

## Research Article

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# Cytotoxic and pro-inflammatory effects of molybdenum and tungsten disulphide on human bronchial cells

<https://doi.org/10.1515/ntrev-2022-0073>

received October 29, 2021; accepted February 9, 2022

**Abstract:** This study aimed to investigate the cytotoxicity and pro-inflammatory responses induced by tungsten disulphide (WS<sub>2</sub>) and molybdenum disulphide (MoS<sub>2</sub>) nanoparticles (NPs) in human bronchial cells (BEAS-2B). For cytotoxicity assessment, the cells were exposed to different concentrations (2.5–200 µg/mL) of WS<sub>2</sub>-NPs or MoS<sub>2</sub>-NPs for 24 and 48 h and then the MTT assay was performed. Afterwards, long-term toxicity was assessed by the colony forming efficiency assay (CFEA) during a 10 days' exposure of the cells. For pro-inflammatory responses, the expression of interleukin-6 (IL-6) and interleukin-1β (IL-1β) mRNA was estimated by the real-time PCR method. Both nanomaterials showed similar cytotoxic effects on BEAS-2B cells assessed by the MTT assay, *i.e.* reduction in cell viability to approx. 60–70% at concentrations of 2.5 and 5 µg/mL after 24 and 48 h. The percentage viability remained relatively constant at this level across all concentrations above 5 µg/mL. In long-term exposure, both nanomaterials inhibited colony formation in a wide range of concentrations up to 100 µg/mL. MoS<sub>2</sub>-NPs were slightly more cytotoxic than WS<sub>2</sub>-NPs. Additionally, MoS<sub>2</sub>-NPs caused an increase in mRNA levels of cytokines, IL-1β, and IL-6 at concentration of 50 µg/mL, while WS<sub>2</sub>-NPs did not cause any changes in the level of mRNA for both cytokines. We also visualised the changes in the cells as a result of WS<sub>2</sub>-NPs or MoS<sub>2</sub>-NPs exposure (2.5 and 25 µg/mL) *via* holotomographic microscopy. This work demonstrates the hazardous potential of both nanomaterials and indicate that WS<sub>2</sub> and MoS<sub>2</sub>

nanoparticles should be included in the occupational risk assessment.

**Keywords:** molybdenum disulphide, tungsten disulphide, cytotoxicity assay, lung cells, holotomographic microscopy

## 1 Introduction

Molybdenum disulphide (MoS<sub>2</sub>) and tungsten disulphide (WS<sub>2</sub>) are a class of layered materials well known for their excellent lubrication performance. They are widely used both as oil additives as well as dry/solid lubricants. The use of molybdenum and tungsten disulphides in dry lubricants is of particular importance in aviation, automotive, paper and food industries, in precision mechanics, electronics, chemical, glass, plastics, and many other industries [1–3]. For the last few years, the use of the nano-form of these compounds has been increasing. Particularly, 2D nanoplates and fullerene-like structures are widely used in many tribological applications [4–6].

At present, there are only a few reports available on the effects of MoS<sub>2</sub> and WS<sub>2</sub> on human bronchial cells. Interestingly, Appel *et al.* [7] investigated the potential toxicological effects of WS<sub>2</sub> nanoparticles prepared by several methods such as mechanical exfoliation and chemical vapour deposition (CVD) toward human epithelial kidney cells (HEK293F). More importantly, Pardo's group [8] presented low cytotoxicity of WS<sub>2</sub> and MoS<sub>2</sub> nanoparticles in human bronchial epithelial cells, which are used in industrial and medical applications to compare to standard environmental particulate matter. Corazzari *et al.* [9] reported evaluation of the impact of commercial 2D WS<sub>2</sub> powders on the viability of other human cell lines, such as NL-20, HEPG2, and macrophages. Promising use of MoS<sub>2</sub> and WS<sub>2</sub> as green components in separation technologies was presented by Köhler's team [10]. They explained the possibility of applying MoS<sub>2</sub> and WS<sub>2</sub> in modern desalination processes based on 2D membranes,

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contributing to increase the availability of clean water in the world. The WS<sub>2</sub>-NPs and MoS<sub>2</sub>-NPs are also used for the synthesis of analogues of such nanomaterials as carbon nanotubes, fullerenes, or graphene, in order to replace them in many consumer products [11,12]. The 2D layered nanomaterials including WS<sub>2</sub>, MoS<sub>2</sub> nanosheets and graphene oxide, reduced graphene oxide, and Ti<sub>3</sub>C<sub>2</sub>T<sub>x</sub> MXene, used as solid lubricants, ensure low wear and friction over the entire component's lifetime. In addition to being applied as a solid lubricant, MoS<sub>2</sub> nanosheets can be used as a reinforcement phase in composites and lubricant additive under extreme conditions, such as vacuum or dry contacts at high temperatures [13–15]. Zhang's team obtained MoS<sub>2</sub> nano-balls fabricated in isopropanol by ultrasonication. This procedure allows to reduce the contact area in steel contacts through extraordinary macro-scale lubricity [16]. The application of WS<sub>2</sub>-NPs or MoS<sub>2</sub>-NPs in lubricants which are used in automotive and aviation is also very beneficial for the environment due to the reduction in particulate matter emission, which results in lesser contamination of air, water, and soil [3,17]. As a result, the reduction in emissions translate into a reduction in human morbidity, as solid particles (especially the fraction of ultrafine dust, with a diameter equivalent to those of nanoparticles) are responsible for the generation of respiratory, cardiovascular, allergic, and cancer diseases. On the other hand, dry lubricants are used in the form of powders and aerosols, which can lead to their significant emission to the environment, in particular the working environment. For this reason, the determination of toxicity of the compounds in the nano-form is extremely important. According to toxicological reports, the use of nanomaterials, although beneficial for technological reasons, is associated with an uncertain health risk [18]. There is a concern that substances with low toxicity may adversely affect the body if used in the form of nanoparticles [19]. Despite the still emerging applications of WS<sub>2</sub>-NPs and MoS<sub>2</sub>-NPs, only a few investigations into their biocompatibility and toxicity have been performed. It is important to note that their modified forms were mainly evaluated [7,20,21]. Considering the increased use of WS<sub>2</sub>-NPs and MoS<sub>2</sub>-NPs in nanoscale, there is the necessity to study their unfunctionalised forms, especially in terms of potential health effects after inhalation. Most of the toxicity studies of nanomaterials are conducted using *in vitro* methods.

The aim of this study was to assess the cytotoxic and pro-inflammatory effects after exposure of human bronchial cells (BEAS-2B cell line) to unmodified WS<sub>2</sub>-NPs and MoS<sub>2</sub>-NPs. Application of relatively new holotomographic technique allowed to observe the morphological alterations in cells exposed to low doses of WS<sub>2</sub>-NPs and MoS<sub>2</sub>-NPs (2.5 and 25 µg/mL), which testified to adverse processes in the cells.

## 2 Materials and methods

### 2.1 Chemicals and reagents

WS<sub>2</sub>-NPs and MoS<sub>2</sub>-NPs with nominal particle size below 90 nm were purchased from Sigma-Aldrich. The media for cell cultures were provided by Gibco BRL (Life Technologies Ltd, Paisley, UK). All reagents for gene expression assay were provided by Thermo Fisher Scientific Inc., Rockford, IL, USA. Other reagents were supplied by Sigma Chemical Co. (St Louis, MO, USA), if not indicated otherwise.

### 2.2 Characterisation of WS<sub>2</sub>-NPs and MoS<sub>2</sub>-NPs

The morphology of WS<sub>2</sub>-NPs and MoS<sub>2</sub>-NPs was determined by scanning electron microscope (SEM, Zeiss Ultra Plus). The particles were scanned at 2 kV. A specific surface area was measured by the Brunauer–Emmett–Teller (BET) technique (Gemini 2360, Micromeritics). The particle properties in a liquid environment, such as hydrodynamic diameter, particle size distribution, particle size, and zeta potential were characterised using the Nanoparticle Tracking Analysis (NTA, NS500, Nanosight Ltd, UK) and Dynamic Light Scattering (DLS, ZetaSizer Nano ZS, Malvern). The measurements were performed both in the phosphate-buffered saline (PBS, Gibco, Invitrogen, Carlsbad, CA, USA) dispersion and in the culture media. The stock solution (1 mg/mL) of WS<sub>2</sub>-NPs and MoS<sub>2</sub>-NPs was prepared by suspending the powder in cold PBS. Then, suspensions were sonicated on ice at a high energy level of 420 J/cm<sup>3</sup> (for 5 min with 90% amplitude) (Sonica Q 700, Qsonica LLC, USA). Working solutions were prepared immediately before the toxicity tests in a serum-free culture medium and vortexed for approx. 1 min to ensure homogeneity prior to use with the cells. A new stock solution was prepared before each experiment.

### 2.3 Cell culture and treatment

The BEAS-2B cells (CRL-9609) were purchased from American Type Culture Collection (LGC Standards Sp. z o.o.). The cells were grown in the LHC-9 serum-free medium in the culture flasks coated with collagen type 1 (Greiner). The cells were maintained at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. To detach cells from the culture plates, 0.25% trypsin-EDTA (Gibco) was used. Cell number and viability were determined

in a Bürker chamber by the trypan blue exclusion method. Additionally, the cells were screened for *Mycoplasma* sp. infection using MycoAlert™ PLUS Mycoplasma Detection Kit (Lonza, Walkersville, Inc.).

## 2.4 Cytotoxicity studies

To detect cytotoxic effects of WS<sub>2</sub>-NPs or MoS<sub>2</sub>-NPs, we performed the MTT-tetrazolium salt reduction assay (MTT), which measures mitochondrial dehydrogenase activity living cells. The BEAS-2B cells were seeded in 96-well microplates at a density of  $2 \times 10^4$ /well and cultured overnight. Then, cells were exposed to WS<sub>2</sub>-NPs or MoS<sub>2</sub>-NPs (2.5–200 µg/mL) for 24 or 48 h. The toxicity was calculated as a ratio of absorbencies of treated and untreated cells. The absorbencies were measured at 570/620 nm using a Synergy 2 microplate reader (BioTek, USA). Cytotoxicity tests were conducted in at least three independent replications.

## 2.5 Colony forming efficiency assay (CFEA)

The CFEA (also referred to as clonogenic assay) was conducted according to the procedure described by Franken *et al.* [22] and adapted from Kruszewski *et al.* [23]. The colony forming efficiency assay is based on the ability of a single cell to grow into a colony. A colony is defined to consist of at least 50 clones of one cell, which corresponds to 6 mitotic divisions. This test is used to detect cells that retained the capacity for producing a large number of progenies after treatments that can cause reproductive death as a result of damage to chromosomes, apoptosis, *etc.* Exponentially growing cells were harvested and seeded in a Petri dish of 60 × 15 mm (21 cm<sup>2</sup>) (Iwaki Cell Biology, Japan) at a density of 500 cells/dish together with a tested compound. Each dish finally contained 5 mL of a cell culture medium with WS<sub>2</sub>-NPs or MoS<sub>2</sub>-NPs in appropriate concentrations (25, 50, and 100 µg/mL) at least in three replicates for each treatment. The cells were exposed to nanoparticles for 10 days. After this period, particle solutions were removed, cells were washed with PBS, fixed with ethanol (Sigma-Aldrich), stained (0.4% Giemza, Sigma-Aldrich), and colonies were counted using a stereomicroscope (IUL, Spain). Then, plating efficiency (PE) and surviving fraction (SF) were calculated as below:

$$\text{PE} = \frac{\text{number of colonies formed}}{\text{number of cells seeded}}$$

$$\text{SF} = \frac{\text{number of colonies formed after treatment}}{\text{number of cells seeded}} \times \text{PE}.$$

PE ratio for BEAS-2B cells calculated from three independent experiments was above 0.6.

## 2.6 Cytokine real-time PCR assays

BEAS-2B cells were seeded into tissue culture 6-well plates (TPP, Techno Plastic Products AG) at a density of 300,000 cells/well and allowed to adhere and establish overnight at 37°C in a humidified atmosphere (5% CO<sub>2</sub>). Cells were treated with WS<sub>2</sub>-NPs or MoS<sub>2</sub>-NPs at different concentrations (5, 25, and 50 µg/mL) for 24 h. Next total cellular RNA was extracted using the ReliaPrep RNA Cell MiniPrep System (Promega), the quantity of RNA obtained from each sample was measured using Quantus fluorometer with the QuantiFluor RNA System (Promega) according to the manufacturer's guidelines.

The 0.5 µg total RNA were reverse transcribed to obtain cDNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Inc.) according to manufacturer's instructions.

All genes were amplified using TaqMan® Gene Expression assays (Thermo Fisher Scientific, Inc.) for interleukin IL-1β (assay ID. Hs01555410\_m1) and IL-6 (assay ID. Hs00174131\_m1). Data were normalised to glucuronidase beta (GUSB) mRNA levels (assay ID. Hs00939627\_m1) as an endogenous control. Quantitative real-time PCR was performed on samples using the 7500 real-time PCR detector (Thermo Fisher Scientific, Inc.).

## 2.7 Holotomographic visualisation of morphological changes

The BEAS-2B cells were seeded into 35 mm culture dishes (IBIDI, Gräfelfing, Germany) at a density of 20,000 cells/dish and incubated for 24 h. After this period, cells were treated with WS<sub>2</sub>-NPs or MoS<sub>2</sub>-NPs (2.5 or 25 µg/mL) and incubated for 24 h. Then, the medium was replaced with MitoView dye (Biotium) at a concentration of 100 nM and incubated for 15 min at 37°C to visualise mitochondrial structures. The cells were also counterstained for DNA with 0.1 µg/mL Hoechst 33258 (Thermo Fisher Scientific). As a positive control we used cells treated with staurosporine (37 nM, Sigma-Aldrich). Then, dishes of control (untreated cells) or exposed cells were placed on the tomographic microscope (3D Cell Explorer, Nanolive S.A., Lausanne, Switzerland) and 3D tomographic images (z-stacks) were created. Further post processing steps such

as background reduction and contrast enhancement were applied to the final figures using STEVE software (Nanolive).

## 2.8 Statistical analyses

Three separate *in vitro* cytotoxicity experiments were conducted in which all samples of nanomaterials were tested simultaneously. At least three independent experiments were performed for cytotoxicity endpoint. The CFEA results were presented as surviving fraction ratio (SF)  $\pm$  standard deviation (SD). SF = 1 was set for the control. CFEA data were analysed by Student's test for comparison between two groups. A value of  $p < 0.05$  indicates a statistically significant difference. The software employed for statistical analysis was Statistica, version 7.1. The results of RT-qPCR are presented as mean values obtained from three experiments. The significance of differences between the mean values was assessed using the paired Student's *t*-test using 7500 Software v2.0.6. (Thermo Fisher Scientific, Inc.). The data were presented as the mean value  $\pm$  standard deviation (SD).

## 3 Results

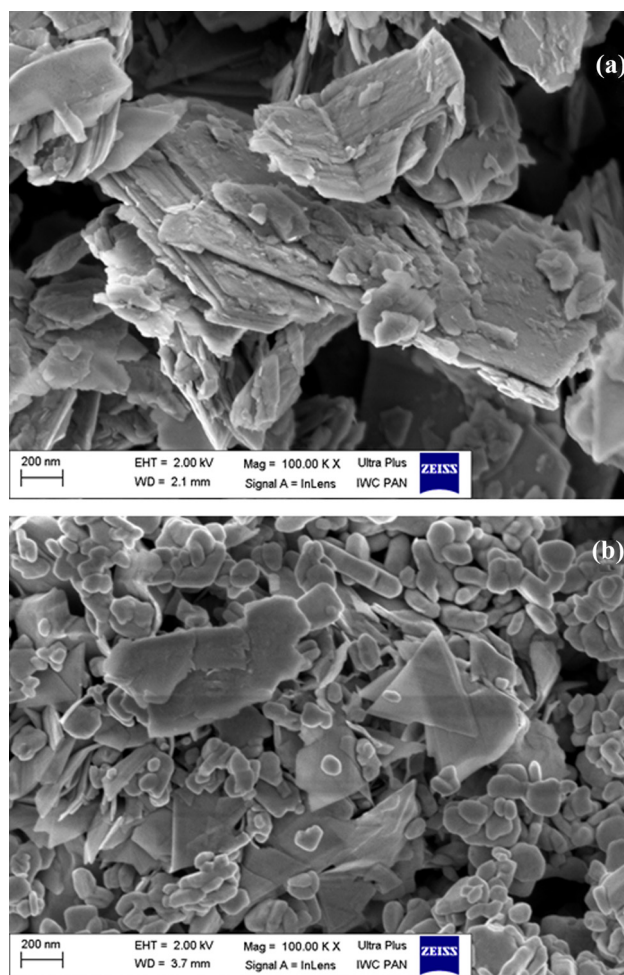
### 3.1 Characterisation of WS<sub>2</sub>-NPs and MoS<sub>2</sub>-NPs

Microscopic analysis (SEM) showed that both nanomaterials had the structure of multilayer plates, whose length and width was on the order of a few micrometres, while the thickness was on the nano scale (Figure 1a and b). WS<sub>2</sub>-NPs were characterised by a larger fraction of particles of smaller dimensions in length, width, and thickness. Specific surface area for MoS<sub>2</sub>-NPs and WS<sub>2</sub>-NPs was 7.8 and 6.2 m<sup>2</sup>/g, respectively.

The particle size distribution analysis by DLS showed that the suspensions of WS<sub>2</sub>-NPs were more homogeneous (PDI  $\approx$  0.3) and more stable ( $\zeta \approx -30$ ) in comparison with MoS<sub>2</sub>-NPs (PDI  $\approx$  0.6 and  $\zeta \approx -10$ ) (Table 1). Both DLS and NTA methods showed that MoS<sub>2</sub>-NPs and WS<sub>2</sub>-NPs existed both as single and as aggregate/agglomerate particles.

### 3.2 Effects of MoS<sub>2</sub>-NPs and WS<sub>2</sub>-NPs on cell viability and proliferation

BEAS-2B cells were exposed to a wide range of concentrations of MoS<sub>2</sub>-NPs or WS<sub>2</sub>-NPs (2.5–200 µg/mL) for 24



**Figure 1:** SEM image of MoS<sub>2</sub>-NPs (a) and WS<sub>2</sub>-NPs (b). Magnification and scale are presented on the picture.

or 48 h. Afterwards, the MTT assays were performed. The results of the MTT assays are summarised in Figure 2. Both nanomaterials caused a reduction in cell viability to approx. 60–70% at concentrations of 2.5 and 5 µg/mL after 24 or 48 h. The percentage viability remained relatively constant at this level across all concentrations above 5 µg/mL.

As shown in Figure 3, both nanomaterials were able to significantly decrease clonogenic survival and cell proliferation in a dose-dependent manner in a wider range of concentrations up to 100 µg/mL, when exposed constantly over 10 days. MoS<sub>2</sub>-NPs in the lowest concentrations (25 and 50 µg/mL) decreased the proliferation of BEAS-2B cells to about 80%. Higher concentration (100 µg/mL) resulted in inhibition of growth of the culture by approx. 30%. In case of WS<sub>2</sub>-NPs, the concentration of 100 µg/mL inhibited the colony formation by about 50%.



Table 1: Physicochemical characteristics of MoS<sub>2</sub>-NPs and WS<sub>2</sub>-NPs

Particles	MoS <sub>2</sub> -NPs	WS <sub>2</sub> -NPs
Specific surface area [m <sup>2</sup> /g] [BET method]	7.80	6.20
Hydrodynamic diameter [nm] [DLS method]	616.17 ± 2.92	226.57 ± 4.84
Polydispersity index [Pdl]	0.59 ± 0.08	0.320 ± 0.03
Zeta potential ζ [mV]	−10.56 ± 0.78	−29.4 ± 2.18
Particle diameter [nm] [NTA method]	Mean value = 294.25 ± 8.7 Mode = 139.9 ± 8.5	Mean value = 218.03 ± 5.2 Mode = 190.37 ± 3.5

3.3 Cellular cytokine response

The expression of mRNAs for cytokines IL-1β and IL-6 was used as a marker of the pro-inflammatory response induced in BEAS-2B cells exposed to different concentrations (5, 25, or 50 µg/mL) of WS<sub>2</sub>-NPs or MoS<sub>2</sub>-NPs for 24 hours. The gene expression of IL-1β and IL-6 increased after treatment with the MoS<sub>2</sub>-NPs in a dose-dependent manner (Figure 4). However, the level of mRNA for both cytokines significantly differed compared to control only in the highest concentration of MoS<sub>2</sub>-NPs (50 µg/mL). We

did not observe significant changes in the IL-1β or IL-6 gene expression after treatment of cells with WS<sub>2</sub>-NPs.

3.4 Holotomographic visualisation of morphological changes

After exposure of BEAS-2B cells to MoS<sub>2</sub>-NPs or WS<sub>2</sub>-NPs at low doses of 2.5 and 25 µg/mL, we observed morphological changes after 24 and 48 h. The untreated cells (negative control) had no alteration in cell morphology or any membrane

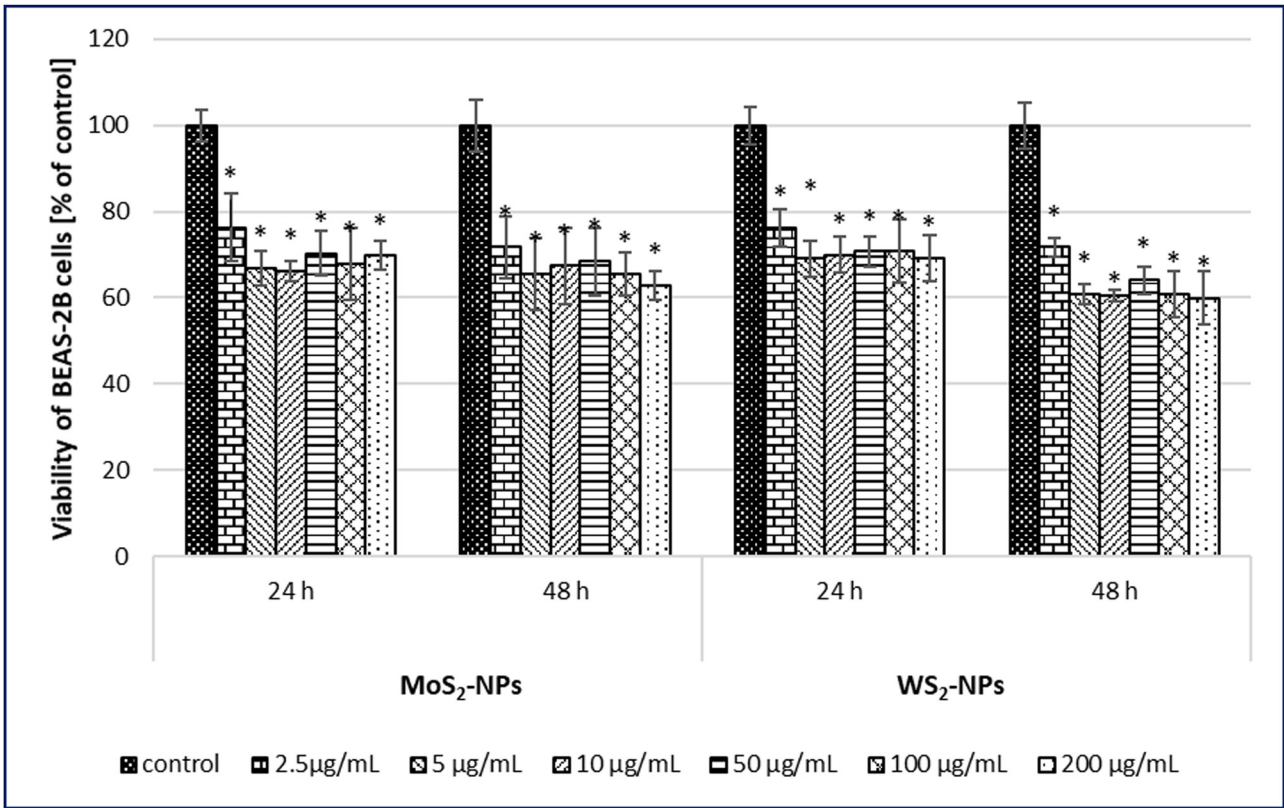
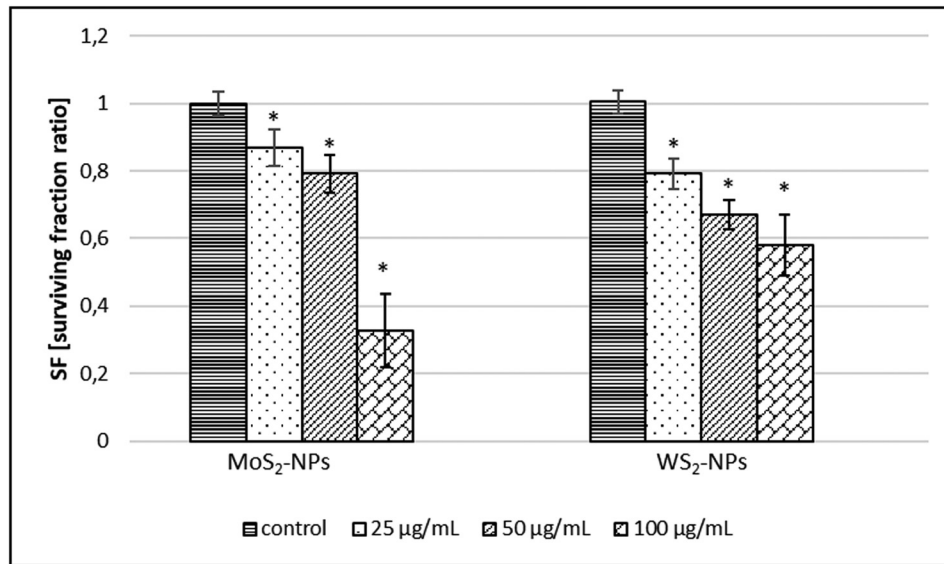


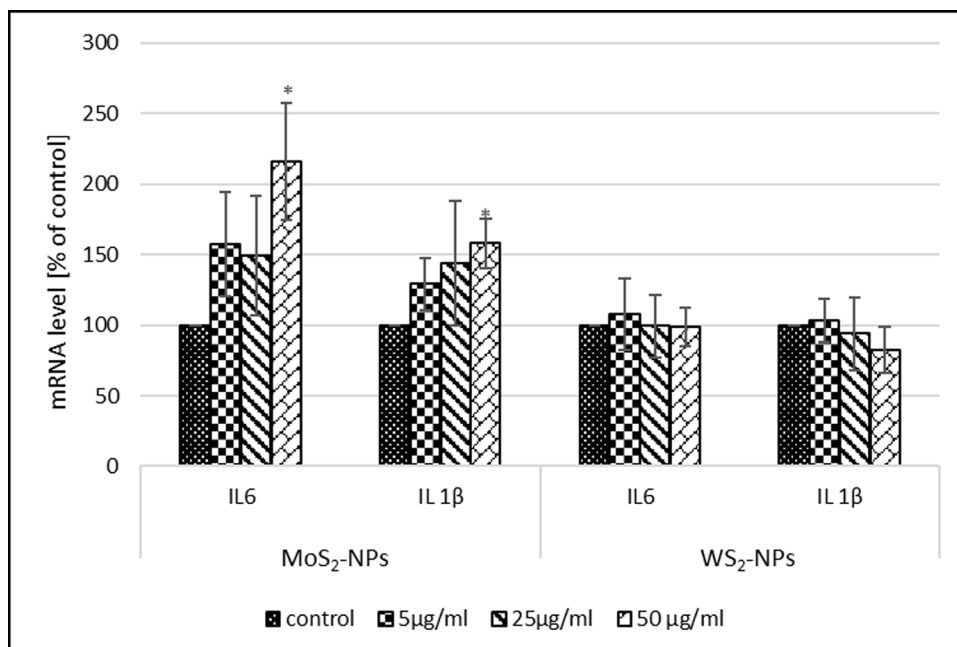
Figure 2: Cytotoxic effect of MoS<sub>2</sub>-NPs or WS<sub>2</sub>-NPs in BEAS-2B cells after 24 and 48 h exposure assessed by MTT assay. Data are expressed as the percentage viability of cells exposed to compounds relative to control cells. Each point represents an average value ± SD from at least three experiments; \*p < 0.05 vs control.



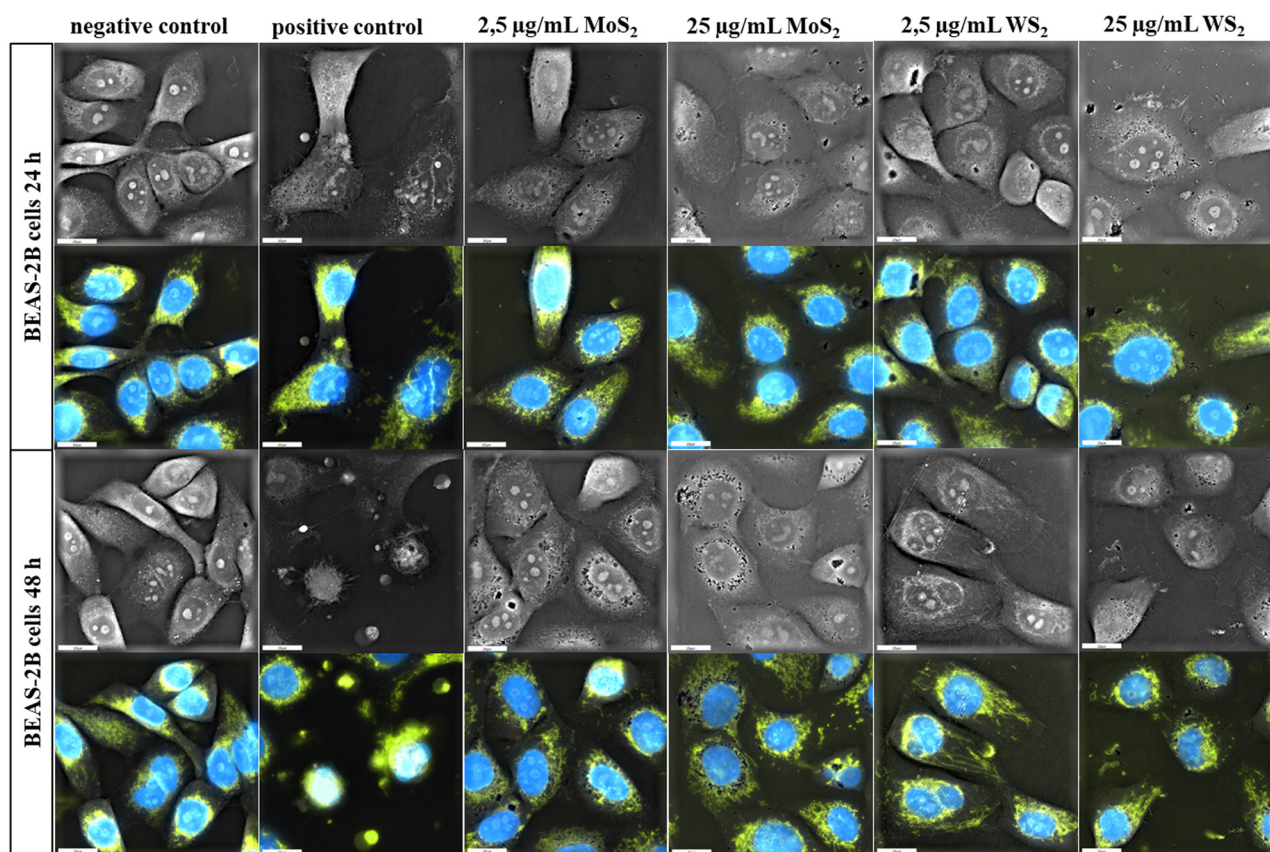
**Figure 3:** Colony forming ability of BEAS-2B cells treated with different concentrations of WS<sub>2</sub>-NPs or MoS<sub>2</sub>-NPs. Each bar represents the mean value  $\pm$  SD of three independent experiments. \* $p < 0.05$  vs control.

damages. In treated cells, we noticed changes in the size and shape of cells and loss of cell-cell contact compared with negative control. During the cells' treatment with MoS<sub>2</sub>-NPs or WS<sub>2</sub>-NPs and time of exposure, the degree of cytoplasmic vacuolisation was increased. Changes in the shape of nucleus and intracellular location of mitochondria and their density

were observed as a result of exposure of cells to NPs. In untreated cells, mitochondria showed a typical cytoplasmic distribution. We noticed that the intensity of the described changes increases in a time – and dose – dependent manner. Morphological changes occurring in cells after exposure are presented in Figure 5.



**Figure 4:** The expressions of cytokine mRNA in BEAS-2B cells exposed to MoS<sub>2</sub>-NPs or WS<sub>2</sub>-NPs. Cells were exposed to 5, 25, or 50 µg/mL for 24 h. The relative mRNA expressions of IL6 and IL-1β were measured by real-time PCR. The expressions were normalised against GUSB. The data represent the mean value  $\pm$  SD of three independent experiments. \* significantly different from control [ $p < 0.005$ ], using Student's *t*-test.



**Figure 5:** Representative images after fluorescence staining to visualise mitochondrial and nucleus location and morphology of living BEAS-2B cells. Negative control (untreated cells) and positive control (Staurosporine 37 nM) cells after exposure to MoS<sub>2</sub>-NPs or WS<sub>2</sub>-NPs (2.5 and 25 µg/mL). Images are presented in bright field and after excitation of FITC fluorescence. Description of changes is presented in the text. Pictures were taken under a magnification of 60×. Scale bar: 20 µm.

## 4 Discussion

The determination of MoS<sub>2</sub>-NPs and WS<sub>2</sub>-NPs toxicity has gained importance due to their expanding application in many fields of industry or biomedicine and the associated increase in the potential health risk in the work environment. Our research focused on two areas. First, we evaluated the ability of WS<sub>2</sub>-NPs and MoS<sub>2</sub>-NPs to induce cytotoxicity and cytokine response in the human lung epithelial cell model – BEAS-2B. These cells are often used to evaluate the toxicity of dust pollution and preferred as an experimental model of the respiratory system due to the normal phenotype of the cells and their ability to release pro-inflammatory cytokines [24,25]. Second, we documented the morphological alterations and changes in organelles after exposure of cells to low doses (2.5 and 25 µg/mL) of NPs.

The crucial role in the *in vitro* toxicity assessment of nanomaterials is played by their proper characterisation, in particular, changes in particle size and particle size

distribution, and formation of aggregates or agglomerates in culture media. These parameters may be a significance to the interpretation of results obtained in toxicological studies [26]. In the present work, the MoS<sub>2</sub>-NPs and WS<sub>2</sub>-NPs formed polydisperse suspensions of different particle sizes in the LHC-9 medium. Both single particles and agglomerates, which were about 2–4 times larger than the nominal particle size, were determined. It has to be taken into consideration that aggregation is a natural process for nanoparticles and is included in the definition of nanomaterials accepted by the European Union [27].

The cytotoxic activity of MoS<sub>2</sub>-NPs and WS<sub>2</sub>-NPs was evaluated with the use of MTT test. The cells were exposed to different concentrations of NPs (2.5–200 µg/mL) for 24 and 48 h. We found little trend in their cytotoxicity profiles: nanomaterials in concentrations of 2.5 and 5 µg/mL reduced the viability of the cells to the level of about 70%, after which there was no further decrease in cell viability, despite being exposed to higher concentrations. We might conclude



that particle aggregation in higher concentrations causes lower availability to the cells. Very similar results of MoS<sub>2</sub>-NPs cytotoxicity were achieved by Chng *et al.* [28] in MTT assay using A549 cells. Pristine MoS<sub>2</sub>-NPs decreased viability of the cells at concentrations below 6.25 µg/mL, whereas higher concentrations did not affect cells viability. In turn, Wang *et al.* [20] showed a slight (up to 80%) decrease in the viability of THP-1 cells and no cytotoxic effect in BEAS-2B cells exposed to various structural forms including aggregates of MoS<sub>2</sub>-NPs in concentrations of 10–50 µg/mL. Importantly, the authors demonstrated that MoS<sub>2</sub>-NPs, despite the lack of cytotoxic effects, caused the release of pro-inflammatory mediators in cells, *in vivo* inflammation, and fibrosis. Also, Qureshi *et al.* [29] observed a minimal decrease in the survival rate of HeLa cells with the increase in MoS<sub>2</sub>-NPs concentration, but they used higher doses, *i.e.* from 32.5 to 300 µg/mL. Likewise, Corazzari *et al.* [9] in studies on A549 cells demonstrated cytotoxic activity of MoS<sub>2</sub>-NPs and WS<sub>2</sub>-NPs in high concentrations only.

In the literature, there are few reports on the toxicity of pristine MoS<sub>2</sub>-NPs and even fewer of those about WS<sub>2</sub>-NPs. Their functionalised or modified forms, such as exfoliated, fullerene-like ones or nanotubes were mainly evaluated. The results of some studies indicate that modifications may change toxicity of MoS<sub>2</sub>-NPs or WS<sub>2</sub>-NPs. Such a tendency was confirmed by Wang *et al.* [20], the data obtained on THP-1 and BEAS-2B cells suggested that exfoliation attenuates the toxicity of MoS<sub>2</sub>-NPs. Other studies [7] also confirm rather low toxicity of a modified form of MoS<sub>2</sub>-NPs or WS<sub>2</sub>-NPs. Exfoliated MoS<sub>2</sub>-NPs and WS<sub>2</sub>-NPs at concentrations of 1–100 µg/mL caused weak cytotoxic and prooxidative activity in HEK293F cells.

MoS<sub>2</sub>-NPs functionalised with polyethylene glycol caused a slight reduction in viability (up to 80–90%) in the culture of RAW264.7 mouse macrophages and human embryonic HEK293F cells at a high concentration of 200 µg/mL [21]. A similar effect, that is a slight decrease in viability to the level 80%, was observed by Liu *et al.* [30] in HeLa cells exposed to exfoliated MoS<sub>2</sub>-NPs at a concentration of 160 µg/mL. Fullerene-like forms (IF-MoS<sub>2</sub>) and nanotubes (INT-WS<sub>2</sub>) remained nontoxic for nontransformed human bronchial cells (NL-20) in concentrations up to 100 µg/mL. However, they induced a relatively low release of the pro-inflammatory cytokines IL-1β, IL-6, IL-8, and TNF-α [8]. The weak cytotoxic effect of IF-MoS<sub>2</sub> and IF-WS<sub>2</sub> nanoplaques was observed by Teo *et al.* [11]. After a 24 h exposure of A549 cells, they observed a reduction in cell viabilities to approx. 30% at a high concentration (400 µg/mL).

In order to study long-term toxic effects of MoS<sub>2</sub>-NPs and WS<sub>2</sub>-NPs, we used the colony forming efficiency

assay. This assay is a suitable and robust *in vitro* method to assess cytotoxicity of nanomaterials, which was confirmed by interlaboratory comparison study performed in the frame of OECD's Working Party of Manufactured Nanomaterials [31]. This test does not require any dyes, which can interact with nanomaterials and lead to invalid results [32,33]. In this experiment, both MoS<sub>2</sub>-NPs and WS<sub>2</sub>-NPs were able to significantly decrease clonogenic survival and cell proliferation in a dose-dependent manner after exposure at doses of 25–100 µg/mL for 10 days. The MoS<sub>2</sub>-NPs were slightly more cytotoxic than WS<sub>2</sub>-NPs.

According to the concept of grouping nanomaterials in terms of risk that they can pose for health, they can be classified as High Aspect Ratio Nanomaterials (HARNs), so they potentially cause the induction pro-inflammatory reaction in the body tissues [34]. Inflammatory reactions induced in lungs under the influence of nanoparticles, especially HARNs, were considered as one of the main reason of their adverse health effects [35,36]. The induction of cytokines by airway cells *in vitro* has been widely used to assess the pro-inflammatory effects of ambient and occupational nanoparticles, *e.g.* particulate matter PM<sub>2.5</sub>, diesel particles, pollution-related metals, and many others [37]. In the present study, the mRNA for cytokine IL-6 and IL-1β was evaluated after exposure of BEAS-2B cells to MoS<sub>2</sub>-NPs or WS<sub>2</sub>-NPs. IL-6 is a pleiotropic cytokine which plays an important role in acute inflammation in the lungs. The activation of IL-6 transcription has been observed in response to exposure to organic extracts from diesel exhaust particles [38] and transition metals [37]. In the present study, there were differences with regard to inflammatory potential of MoS<sub>2</sub>-NPs and WS<sub>2</sub>-NPs. The MoS<sub>2</sub>-NPs caused an increase in the level of both IL-6 and IL-1β mRNA at a concentration of 50 µg/mL, while in the case of WS<sub>2</sub>-NPs, mRNA expression was at control level.

Treatment of cells with NPs may also affect changes in their morphology [39]. In previous report [40], we showed that holotomographic microscopy (HTM) is an excellent tool for visualisation of the morphological alterations in cells after exposure to NPs. In this work, we also used HTM to document changes in morphology and organelles of cells treated with MoS<sub>2</sub>-NPs or WS<sub>2</sub>-NPs (2.5 and 25 µg/mL) for 24 and 48 h (Figure 5). After exposure of cells to nanoparticles and staurosporine, we observed cell contraction due to the loss of intracellular waters and electrolytes compared to negative control. The vacuolisation of cytoplasm in both, positive control and treatment cells, reflected the effect of NPs. The characteristic impact of NPs was time dependent loss of cell-cell contact and changes in the shape of nucleus. We noticed swelling of mitochondria, changes in their intracellular location, their density, and dense granules in the



matrix as a result of cells' exposure to staurosporine or NPs. We might conclude that even low dose (2.5 and 25 µg/mL) of NPs can cause morphological alterations and induce disruption of cells.

Summarising, cytotoxic and pro-inflammatory effects of MoS<sub>2</sub>-NPs and WS<sub>2</sub>-NPs suggest their hazardous potency. The inclusion of WS<sub>2</sub> and MoS<sub>2</sub> nanoparticles in the occupational risk assessment is advisable. Holotomographic images show the morphological changes and alterations of organelles in BEAS-2B cells as a result of exposure to low concentrations of MoS<sub>2</sub>-NPs or WS<sub>2</sub>-NPs. Our findings highlight the importance of cytotoxicity of MoS<sub>2</sub>-NPs and WS<sub>2</sub>-NPs investigations, which in the future allow to improve the safety in the work environment.

**Acknowledgements:** The authors thank Lilianna Marciniak for her assistance, time and effort.

**Funding information:** This article has been based on the results of a research task carried out within the scope of the fourth stage of the National Programme "Improvement of safety and working conditions" partly supported in 2017–2019 – within the scope of research and development – by the Ministry of Science and Higher Education/National Centre for Research and Development. The Central Institute for Labour Protection – National Research Institute is the Programme's main co-ordinator.

**Author contributions:** Conceived and designed the experiments: L.Z., L.Ch.P., D.S., K.M.D., and J.S. Performed the experiments: L.Z., L.Ch.P., and D.S. Analysed the data: L.Z., L.Ch.P., and D.S. Wrote the paper: L.Z., L.Ch.P., and D.S. Corrected and edited the manuscript: L.Ch.P and D.S. All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

**Conflict of interest:** The authors state no conflict of interest.

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