dose rates - may result in non-linearity of responses, effects occurring from saturated receptor pathways (for both activating and detoxifying interactions), and inflammation due to conditions of overwhelming defenses [5] that are not representative of effects from realistic dose exposures.

The lack of negative and positive controls of known agents, together with the lack of different doses to characterize dose–response relationships, limits the ability to conclude there are substance-specific effects rather than as result of inflammation due to simple foreign particle introduction resulting from a bolus, high-dose exposure; similar outcomes are known to occur from high doses of any poorly soluble dust. The study design incorporates interesting measures of gene and protein expression following exposure, however the issue is not discussed regarding whether these high dose responses relate specifically to CNC, or might similarly occur due to common respiratory triggers (such as other fibrous and non-fibrous particle types) at these exposure levels. The lack of low dose testing similarly limits interpretation of the gene and cytokine responses.

We question, further, why the abstract includes an unexpected statement comparing CNC to both carbon nanotubes and asbestos, never to be mentioned again in the paper. A comparison to asbestos as a positive benchmark control was not part of the study design, neither was a negative benchmark included. Adding such benchmarks - combined with a dose–response approach - would have enhanced the study beyond a simple design by allowing to rank CNC against well-characterized benchmarks. Without this, referring to asbestos solely in the abstract is unwarranted and unjustified.

The sulfated CNCs examined by Shvedova et al. were 158 nm long and are not classifiable as World Health Organization (WHO) fibers [6]. Additionally, they are 1–2 orders of magnitude shorter than fibers we would expect to induce mechanisms that lead to toxicity under the asbestos fiber toxicity paradigm, where fibers longer than 15–20 μm are critical [7]. We are not suggesting that inhalation of high doses of CNCs would not cause the inflammatory response observed by Shvedova et al., however the toxicity observed is better expressed in terms of the very high lung burden, rather than comparing it in the abstract to asbestos.

In summary, readers of "Particle and Fibre Toxicology" should recognize that the study outlined by Shvedova et al. was an investigation into gender differences of pulmonary toxicity from bolus-type lung exposure at a high dose of a poorly soluble dust. The conclusions that can be drawn about the pulmonary toxicity of CNCs - that very high doses of CNCs cause inflammation - are common to even benign fibrous and non-fibrous particles.

Authors' response to: comment on Shvedova et al. (2016), "gender differences in murine pulmonary responses elicited by cellulose nanocrystals"

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We would like to thank Drs. Shatkin and Oberdörster for their interest and comments on our paper [1] and we would like to take this opportunity to clarify some of the issues raised. One of the specific comments is focused on the relevance of our original findings to differences in the total body weights between the female and male mice. Our study determined the respiratory toxicological endpoints after several pharyngeal aspiration exposures to cellulose nanocrystals (CNC) in male and female mice with a specific focus on the comparison of the relative responses associated with gender differences. Significant differences in the responses to respirable CNC with a higher pulmonary toxicity in female mice were described. However, Shatkin and Oberdörster suggest that these differences might be due to the lower body weights of female mice, resulting in higher relative doses vs males. Accumulating evidence suggests that gender can have a profound effect on incidence and severity of a variety of pulmonary diseases [8–10]. It is well known that changes in the lung volume/mass, rather than body weight, define the respiratory abnormalities. Additionally, it has been established that the lung volume/mass of male and female mice of the same age are not different, in spite of the differences in the body weights [11, 12]. As per Environmental Health Criteria 239 [13], the tissue dose which is the amount distributed to and present in a specific tissue of interest, in this case – the lung, would be in fact, the same for male and female mice. In our study, only the *pulmonary* responses were compared between male and female mice: inflammation and damage, TGF-β, and collagen, oxidative stress and pulmonary functions as well as the global mRNA expression were measured in lung. Thus, the comment on the employed doses with respect to the total weight differences between males and females is without merit. We maintain that the stronger responses to CNC documented in female mice were due to the gender associated differences in the pulmonary reactivity, rather than to 14 % variance in the total body weight between the female and male mice. We thank Shatkin and Oberdörster for raising this issue in their letter [14], and the opportunity to expand our discussions. Further the comment by Shatkin and Oberdörster was useful as it allowed us to explain an important point: that in spite of the slight differences in the total body weight between female and male mice – there was essentially no gender differences between the lungs of the animals either in terms of their weight/area or functions.

Shatkin and Oberdörster expressed concerns that the dosages employed in this study exceeded the estimated human daily deposited dose – at the Permissible Exposure Limit allowable by OSHA - by a factor of 12. It has to be acknowledged that direct quantitative comparisons between rodent and human toxicological assessments are difficult to make. This is due to uncertainties associated with various methods/tools available for modeling nanomaterial deposition in the lungs and/or differences in the physicochemical characteristics inherent to each material being investigated, as well as those related to each species. With full understanding of these limitations, the dose responses in mice can still provide useful information for meaningful modeling and approximate evaluations relevant to realistic human exposure scenarios. Several mathematical models, including the MPPD method preferred by Shatkin and Oberdörster, have been developed and used by various groups to improve translation of the in vivo rodent assessments to corresponding human equivalent exposures [15–18]. In our study, mice were exposed over an 18 day period to 6 single doses (once every 3 days) of 40 μg of CNC materials using a pharyngeal aspiration technique (cumulative dose ~240 μg/mouse) in lieu of a single bolus dose. Therefore, for estimating human equivalent exposures to attain similar lung burdens, we opted to make several assumptions including no clearance over the 18 day period and the equivalency of the exposure dose to the dose deposited in the alveolar region. In our comparisons, we first estimated lung burden in rodent models, then normalized it to lung burden/alveolar epithelial surface area to further estimate human equivalent exposure time period as follows.

Human Alveolar Deposition = Exposure Concentration x Ventilation/8 h working x Deposition Fraction

- OSHA Conc. of allowable exposure to Cellulose = 5 mg/m^3 (respirable fraction)
- Ventilation / 8 h working (20 L/min * 0.001 m^3/L * 60 min/h * 8 h/day) = 9.6 m^3/d ([19])
- Deposition fraction for CNC having largest dimension ~100-300 nm [13] = ~15 % ([19, 20])
- Total alveolar surface area of human lung = 634,620 cm^2 at functional residual capacity [FRC]
- Mouse alveolar surface area = 526 cm^2 at FRC.

Based on this, the lung burden in humans per day can be estimated as 7200 μg or 11.4 ng/cm^2 per day. Thus, the accumulated alveolar lung burden in humans ignoring the clearance and other potential factors over 18 days will be 18*11.4 = ~205 ng/cm^2. The $T_{1/2}$ for alveolar clearance in humans is ~ 1 year and can be ignored in these estimates, as the clearance would be insignificant over the 18 days required to achieve the equivalent worker lung burden. Assuming that the CNC particles administered through pharyngeal aspiration deposit predominantly in the deep lungs (the alveolar region), the dose employed in mice would result in a lung burden of ~0.456 $μg/cm^2$ or 456 ng/cm^2, i.e. only ~ 2.3 times higher than can be expected from equivalent exposures per day in humans. Thus we estimated that the human equivalent lung burden would be achieved in

~42 days (2.3 x 18 days = 41.4 days). It has to be noted that if the total dust concentrations, instead of respirable fraction, of OSHA Permissible Exposure Limit (PEL – 15 mg/m^3) or NIOSH Recommended Exposure Limit (REL – 10 mg/m^3) were considered, then the respective human alveolar lung burden would be equivalent to 34 ng/cm^2 or 23 ng/cm^2, respectively. Over 18 days, this would result in the accumulation of 612 or 414 ng/cm^2, which are slightly higher than or closer to the average CNC dose in mice per day employed in our study. Moreover, the employed exposure regimen and concentrations over 3-weeks of exposure are further justified as one considers that humans may be exposed chronically for longer periods of times.

Can the effects reported in our study be due to non-linear responses stemming from receptor oversaturation pathways and/or conditions of overwhelming the defenses—lung overload phenomenon? So far, this has been demonstrated in rats, but not in mice or humans. Porter et al. [21, 22] demonstrated that a lung burden of 6 mg/rat of exposure to silica particles (alveolar surface area ~0.4 m^2 of rat lung [23, 24] had not reached overload and had not decreased the clearance rate. This is equivalent to 0.9 mg/mouse lung (alveolar surface area ~0.06 m^2 of mouse lung [23, 24]) which is ~4 times higher than the concentrations of CNC (up to 240 μg/mouse) we have investigated in several of our studies [1, 25, 26]. Importantly, several studies indicated that the dose dependent effects of nanomaterials were mostly linear within this dose range.[25].

Shatkin and Oberdörster overlooked one of the most essential experimental features of our study [1]: the employment of chronic treatments achieved by scheduled repeated exposures (twice a week for 3 weeks resulting in an accumulated dose of 240 μg) to deliver CNC by pharyngeal aspiration. This regimen achieves the accumulation of particles in the lung over time, which is closer to potential occupational exposures than a single daily bolus dose (as supposed by Shatkin and Oberdörster). Importantly, this protocol may be one of the best ways to dose animals in cases when generation of nanomaterial aerosol for inhalation studies (eg, nano-cellulose materials) could represent technical difficulties [27]. Several studies have documented the noninvasive and reproducible character of particle deposition and clearance from the mouse lower respiratory tract after pharyngeal aspiration [28, 29]. Moreover, direct comparisons of bolus inhalation vs aspiration exposures of SWCNTs further demonstrated the efficiency of the aspiration technique in studies of fibrous particles [30]. The repeated exposure regimens have been employed by others for pharyngeal aspiration [18, 31–33] and intra-tracheal instillation exposures [34–37]. Furthermore, the deposition estimates using MPPD model (v3.0) preferred by Shatkin and Oberdörster also have several limitations. These include (a) deposition models for mouse are based on only two strains (BALBc and B6C3F1)—both of which are models for either asthmatic or polygenic diseases, (b) calculations on mouse extrapolation are based on experimental data for spherical particles, whereas CNC particles with elongated structures could exhibit different kinetics, (c) generally low estimates of alveolar surface area (64.5 m^2 vs 102 m^2 for humans and 0.03 m^2 vs 0.06 m^2 for mice), and (d) consideration of endotracheal vs nose-only exposure.

Why did we choose a cumulative dose of 240 μg/mouse? We agree that determining the slope of a dose–response relationship is important for the assessment of the relevance of the mouse doses used in our study to human exposures. The dose response of bolus adverse effects of CNC exposure (50 – 200 μg/mouse) in mice by pharyngeal route has been published previously [25]. Our study described dose–dependent effects assessed by several outcomes including inflammation, cytokine/chemokine release, pulmonary damage, and oxidative stress markers - protein carbonyls and 4-hydroxynonenal. Accordingly, the doses selected for repeated exposure regimen in this study were similar to our low-dose bolus exposure (40 μg/mouse) given to mice twice a week for 3 weeks thus reaching cumulative dose of 240 μg/mouse closer to the highest CNC bolus dose.

Shatkin and Oberdörster [14] further commented on the lack of positive and/or negative controls in our study. Our previous work demonstrated that asbestos administration (employed as a positive control) at the same lowest CNC dose (50 μg/mouse) demonstrated lower acute toxicity compared to CNC particles at equal mass concentration [38]. While detailed comparisons of pulmonary toxicity and long term effects of cellulose nanocrystals with asbestos and other fibrous materials (e.g., CNTs, CNF…) are definitely of great interest, they were not the topic of this study and we are not suggesting from our present data that CNC are asbestos-like with respect to the spectrum of asbestos induced diseases. We share the common opinion that such studies, together with the comparison of inhalation studies, are essential for the further assessment of possible human toxicity of CNCs.

Finally, Shatkin and Oberdörster [14] question whether comparisons of CNC to carbon nanotubes and asbestos was necessary in the abstract. It is common knowledge in the field of nanotoxicology that high aspect ratio materials, particularly carbon nanotubes, can be potentially pathogenic like asbestos. The similarity in mechanisms and pathways of toxicity have been articulated and emphasized in many published papers and included in their titles or abstracts [39–46] as well as in studies of nanocellulose materials [25, 47–49]. Thus, while not directly investigated in the current

study, this paradigm has been widely accepted by the toxicology community, it was not directly investigated in the current study and we agree that this inference should have been left out.

In summary, we do not believe that the slight differences in the total body weights of female and male C57BL/6 mice contributed to the elevated responses in female mice upon exposure to CNC. Our study was aimed at the investigation of gender differences of pulmonary toxicity from repeated pharyngeal aspiration exposures to CNC materials in mice, rather than exploration of dose–response effects of CNCs. We believe this study representing a "proof of principle" or "hypothesis forming" study [27], was an investigation into gender differences of pulmonary toxicity from repeated pulmonary aspiration exposure to CNC and needs to be followed up by long term inhalation studies, as rightfully emphasized by Shatkin and Oberdörster [14]. Further, the specific gene expression changes related to carbohydrate/pattern /polysaccharide and glycosaminoglycan binding and signaling, as detailed in our paper, support a biological response as a result of CNC exposure. Taken together, our data provide evidence that raise doubts concerning the validity of the conclusion drawn by Shatkin and Oberdörster [14] that very high doses of CNCs cause inflammation and that such pulmonary responses are common to even benign fibrous and non-fibrous particles. However, we do agree that there is a critical need for further detailed research aimed at mechanistic understanding of potential risks of human exposure to CNCs. Studies in our research group, detailing the pulmonary effects of CNCs via inhalation exposure route at relevant exposure limits, are currently underway. Finally, we would like to thank Shatkin and Oberdörster for their thought provoking commentary [14] highlighting the critical need for continuous research aimed at better understanding of the potential significance and risk for human exposures to CNCs.

Abbreviations

CNC: Cellulose nanocrystals; FRC: Functional residual capacity; GSD: Geometric standard deviation; MMAD: Mass median aerodynamic diameter; MPPD: Multiple-Path Particle Dosimetry; OSHA: Occupational Safety & Health Administration; WHO: World Health Organization

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Availability of data and materials

All data generated or analyzed during this study are available from the corresponding author on reasonable request.

Authors' contributions

JAS prepared the first draft of this commentary; GO provided additional comments specifically related to dosimetry. Both authors read and approved the final manuscript.

Competing interests

JAS is president of Vireo Advisors LLC, an advisory firm to public and private organizations, including those seeking to commercialize nanomaterials.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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