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Reviewer's opinion

Thesis of Mrs. Faria Khan

"Chemical Profiling and Toxicological Assessment of Atmospheric Aerosol Using Human Lung Cells"

Airborne fine particulate matter of aerodynamic diameters < 2.5 μm (PM2.5) contributes to poor air quality, climatic change and exhibits adverse health effects. After inhalation, PM2.5 trigger different pathologies, particularly (but not only) in respiratory system, including exacerbation of chronic diseases (asthma, chronic obstructive pulmonary disease, allergic rhinitis), increased infection susceptibility, or decreased lung function parameters.

Chemical pollutants present in atmosphere in gaseous form and in particulate matter form a dynamic, interactive matrix, with properties changing with time. All pollutants are subject of ageing and accumulation during atmospheric lifetime. However, exact mechanism of their detrimental action on humans is not well understood.

In this context, the topic selection of the doctoral thesis by Mrs. Faria Khan should be assessed positively, as it is accurate not only in the scientific aspect, but it may have practical applications in the future. Taking up the topic by Mrs. Faria Khan, made in her doctoral dissertation, creates an opportunity to significantly expand knowledge in this field.

The title of the work accurately and concisely reflects its content.

I. Assessment of the form of work

Mrs. Faria Khan's essay for the PhD degree of medical sciences consists of 230 pages of the main text, divided into chapters, which are clearly presented in the ***Table of Contents***. Additionally, we find the ***List of Abbreviations*** at the beginning. The ***Abstract*** of the thesis supplements the text of the dissertation, summarizing the most important elements of the study (similar to the ***Abstrakt*** in Polish). This summary helps to quickly familiarize with the most important parts of the content of

the work, before starting to study it carefully. Nothing important in the paper is omitted from this summary.

Interestingly written, though quite short (8% of the total dissertation) ***Literature Review*** efficiently introduces the presented issue and provides an opportunity to rationally argue the purpose of the study.

The ***Objectives of the Thesis*** are presented as one general leading hypothesis and 4 specific aims.

Research methodology section (***Experimental section***) is presented succinctly as well as in a detailed manner. It is divided into ***Materials and Chemicals*** and ***Methodology and Experimental Procedure***. In total, it takes up 14% of the work. Presentation of the **Results and Discussion** takes 78% of the Thesis. Individual parts of the four works are divided into sub-chapters, which increases the clarity of the presentation. The short discussion that follows each section gives an overview of the most important results.

The **Research Summary and Future Perspective** answer most of the detailed questions mentioned in the purpose of the paper, although they are rather a summary of the results.

The list of ***References***, constituting a list of 395 English-language items, is arranged in the order of appearance in the text. The items listed in the ***References*** are cited in the relevant fragments of the text, which proves their precise use.

In the external evaluation of the form of the doctoral dissertation, it is a pleasure to praise its clear layout and careful publication. Tables and color figures are placed continuously in the text stream, which makes it easier to read the work.

II. Substantive assessment

Most of the abbreviations used in the text of the work are described for the first time and also included in the ***List of Abbreviations*** at the beginning of the thesis. The abbreviations used comply with the applicable terminology.

The considerations in the ***Literature Review*** present an overview of the toxic effects of aerosols, which efficiently introduces the topics discussed in the doctoral dissertation.

Against the background of these considerations, the aim of the study is fully justified, with the author singling out 4 detailed problems with sub-points, describing the planned analyzes.

The main objective was to determine the exposure health effects of various types of atmospherically relevant submicron organic aerosol (OA) at different biological levels: genomic, molecular, biochemical, and/or cellular levels. Biological changes were induced by submicron OA exposures originating from four different atmospheric sources:

- monoterpene-derived secondary organic aerosol (SOA) obtained through the ozonolysis of α-pinene, an abundantly emitted monoterpene from terrestrial vegetation.
- heterogeneously-aged isoprene-derived particulate 2-methyltetrol sulfates (2-MTSs). Isoprene, the most abundant reactive hydrocarbon released into Earth's atmosphere from vegetation, in the presence of inorganic sulfates, yields high quantities of gaseous epoxy diols, interacting with acidic sulfate aerosol to afford a wide variety of products, which undergo further chemical changes. 2-MTSs are the most abundant particulate organosulfates (OS) detected in ambient PM2.5
- atmospheric-relevant mono-nitrophenols (NPs), are found as trace pollutants in various environmental matrices, including PM2.5, agricultural residues, cloud water, rainwater, wildfires, and industrial wastes.
- emissions from biomass burning aerosol (BBA). BB is a major pollution source, particularly in urban, suburban, and rural areas, and related to increased morbidity and mortality through long-term inhalation. The four analysed BBA components included levoglucosan (LG), 3-nitrosalicylic acid (NS), 4-nitrocatechol (NC), and 4-nitroguaiacol (NG)

All those chemicals are present in atmophere. However, its impact on pulmonary pathophysiology remains uncertain.

When conducting her experiments, Mrs. Faria Khan used an oxidation flow reactor to produce secondary organic aerosols (SAO) from α-pinene ozonolysis, and the heterogeneous hydroxyl radical (•OH)-mediated oxidation of particulate 2-MTSs. This equipement give biochemical results corresponding to 0 - 22 days of atmospheric aging. The aerosol mixtures were analysed using liquid chromatography interfaced to high-resolution electrospray ionization tandem mass spectrometry (LC/ESI-HR-MS/MS) to detect organic acids and peroxides from α-pinene ozonolysis SOA, and from heterogeneously aged particulate 2-MTSs. The description of the laboratory methods is sufficiently detailed and precise.

To decipher changes induced by above mentioned pollutants, Mrs. Faria Khan used different biological targets. Cellular proliferation, cell viability, and oxidative stress were assessed as toxicological endpoints in this study. Time- and concentration-dependent viability values were used to determine the inhibitory concentration-50 (IC50) of each atmospheric OA system.

In order to determine acute exposure cellular effects, she used 2 *in vitro* cell models, immortalized human bronchial epithelial cells (BEAS-2B) and adenocarcinoma human alveolar epithelial cells (A549), using several ROS, mtROS, cellular viability, and cellular death assays.

Real-time quantitative polymerase chain reaction (RT-qPCR) was utilized to evaluate genomic changes (modulation of oxidative stress and inflammatory genes) that could result from exposures to heterogeneously aged particulates.

Functional assays with fluorescent probes were used to detect cellular reactive oxygen species (ROS) and mitochondrial ROS (mtROS) effects using flow cytometry and confocal microscopy to predict altered biochemical pathways at different exposure concentrations and times. Changes in cellular viability were analysed through live/dead staining using fluorescent microscopy, whereas cells death mechanisms were determined with flow cytometry.

The description of statistical analysis is concise, but sufficiently precise. Statistical methods used by the author are accurately selected and results of the analyzes are presented clearly.

Mrs. Faria Khan attempted to solve the problem specified in the Thesis title based on the implementation of several grants. She studied 4 independent, but logically related and complementary problems. Detailed presentation of the results constitute the essential and dominant part of the dissertation.

In the **first part** of the thesis, Mrs. Faria Khan quantified an increasing concentration response of three well-established α-pinene SOA tracers (pinic, pinonic, and 3-methyl-1,2,3-butanetricarboxylic acids) and a complete mixture of α-pinene ozonolysis SOA in cell lines.

BEAS-2B cells exposed to high concentration of α-pinene SOA presented decreased cellular proliferation (to ~70% and 44% at 24 – 48% post-exposure). This was due to cellular death but not changes in cellular growth or metabolic inhibition. Cells did not undergo any morfological changes, but a 4-fold increase in cellular oxidative stress was observed in this cell line following exposure, compared to controls. This reactive oxygen species build-up resulted from cytotoxicity after exposure to α-pinene ozonolysis products.

The three aforementioned tracers contributed to ~57% of the α-pinene ozonolysis SOA mass; however, other α-pinenen ozonolysis SOA components (multifunctional hydroperoxides) could have contributed more than these individual SOA tracers to the toxicological changes observed. Both aerosol exposure concentration and time had a significant role in determining the cellular response in BEAS-2B cells.

Contrary to this, no apparent changes in A549 cells and neither increase in ROS signal were observed after aerosol exposure, probably due to differences in metabolic activity of neoplastic cells. This different cell lines susceptibility to pollutants was apparent also in further parts of the Thesis. This observation of different cells fragility underscores the importance of careful selection of proper subject / target and careful interpretation of cellular model studies.

In the **second part** of the Thesis, Faria Khan focused on examining the inhalation toxicity associated with the isoprene-derived aerosol particles. She studied how multifunctional

organosulfates alters the anti-inflammatory and anti-oxidative – stress gene responses in BEAS-2B cells. First, she documented that chemical ageing under heterogenous *OH exposure caused the particulate toxicity to increase, as documented by mostly decreasing IC50 values in BEAS-2B cells. In gene-expression study, she observed that BEAS-2B cells were shifting between pro-inflammatory and anti-inflammatory responses depending on concentration and chemical composition of exposures (downregulation or upregulation of studied genes). This confirms dynamic genomic – level toxicological response of BEAS-2B cells following exposure to analysed chemicals, particularly evident in genes involved in glutathione detoxification pathway (*GDT, GSR, GCLC, GCLM*) and genes of interleukin cluster (*IL-6, IL-8, IL-10*). This corresponds to up-regulation of second-phase detoxification enzyme genes upon exposure to particulate 2-MTSs. In general, the results of this part od thesis underlies how atmospheric chemical aging of isporene -derived particules increases cellular toxicity in lungs.

In the **third part** of the Thesis, toxicological profiling of atmospherically relevant nitro phenols was studied to highlight the early biological changes in the lung cells. First, 2-nitrophenol (2NP), 3-nitrophenol (3NP), 4-nitrophenol (4NP), and their equimolar mixture was exposed to the eukaryotic lipid bilayer membrane model to determine the exposure effects on the cell membrane surface. Total disintegration of PC bilayer was observed only after exposure to high NPs concentrations (2 mg/ml), about tenfold more elevated than measured in the atmosphere. However, after exposure to NPs concentration at 200 ug/mL, the membrane thickness decreased and developed pores allowing the internalization of NPS. At lower concentrations, NPs do not disintegrate the membrane, but pass through it, changing its properties.

NPs leads to rearrangement of cell membrane structures, allowing for the internalization of NPs and changes in cellular morphology. The 3NP- and 4NP-treated BEAS-2B cells induced the highest growth inhibition and mtROS build-up, followed by apoptosis after 24 – 48 h of exposure. Consequently, the inhibitory concentration-50 (IC50) was highest in the 2NP and lowest in 4NP-treated BEAS-2B cells at 24 and 48 h of exposure.

Comparative toxicology documented, that 2-nitrophenol (2NP) was relatively safe, but highest toxicological response was observed for 4-nitrophenol (4NP) as well as NPs mixture. It is an important finding, as 4NP is one of the most abundant in most environmental PM2,5 samples.

Other important observation is that increased susceptibility and increased mitochondrial ROS signal after NPs exposure was observed only in BEAS-2B cells. However, further chronic studies are necessary to assess NPs contribution to lung associated pathologies.

Finally, in the **fourth part** of the Thesis, Mrs, Faria Khan conducted a detailed toxicological analysis of four important biomass burning aerosols (BBA) components in the A549 and BEAS-2B cell lines (levoglucosan LG, 3-nitrosalicylic acid NS, 4-nitrocatechol NC and 4-nitroguaiacol NG).

NC was the most toxic of all nitro aromatic components studied in BEAS-2B lines and negative effects (for example increased inhibition response, apoptosis) were detected at concentrations corresponding to real-life exposures achieved after few years. Very disturbing is that antropogenic and natural emissions of NACs included in this study often exceed measured threshold and may induce harmful effect at shorter time that one estimated in the study. Mrs. Faria Khan recommends that NC concentrations from ambient BBA emissions can be used as a predictor of adverse effects following inhalation.

This part of the study was concluded by proposing cellular death mechanisms upon exposure to these chemicals. These results suggest that exposure to NC, NG, LG and 4BBA lead to collapse of mitochondrial membrane potential and mitochondrial dysfunction was a predominant factor inducing cellular death.

The very brief discussion immediately follows the presentation of the results of each study providing an overview of their own observations, compared to the available literature on the subject. It shortly presents possible interpretations of the results, paying attention to the potential causes of discrepancies in the results reported by several authors. The reasoning is convincing, and the discussion is carried out logically and with great freedom, which proves the knowledge of the scientific basis of the issue and the ability to critically analyze.

Out of the 395 references cited, 125 were published between 2017 and 2021, and 5 are websites. References are correctly quoted in the text of the doctoral dissertation.

Mrs. Faria Khan's results are of great scientific importance, but will also constitute a starting point for further research. A well-carried out study brought important insights into the mechanisms of pollutants toxicity. The profiling of atmospheric aerosol mixtures and their individual markers from four atmospherically relevant systems provide a comparative toxicology in lung cells. Faria Khan presents a prediction system for the highest adverse effects following inhalation using the IC50 values and the number of atmospherically relevant years required to achieve that effect.

As she mentions, chronic exposure to heterogenous *OH oxidation products may lead to underlying pathology and there is a need for chronic exposure studies at atmospherically – relevant particulate concentrations. In particular, she highlights the need to study the aerosol chemical aging process in the atmosphere to predict the long-term exposure responses. Mrs. Faria Khan is aware that observed genomic – level changes after exposure could be different from proteomic changes, and *in vivo* models can help better predict the final inflammatory or irritative effect in the respiratory system.

This thesis determines the pathophysiological changes in the lungs at the molecular and cellular level after exposure, which varied significantly with the chemical composition and chemical structure of the markers, as well as time and concentration of exposure. The study further highlights

the urgent need to develop regulations and control strategies to mitigate the emission rates of some emission types due to their potential inhalation toxicity following acute exposure. Mrs. Faria Khan emphasizes the need to control the emission of particularly toxic ingredients, as nitro-aromatic components.

Results of the research presented in detail will undoubtedly be used in future publications. The author fills in important gaps in understanding the mechanisms of harmful effects of air pollutants. These results also indicate the need for further research in this area. Consequently, chronic exposure studies are warranted as human lungs are more complex than the *in vitro* system.

The most important for me as a clinician is definitive demonstration by Mrs. Faria Khan of the toxic influence of pollutants on physiological processes. One can expect that these observations will be confirmed in *in vivo* studies, as the findings of Mrs. Faria Khan may have practical application in the future.

From the reviewer's duty, I would like to point out that despite the apparent diligence in the preparation of the text edition, there are few editorial errors. The same abbreviation is used twice: PC for Phosphatidylcholines and Principal Components (see *List of abbreviations*). On the other hand, the *List of abbreviations* does not include some terms frequently used in the dissertation, such as OA organic aerosol, NP nitrophenol, SOA secondary organic aerosol, or tricarboxylic acid TCA.

The aforementioned minor editorial flaws do not diminish in any way the substantial value of Mrs. Faria Khan's doctoral dissertation. I believe that Mrs. Faria Khan showed excellent preparation for scientific work and proved that she has the ability to solve problems independently. The reviewed doctoral dissertation of Mrs. Faria Khan entitled "***Chemical Profiling and Toxicological Assessment of Atmospheric Aerosol Using Human Lung Cells***" is positively assessed by me because it contributes information important for science, but also meets the conditions set out in Polish regulations on academic degrees and academic titles [art. 187 ustawy z dnia 20 lipca 2018 r. Prawo o szkolnictwie wyższym i nauce (Dz.U. z 2018 r., poz. 1668 ze zm.)].

For the above-mentioned reasons, I submit an application to the High Council of the Institute of Physical Chemistry of Polish Academy of Sciences for the admission of Mrs. Faria Khan to the next stages of the doctoral dissertation.

I am also applying for distinction for the thesis of Mrs. Faria Khan.

Henryk Mazurek, MD, PhD