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Toxicological appraisal of the chemical fractions of ambient fine (PM_{2.5-0.3}) and quasi-ultrafine (PM_{0.3}) particles in human bronchial epithelial BEAS-2B cells

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1 **TOXICOLOGICAL APPRAISAL OF THE CHEMICAL FRACTIONS OF AMBIENT**
2 **FINE (PM_{2.5-0.3}) AND QUASI-ULTRAFINE (PM_{0.3}) PARTICLES IN HUMAN**
3 **BRONCHIAL EPITHELIAL BEAS-2B CELLS**

4
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24 **ABSTRACT**

25

26 New toxicological research is still urgently needed to improve the current knowledge about
27 the induction of some underlying mechanisms of toxicity by the different chemical fractions of
28 ambient particulate matter (PM). This *in vitro* study sought also to better evaluate and compare the
29 respective toxicities of fine particles (PM_{2.5-0.3}) and their inorganic and organic chemical fractions,
30 and the respective toxicities of the organic chemical fractions of PM_{2.5-0.3} and quasi-ultrafine
31 particles (PM_{0.3}). Human bronchial epithelial BEAS-2B cells were also exposed for 6 to 48 h to
32 relatively low doses of PM_{2.5-0.3} and their organic extractable (OEM_{2.5-0.3}) and non-extractable
33 (NEM_{2.5-0.3}) fractions, and the organic extractable fraction (OEM_{0.3}) of PM_{0.3}. We reported that not
34 only PM_{2.5-0.3}, but also, to a lesser extent, its inorganic chemical fraction, NEM_{2.5-0.3}, and organic
35 chemical fraction, OEM_{2.5-0.3}, were able to significantly induce ROS overproduction and oxidative
36 damage notwithstanding the early activation of NRF2 signaling pathway. Moreover, for any
37 exposure, inflammatory and apoptotic events were noticed. Similar results were observed in
38 BEAS-2B cells exposed to OEM_{0.3}, rich of polycyclic aromatic hydrocarbons and their nitrated
39 and oxygenated derivatives. In BEAS-2B cells exposed for 24 and 48 h to OEM_{2.5-0.3} and OEM_{0.3},
40 to a higher extent, there was an alteration of the levels of some critical proteins even though crucial
41 for the autophagy rather than a real reduction of autophagy. It is noteworthy that the toxicological
42 effects were equal or mostly higher in BEAS-2B cells exposed for 6 and/or 24 h to PM_{2.5-0.3} from
43 those exposed to NEM_{2.5-0.3} or OEM_{2.5-0.3}, and in BEAS-2B cells exposed for 6 and/or mostly 24
44 h to OEM_{0.3} from those exposed to OEM_{2.5-0.3}. Taken together, these results revealed the higher
45 potentials for toxicity, closely linked to their respective physical and chemical characteristics, of
46 PM_{2.5-0.3} vs NEM_{2.5-0.3} and/or OEM_{2.5-0.3}, and OEM_{0.3} vs OEM_{2.5-0.3}.

47 **CAPSULE:** Inorganic and organic chemical fractions from ambient fine and ultrafine particles
48 differentially triggered some critical underlying mechanisms of toxicity in BEAS-2B cells.

49

50 **KEYWORDS:** Fine particles; quasi-ultrafine particles; extractable and non-extractable chemical
51 fractions; normal human bronchial epithelial BEAS-2B cells; toxicity; underlying mechanisms

52

53 INTRODUCTION

54

55 Epidemiological evidence supported the association between exposure to ambient particulate
56 matter (PM) and hospital admissions, morbidity and mortality related to chronic inflammatory
57 lung diseases (e.g., asthma and chronic obstructive pulmonary disease) and lung cancer (Beelen et
58 al. 2013; Doiron et al. 2019; Hamra et al. 2014; Jerrett et al. 2016; Lelieveld et al. 2019; Raaschou-
59 Nielsen et al. 2016; Loomis et al. 2014). Fine (i.e., PM_{2.5}) and mostly ultrafine (i.e., PM_{0.1}) particles
60 have generally been reported as the major contributors to the adverse health effects of air pollution
61 (Abbas et al. 2018). Several experimental studies have notified the harmful lung effects of ambient
62 PM, mostly through their ability to trigger some critical underlying mechanisms of toxicity, such
63 as oxidative stress-induced airway inflammation (e.g., Abbas et al. 2013, 2016, 2019; Bocchi et
64 al. 2016; Garçon et al. 2006; Gualtieri et al. 2010, 2011; Halonen et al. 2015; Leclercq et al. 2016,
65 2017, 2018; Longhin et al. 2013, 2016, Sotty et al. 2019). However, although significant progress
66 has been made during the last decade, a considerable effort will continue to be required to better
67 identify some typical features of *in vitro* toxicological responses, which could be related to some
68 of the specific physical and chemical characteristics of ambient PM.

69 A careful consideration has already been devoted to the types of toxicological responses
70 induced by ambient PM, since their knowledge is greatly complicated by the fact that it is a very
71 heterogeneous and often poorly described pollutant (Perrone et al. 2013). Despite the particular
72 attention of the scientific community about the types of toxicological responses depending on the
73 sources of ambient PM, this question is still far from being completely resolved. Gualtieri et al.
74 (2010), Lonkar and Dedon (2011), and Perrone et al. (2013) have all used the inclusion of the
75 grouping of inorganic constituents to provide useful information on the differential effects of the

76 components and/or the sources of ambient PM. Hence, it is worthy to extend this approach to
77 organic components. Among them, polycyclic aromatic hydrocarbons (PAH) were identified as
78 major redox-active components, which, through their metabolic activation into highly biologically
79 reactive electrophile metabolites, largely contribute to the overall toxicity of ambient PM (Abbas
80 et al., 2018; Boublil et al. 2013; Longhin et al. 2013, 2016; Sotty et al. 2019; Zhou et al. 2016).
81 Other authors reported the critical role of the organic extractable matter (OEM) derived from
82 ambient PM in its overall toxicity but failed to fully clarify them (Akhtar et al. 2010; Alessandria
83 et al. 2014; De Brito et al. 2013, Oh et al. 2011, Xiang et al. 2018).

84 New toxicological research is therefore requested to improve the current knowledge about the
85 specific involvement of the inorganic and organic chemical fractions of ambient PM in its overall
86 toxicity. Recently, OEM derived from ambient $PM_{2.5-0.3}$ was recognized as triggering oxidative,
87 inflammatory, and genotoxic events: a combination thereof may be the same or different in
88 comparison with those triggered by the whole $PM_{2.5-0.3}$. However, further works are still needed to
89 better compare the respective roles of each chemical fraction: it might also be more appropriate to
90 study the toxicity of $OEM_{2.5-0.3}$ together with this of the whole $PM_{2.5-0.3}$, from which it was
91 extracted, and the toxicity of non-extractable matter ($NEM_{2.5-0.3}$; i.e., residual $PM_{2.5-0.3}$ after
92 $OEM_{2.5-0.3}$ extraction). The comparison between the specific toxicities of $OEM_{2.5-0.3}$ and $OEM_{0.3}$,
93 respectively prepared from fine ($PM_{2.5-0.3}$) or quasi-ultrafine ($PM_{0.3}$) particles, could also be very
94 interesting to better assess the chemical compounds mainly involved. Hence, in this work, we
95 sought to better investigate: (i) the toxicological effects of $OEM_{2.5-0.3}$ and $NEM_{2.5-0.3}$ with a
96 particular attention being paid to oxidative, inflammatory, autophagic, and apoptotic events, and
97 thereafter compare them to those of $PM_{2.5-0.3}$ in its entirety, and (ii) the toxicity of $OEM_{0.3}$ which
98 would lastly be related to that of $OEM_{2.5-0.3}$.

100 MATERIALS AND METHODS

101

102 Chemicals

103 Sigma-Aldrich (Saint-Quentin Fallavier, France) provided BEAS-2B cells (ATCC® CRL-
104 9609™) and chemicals. ThermoFisher scientific (Villebon-sur-Yvette, France) provided hydro-
105 ethidium (HE), 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA),
106 Pierce™ BCA protein assay kits, Click-iT™ Plus EdU Alexa Fluor™ 647 flow cytometry assay
107 kits, Single-channel dead cell apoptosis kits with Annexin V alexa fluor™ 488, and SYTOX™
108 green dyes, and other biological reagents. Qiagen (Courtaboeuf, France) provided RNeasy mini
109 Kits. Promega (Charbonnière-les-Bains, France) provided CellTiter-Glo® luminescent cell
110 viability, GSH/GSSG-Glo™ assay, and Caspase Glo® 3/7, Caspase Glo® 8, and Caspase Glo® 9
111 assays. Gentaur (Paris, France) provided Highly sensitive 8-OHdG check kits. Abcam (Cambridge,
112 UK) provided Protein carbonyl content assay kits and 8-isoprostane ELISA kits. Active Motif (La
113 Hulpe, Belgium) provided Nuclear extract and TransAM® NRF2 kits. Merck-Millipore (St
114 Quentin-en-Yvelines, France) provided MILLIPLEX® MAP human cytokine/chemokine
115 magnetic bead panel-immunology multiplex assays. Bio-techne (Rennes, France) provided
116 TACS® TdT *in situ*-fluorescein system and mouse monoclonal anti-human actin antibody
117 (MAB8929), rabbit polyclonal anti-human ATG5 (NB110-53818), rabbit polyclonal anti-human
118 LC3-B antibody (NB100-2220), rabbit polyclonal anti-human beclin antibody (NB500-249),
119 mouse monoclonal anti-human p62/SQSTM1 antibody (H00008878-M01), and goat polyclonal
120 anti-rabbit IgG, HRP-linked antibody (NB7160). Cell Signaling Technology (Montigny-le-
121 Bretonneux, France) provided rabbit monoclonal anti-mouse IgG, HRP-linked antibody (S7076).

122

123 **Sampling site description, PM sampling methods, PM physicochemical characterization,**
124 **and PM fraction preparation**

125 Sampling site description, sampling procedure, physicochemical characterization of PM_{2.5-0.3}
126 and PM_{0.3}, and preparation of NEM_{2.5-0.3}, OEM_{2.5-0.3}, and OEM_{0.3} have been published elsewhere
127 (Badran et al. 2020; see also supplemental data: Tables S1 and S2, and Figure S1).

128

129 **Cell culture and exposure**

130 Normal human bronchial epithelial BEAS-2B cells (ATCC® CRL-9609™) were cultured as
131 published elsewhere (Leclercq et al. 2017). Just before reaching the confluence (< 80%), BEAS-
132 2B cells were incubated either with LHC-9 with dimethylsulfoxide (DMSO) at 0.5% (v/v), as a
133 negative control, or with PM_{2.5-0.3}, NEM_{2.5-0.3}, OEM_{2.5-0.3}, and OEM_{0.3}, prepared in LHC-9 with
134 DMSO ≤ 0.5% (v/v), at concentrations ranging from 3 to 96 µg Eq. PM/cm², as detailed elsewhere
135 (Badran et al. 2020). Aliquots of cell-free culture supernatants were collected after 6, 24 and/or 48
136 h, and quickly frozen at -80°C. Adherent cells were washed twice with 1 mL-aliquots of cold
137 sterile PBS, and either immediately fixed or quickly frozen at -80°C, as published elsewhere (Sotty
138 et al. 2019).

139

140 **Cytotoxicity**

141 Intracellular ATP concentrations of BEAS-2B cells were determined using the CellTiter-
142 Glo® luminescent cell viability (Promega). This assay system used the properties of a proprietary
143 thermostable luciferase to enable reaction conditions that generate a stable “glow-type”
144 luminescent signal. The mono-oxygenation of luciferin was catalyzed by luciferase in the presence

145 of Mg²⁺, ATP, and molecular oxygen, and resulted in the emission of light, directly linked to the
146 ATP concentration and also the number of living cells.

147

148 **Oxidative stress**

149 Firstly, the fluorescence of either HE (1 μM) or carboxy-H₂DCFDA (1 μM) was kinetically
150 monitored at 37°C within BEAS-2B cells. Secondly, the nuclear factor erythroid 2-related factor
151 2 (NRF2) DNA binding activity was studied using TransAM® NRF2 from Active Motif.
152 Associated gene expressions of some members of the NRF2 signaling pathway (i.e., *NRF2*; *Kelch-*
153 *like ECH-associated protein 1*, *KEAP-1*; *heme oxygenase 1*, *HMOX*; *NAD(P)H quinone*
154 *dehydrogenase 1*, *NQO1*; *superoxide dismutase*, *SOD*) were evaluated by RT-qPCR using specific
155 Taqman™ gene expression assays (*NRF2*: hs00975961_g1, *KEAP-1*: hs00202227_m1, *HMOX*:
156 Hs01110250_m1, *NQO1*: Hs01045993_g1, *SOD*: hs00162090_m1, *ARN18S*: Hs99999901_s1), a
157 StepOnePlus™ Real-Time PCR System, and the Expression Suite Software (ThermoFisher
158 scientific). Thirdly, the status of glutathione (GSSG/GSH), and the concentrations of carbonylated
159 protein (CO-protein), 8-isoprostane (8-Isop), and 8-hydroxy-2'-deoxyguanosine (8-OHdG) were
160 carried out using GSH/GSSG-Glo™ assay (Promega), Protein carbonyl content assay kits
161 (Abcam), 8-isoprostane ELISA kits (Abcam), and Highly sensitive 8-OHdG check enzyme
162 immunoassays (Gentaur), respectively, as published elsewhere (Leclercq et al. 2016). Menadione
163 (2.5 mM, 4 h)-treated BEAS-2B cells served as a positive control.

164

165 **Inflammation**

166 The secretion of tumor necrosis factor (TNF-α), interleukin-6 (IL-6), interleukin-8 (IL-8), and
167 monocyte chemoattractant protein 1 (MCP-1) in the cell-free culture supernatants of BEAS-2B

168 cells was detected by MILLIPLEX® MAP Human Cytokine/Chemokine Magnetic Bead Panel-
169 Immunology Multiplex Assay (Merck-Millipore). Lipopolysaccharide (LPS from E. coli, 50
170 µg/mL, 24 h)-treated BEAS-2B cells served as a positive control.

171

172 **Autophagy**

173 ATG5, BECN1/Beclin 1, SQSTM1/p62 protein, and microtubule-associated protein 1 light
174 chain 3 b (MAP1LC3B/LC3B) were studied within BEAS-2B cells. Protein extracts (20 µg) of
175 BEAS-2B cells were prepared using protease and phosphatase inhibitor cocktail (Sigma-Aldrich)-
176 supplemented RIPA lysis/extraction buffer (ThermoFisher scientific). Western-blot method
177 consisted of: separation by electrophoresis on SDS-PAGE gels, transfer on nitrocellulose
178 membranes, blockage in 5 % (m/v) non-fat dry milk for 45 min, primary antibody incubation
179 overnight at +4°C (i.e., rabbit polyclonal anti-human ATG5, rabbit polyclonal anti-human LC3-B
180 antibody, rabbit polyclonal anti-human Beclin antibody, mouse monoclonal anti-human
181 p62/SQSTM1 antibody, mouse monoclonal anti-human actin antibody (Bio-technie), secondary
182 antibody incubation for 1 h at room temperature (i.e., rabbit monoclonal anti-mouse IgG, HRP-
183 linked antibody, Cell Signaling Technology; goat polyclonal anti-rabbit IgG, HRP-linked
184 antibody, Bio-technie), and revelation by ECL prime Western Blotting Detection reagent (GE
185 Healthcare). Chemiluminescence was recorded by Fusion FX Spectra (Vilbert-Lourmat, Marne-
186 la-Vallée, France). Rapamycin (10 µM, 24 h)-treated BEAS-2B cells served as a positive control.

187

188 **Apoptosis**

189 Caspase Glo® 3/7, Caspase Glo® 8, and Caspase Glo® 9 Assays (Promega) were used as
190 recommended by the manufacturer. TACS® TdT *in situ*-Fluorescein System (Bio-technie) was

191 used according to the manufacturer's recommendations before BEAS-2B cell observations using
192 an EVOS® FL Cell Imaging System (ThermoFisher scientific). Single-channel dead cell apoptosis
193 kit with Annexin V Alexa Fluor™ 488 and SYTOX™ green dyes, and Click-iT™ Plus EdU Alexa
194 Fluor™ 647 flow cytometry assay kits (ThermoFisher scientific) were used as recommended by
195 the manufacturer, before BEAS-2B cell analysis using an Attune™ NxT Acoustic Focusing
196 Cytometer (ThermoFisher scientific) to study apoptosis and cell cycle, respectively. Staurosporine
197 (2.5 µM, 24 h)-treated BEAS-2B cells served as a positive control.

198

199 **Statistical analysis**

200 Results were expressed as mean values and standard deviations. Comparisons were performed
201 between data from exposed BEAS-2B cells (i.e., PM_{2.5-0.3}, NEM_{2.5-0.3} OEM_{2.5-0.3}, or OEM_{0.3}) and
202 those from negative controls. Significant differences between results from BEAS-2B cells exposed
203 either to NEM_{2.5-0.3} or OEM_{2.5-0.3} vs PM_{2.5-0.3}, and to OEM_{0.3} vs OEM_{2.5-0.3} were reported. The non-
204 parametric Mann-Whitney U-test with correction for multiple comparisons ($p < 0.05$) was used to
205 perform statistical analyses (Software: SPSS for windows).

206

207 **RESULTS AND DISCUSSION**

208

209 **PM_{2.5-0.3}, NEM_{2.5-0.3}, OEM_{2.5-0.3}, and OEM_{0.3}-induced cytotoxicity**

210 As shown in Figure S2 (see also supplemental data), significant decreases of intracellular
211 ATP concentrations were reported in BEAS-2B cells 6, 24 and 72 h after their exposure to PM_{2.5-}
212 _{0.3}, from the dose of 6 µg/cm². Almost a similar profile of intracellular ATP concentrations was
213 seen in BEAS-2B cells after their exposure to NEM_{2.5-0.3}. In contrast, both OEM_{2.5-0.3} and
214 particularly OEM_{0.3} significantly altered intracellular ATP concentrations of BEAS-2B cells only
215 for the highest doses, almost since 48 µg Eq. PM/cm². The dose- and/or time-dependent
216 cytotoxicity of PM_{2.5-0.3}, NEM_{2.5-0.3}, OEM_{2.5-0.3}, and OEM_{0.3} observed in BEAS-2B cells allowed
217 to choose the concentrations (i.e., 3 and 12 µg Eq. PM/cm²) and kinetic (from 6 to 48 h) to apply
218 to the further study of the other toxicological endpoints. It is noteworthy that both these
219 concentrations were among the lowest shown as toxicologically active without, however,
220 triggering massive regulated cell death (RCD) (Abbas et al. 2019; Badran et al., 2020; Gualtieri et
221 al. 2010, 2011; Leclercq et al. 2018; Longhin et al., 2013, 2016; Platel et al. 2019; Sotty et al.
222 2019).

223

224 **PM_{2.5-0.3}, NEM_{2.5-0.3}, OEM_{2.5-0.3}, and OEM_{0.3}-induced oxidative stress**

225 Oxidative stress is one of the primary critical underlying mechanisms of toxicity activated by
226 the inhalation of ambient PM. A ROS overproduction by the BEAS-2B cells 6 and 24 h after their
227 exposures to PM_{2.5-0.3}, NEM_{2.5-0.3}, OEM_{2.5-0.3}, or OEM_{0.3} was shown in Figure 1. Indeed, HE
228 fluorescence intensity increased in a dose-dependent manner in BEAS-2B cells 6 and 24 h after
229 their exposures to NEM_{2.5-0.3} and OEM_{2.5-0.3}, and more markedly PM_{2.5-0.3} and OEM_{0.3} (Figure 1-

230 A, B). It is noteworthy that, after 6 and 24 h, HE fluorescence was significantly higher in BEAS-
231 2B cells exposed to $PM_{2.5-0.3}$ from those exposed to $NEM_{2.5-0.3}$ or $OEM_{2.5-0.3}$, and in BEAS-2B cells
232 exposed to $OEM_{0.3}$ from those exposed to $OEM_{2.5-0.3}$. Carboxy- H_2DCFDA fluorescence revealed
233 almost similar changes (Figure 1-C, D). The ahead-reported intracellular overproduction of ROS
234 in BEAS-2B cells could be related either to the inorganic chemicals (e.g., Al, Cr, Cu, Fe, Mn, Ni,
235 Pb, Ti, and Zn) detected within $PM_{2.5-0.3}$ and $NEM_{2.5-0.3}$, or to the organic chemicals (i.e., PAH, O-
236 PAH, and N-PAH) found within $PM_{2.5-0.3}$, $OEM_{2.5-0.3}$, and rather $OEM_{0.3}$, also usually associated
237 with redox reactions (Abbas et al. 2019, Badran et al. 2020; Gualtieri et al. 2011, Leclercq et al.
238 2018; Longhin et al. 2013, Oh et al. 2011, Saint-Georges et al. 2008, 2009). Accordingly, due to
239 the presence of both inorganic and organic chemical fractions within the whole $PM_{2.5-0.3}$, there was
240 also a higher ROS overproduction in BEAS-2B cells exposed to $PM_{2.5-0.3}$ from those exposed only
241 to $NEM_{2.5-0.3}$ or $OEM_{2.5-0.3}$. Interestingly, the higher ROS overproduction reported in BEAS-2B
242 cells exposed to $OEM_{0.3}$ from those exposed to $OEM_{2.5-0.3}$ could be explained by its higher contents
243 of total carbon, PAH, O-PAH, and N-PAH (Badran et al. 2020, see also supplemental data: Figure
244 1, Tables S1 and S2).

245 In addition, significant increases of the gene expression and binding activity of NRF2 were
246 reported in BEAS-2B cells exposed to $NEM_{2.5-0.3}$ or $OEM_{2.5-0.3}$, and more markedly to $PM_{2.5-0.3}$ or
247 $OEM_{0.3}$ (Figure 1-E, F, G, H). All these changes were generally higher at 6 than 24 h. NRF2,
248 because of its crucial function as a primary regulator of cellular redox homeostasis, is devoted to
249 counteract the cytosolic overproduction of ROS and, therefore, to maintain redox balance within
250 the cell (Wardyn et al. 2015). However, 24 h after any exposure, there was only a relatively low
251 activation of NRF2 transcription factor, which could be explained by its possible relocalization,
252 binding inactivation or degradation (Leclercq et al. 2018). It is important to note that, according to

253 ROS overproduction, the activation of the NRF2 signaling pathway was significantly higher in
254 BEAS-2B cells exposed for 6 and 24 h to PM_{2.5-0.3} from those exposed to NEM_{2.5-0.3} or OEM_{2.5-0.3},
255 and in BEAS-2B cells exposed for 6 h to OEM_{0.3} from those exposed to OEM_{2.5-0.3} (Figure 2).
256 Once again, all these results underlined the higher toxicity potentials of PM_{2.5-0.3} vs NEM_{2.5-0.3}
257 and/or OEM_{2.5-0.3}, and OEM_{0.3} vs OEM_{2.5-0.3}, also closely linked to their respective physical and
258 chemical characteristics.

259 Remarkably, for any exposure, despite the early activation of NRF2 signaling pathway,
260 oxidative alterations of glutathione, DNA and proteins significantly occurred in a dose- and time-
261 dependent manner (Figure 3). Overall, the highest oxidative damage to target macromolecules was
262 reported in BEAS-2B cells 24 h after their exposures to PM_{2.5-0.3}, and interestingly OEM_{0.3} rather
263 than NEM_{2.5-0.3} and OEM_{2.5-0.3}, thereby underlining the higher toxicity potentials of PM_{2.5-0.3} vs
264 NEM_{2.5-0.3} and/or OEM_{2.5-0.3}, and OEM_{0.3} vs OEM_{2.5-0.3}. Overall, these results supported the
265 occurrence of a remarkable cytosolic oxidative stress in BEAS-2B cells after their exposures to
266 PM_{2.5-0.3} and OEM_{0.3}, and NEM_{2.5-0.3} and OEM_{2.5-0.3}, to a lesser extent, and the inability of the
267 NRF2 signaling pathway to totally counteract ROS overproduction. Oxidative damage to DNA,
268 proteins and/or lipids can also disrupt the cell homeostasis, thereby contributing to gene expression
269 deregulation, DNA mutation, protein alteration with loss of function, and lipid peroxidation with
270 loss of membrane integrity and/or fluidity (Abbas et al. 2019; Dergham et al., 2012, 2015; Garçon
271 et al., 2006; Gualtieri et al., 2010, 2011; Leclercq et al. 2016, 2017, 2018; Longhin et al. 2013,
272 2016).

273

274 **PM_{2.5-0.3}, NEM_{2.5-0.3}, OEM_{2.5-0.3}, and OEM_{0.3}-induced cytokine secretion**

275 The four cytokines under study, TNF- α , IL-6, IL-8 and MCP-1, were significantly secreted
276 by BEAS-2B cells 6 and 24 h after their exposures to PM_{2.5-0.3}, and NEM_{2.5-0.3}, to a lesser extent,
277 but only 6 h after their exposure to OEM_{2.5-0.3}, and OEM_{0.3} (Figure 4). Once again, except for TNF-
278 α , the highest concentrations of the three other cytokines have been secreted by BEAS-2B cells
279 exposed for 24 h to PM_{2.5-0.3} from those exposed to NEM_{2.5-0.3} and/or OEM_{2.5-0.3} (Figure 4-D, F,
280 H). In keeping with the current literature, a significant secretion of proinflammatory cytokines by
281 BEAS-2B cells or other lung cell models, acutely or repeatedly exposed to relatively low doses of
282 ambient PM, has already been reported (Abbas et al. 2019; Dergham et al., 2012, 2015; Boulbil et
283 al. 2013; Garçon et al., 2006; Ghio et al. 2013; Gualtieri et al. 2010, 2011; Leclercq et al. 2016,
284 2018; Longhin et al. 2013, 2016; Loxham et al. 2015; Sotty et al. 2019; Zhou et al. 2016). The
285 relationship between the contents of redox-active compounds in ambient PM and the secretion of
286 proinflammatory cytokines by lung cells might closely rely on the regulation of NF- κ B (Lodovici
287 and Bigagli, 2011).

288 However, in this work, despite the significant secretion of these cytokines by BEAS-2B cells
289 exposed for 6 h to OEM_{2.5-0.3} and OEM_{0.3}, no significant secretion of them was still observed after
290 24 h. Interestingly, other authors, contributing to better decipher the underlying mechanisms of
291 toxicity of PAH-rich PM in different mouse or human cell models reported a negative association
292 between the PAH contents of ambient PM and the secretion of inflammatory cytokines (Jalava et
293 al. 2009; Kaur et al., 2019; Manzano-León et al. 2016).

294 Moreover, although the endotoxin contents have not been determined in PM_{2.5-0.3}, PM_{0.3}, nor
295 their NEM nor OEM, their possible influences on the secretion of the proinflammatory mediators
296 could not be excluded.

297 Another explanation supporting the difference between the results arising from cells exposed
298 to metal-rich PM ($PM_{2.5-0.3}$ and $NEM_{2.5-0.3}$) vs PAH-rich PM ($OEM_{2.5-0.3}$ and $OEM_{2.5-0.3}$) in terms
299 of cytokine secretion could be related to the bioavailability and the bioaccessibility of the organic
300 compounds, whether those adsorbed-on core PM, or found in OEM (Garçon et al. 2004a, 2004b).
301 The cell absorption of native particles by endocytosis, and the vector role of core PM could also
302 facilitate the absorption of organic compounds, while PAH within OEM could be directly diluted
303 in the culture medium. At once, PAH-coated onto PM could be only progressively released in cell
304 cytoplasm after particle internalization, and progressively metabolized, thereby contributing to
305 prolong their adverse effects within target cells. In contrast, the specific chemical properties of
306 OEM, which regrouped lipophilic compounds that can easily and even rapidly go through the
307 cytoplasmic membrane and could be freely, quickly, metabolized, within the cell, could contribute
308 to shorten their adverse effects within the target cells.

309

310 **$PM_{2.5-0.3}$, $NEM_{2.5-0.3}$, $OEM_{2.5-0.3}$, and $OEM_{0.3}$ -induced autophagy**

311 Mounting evidence further suggests that the autophagy machinery is closely involved in
312 extensive crosstalk with several chronic inflammatory lung diseases, including asthma and COPD,
313 and several, if not all forms of RCD, either by hyperactivation or by inhibition of the autophagy
314 flow (Racanelli et al. 2016; Napoletano et al. 2019; Zhou et al. 2016). The evaluation of some
315 hallmarks (i.e., BECN1/Beclin 1, ATG5, SQSTM1/p62 protein, and MAP1LC3B/LC3B) was also
316 undertaken to better decipher the possible alteration of the autophagy process within BEAS-2B
317 cells 24 and 48 h after their exposures to $PM_{2.5-0.3}$, $NEM_{2.5-0.3}$, $OEM_{2.5-0.3}$, or $OEM_{0.3}$ (Figure 5).
318 Significant decreases of ATG5 and Beclin1 were noted in BEAS-2B cells 24 h after their exposures
319 only to $OEM_{2.5-0.3}$ and $OEM_{0.3}$, thereby supporting the reduction of two critical proteins, even

320 though crucial for the autophagy process. Remarkably, the highest decreases of these autophagy
321 hallmarks have been observed in BEAS-2B cells exposed for 24 h to OEM_{0.3} from those exposed
322 to OEM_{2.5-0.3} (Figure 5-B, D). Two ubiquitin-like conjugation systems, LC3B and ATG5-12, up-
323 regulated by another critical autophagy protein, Beclin, are generally highly required prior to
324 autophagosome formation (An et al. 2019). One of the well-described hallmarks of autophagosome
325 formation consists of the conversion of LC3-I unconjugated cytosolic form to LC3-II
326 autophagosomal membrane-associated phosphatidylethanolamine-conjugated form.
327 Interestingly, LC3B-II/LC3B-I protein ratio appeared to be specifically reduced in BEAS-2B cells
328 only 48 h after their exposures to OEM_{2.5-0.3} and mostly OEM_{0.3}. Overall, the alterations of these
329 critical proteins closely involved in the autophagy process revealed differential responses of whole
330 PM or NEM towards PAH-rich OEM. However, according to Yoshii and Mizushima (2017), while
331 LC3 is currently the most widely used autophagosome marker, one must keep in mind that the
332 LC3-II amount at a given time point does not necessarily estimate the autophagic activity, because
333 not only autophagy activation but also inhibition of autophagosome degradation could greatly
334 increase the amount of LC3-II. Also, LC3-II can ectopically localize on non-autophagosome
335 structures that are not turned over in the lysosome. Taken together, the present results supported
336 rather an alteration of the levels of some critical proteins even though crucial for the autophagy
337 rather than a real reduction of autophagy itself. Nevertheless, the essential role of autophagy in
338 regulating inflammation induced by PM containing environmentally persistent free radicals was
339 reported by Chen et al. (2016), and Xu et al. (2017). Their statements supported the alteration of
340 some current hallmark proteins of autophagy and at once the relatively low secretion of
341 proinflammatory cytokines, both noticed in this work in BEAS-2B cells exposed to OEM_{2.5-0.3} and

342 OEM_{0.3}, at a higher extent, despite the absence of additional observations proving the alteration of
343 the autophagy itself.

344

345 **PM_{2.5-0.3}, NEM_{2.5-0.3}, OEM_{2.5-0.3}, and OEM_{0.3}-induced apoptosis**

346 Recent data suggested divergent cell responses towards ambient PM resulting in RCD (i.e.,
347 apoptosis, autophagy, and necrosis) (Peixoto et al. 2017). To go further, apoptosis was also
348 investigated. As depicted by Figure 6-A, B, C, the caspases 8, 9, and 7/ activities showed almost
349 similar changes in BEAS-2B cells 24 and 48 h after any exposure, thereby supporting the
350 progressive occurrence of molecular apoptotic events.

351 However, as shown by Figure 5-D, E, F, TUNEL, Annexin-V immunostaining, and cell cycle
352 phases of BEAS-2B cells, indicated rather no clear morphological characteristics of RCD, even
353 after 48 h. An et al. (2019), studying the critical interplays between oxidative stress, autophagy
354 and apoptosis induced by oxidized black carbon, noticed that it was able to activate each of them
355 in a dose-dependent manner. Other authors reported the proapoptotic effects of ambient PM_{2.5}
356 within different human epithelial bronchial cell models, including BEAS-2B cells (Boublil et al.
357 2013; Gualtieri et al. 2011; Leclercq et al. 2018; Longhin et al. 2013; Lee et al. 2019).

358 Peixoto et al. (2017) recently published relevant data about the relationships between the
359 exposure to ambient PM and the underlying mechanisms related to RCD, with a particular attention
360 devoted to the pivotal role played by oxidative stress in the determination of cell fate. Indeed, the
361 cellular fate was progressively directed toward RCD, and notably autophagy, when the levels of
362 ROS were overwhelming for the cells, and lead to an inhibition of the apoptotic pathway. **In this**
363 **work, for any exposure, while no clear autophagy process has been identified, molecular but not**
364 **morphological evidence of apoptosis has been noted.**

365

366 **CONCLUSION**

367

368 Overall, the present results clearly demonstrated that whole $PM_{2.5-0.3}$ and both its inorganic
369 and organic chemical fractions were able to induce some underlying mechanisms of toxicity
370 relying on ROS overproduction, inflammation, and, with regards to RCD, apoptosis rather than
371 autophagy. Despite the capacity of $NEM_{2.5-0.3}$, and $OEM_{2.5-0.3}$ to activate them, their combination
372 thereof may generally be lower comparing with those triggered by the whole $PM_{2.5-0.3}$. Similar
373 data, even with equal or higher extent from those of $OEM_{2.5-0.3}$, were reported in BEAS-2B cells
374 exposed to $OEM_{0.3}$. Taken together, these results highlighted the higher toxicity potentials, closely
375 linked to their physical and chemical characteristics, of the $PM_{2.5-0.3}$ vs $NEM_{2.5-0.3}$ and/or $OEM_{2.5-0.3}$,
376 and $OEM_{0.3}$ vs $OEM_{2.5-0.3}$. Future works are still needed to go further in the better knowledge
377 of the toxicity potentials of the different chemical fractions of ambient PM.

378

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555

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557

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563

564 **Figure Legend**

565

566 **Figure 1:** Fluorescence intensity of hydroethidium (HE) (Figure 1-A, B) and 6-carboxy-2',7'-
567 dichlorodihydrofluorescein diacetate (carboxy-H₂DCFDA) (Figure 1-C, D), and gene expression
568 and/or protein binding activity of nuclear factor erythroid 2-related factor 2 (NRF-2) (Figure 1-E,
569 F and Figure 1-G, H, respectively), 6 (Figure 1-A, C, E, G) and 24 h (Figure 1-B, D, F, H) after
570 BEAS-2B cell exposure to fine particles (PM_{2.5-0.3}) and their extractable (OEM_{2.5-0.3}) and non-
571 extractable (NEM_{2.5-0.3}) fractions, and to the extractable fraction (OEM_{0.3}) of the ultrafine particles
572 (PM_{0.3}). Menadione (100 μM, 4 h) was used as positive control. Results are normalized to controls
573 and presented as means and standard deviations of 6 or 3 replicates from three independent
574 experiments. (Mann-Whitney U-test; vs. controls: * = $p < 0.05$, and *** = $p < 0.001$; vs. PM_{2.5-0.3}
575 or vs OEM_{0.3}: c = $p < 0.05$).

576

577 **Figure 2:** Gene expression of Kelch-like ECH-associated protein 1 (KEAP-1) (Figure 2-A, B),
578 heme oxygenase 1 (HMOX) (Figure 2-C, D), NAD(P)H quinone dehydrogenase 1 (NQO1) (Figure
579 2-E, F), and superoxide dismutase (SOD) (Figure 2-G, H) in BEAS-2B cells, 6 (Figure 2-A, C, E,
580 G) and 24 h (Figure 2-B, D, F, H) after BEAS-2B cell exposure to fine particles (PM_{2.5-0.3}) and
581 their extractable (OEM_{2.5-0.3}) and non-extractable (NEM_{2.5-0.3}) fractions, and to the extractable
582 fraction (OEM_{0.3}) of the ultrafine particles (PM_{0.3}). Results are normalized to controls and
583 presented as means and standard deviations of 3 replicates from three independent experiments.
584 (Mann-Whitney U-test; vs. controls: * = $p < 0.05$; vs. PM_{2.5-0.3} or vs OEM_{0.3}: c = $p < 0.05$).

585

586 **Figure 3:** Glutathione status (Figure 3-A, B), 8-hydroxy-2'-deoxyguanosine (8-OHdG) (Figure 3-
587 C, D), carbonylated protein (CO-protein) (Figure 3-E, F), and 8-isoprostane (8-Isop) (Figure 3-G,

588 H) in BEAS-2B cells, 6 (Figure 3-A, C, E, G) and 24 h (Figure 3-B, D, F, H) after BEAS-2B cell
589 exposure to fine particles ($PM_{2.5-0.3}$) and their extractable ($OEM_{2.5-0.3}$) and non-extractable
590 ($NEM_{2.5-0.3}$) fractions, and to the extractable fraction ($OEM_{0.3}$) of the ultrafine particles ($PM_{0.3}$).
591 Results are normalized to controls and presented as means and standard deviations of 3 replicates
592 from three independent experiments. (Mann-Whitney U-test; vs. controls: $* = p < 0.05$; vs. $PM_{2.5-0.3}$
593 0.3 or vs $OEM_{0.3}$: $c = p < 0.05$).

594

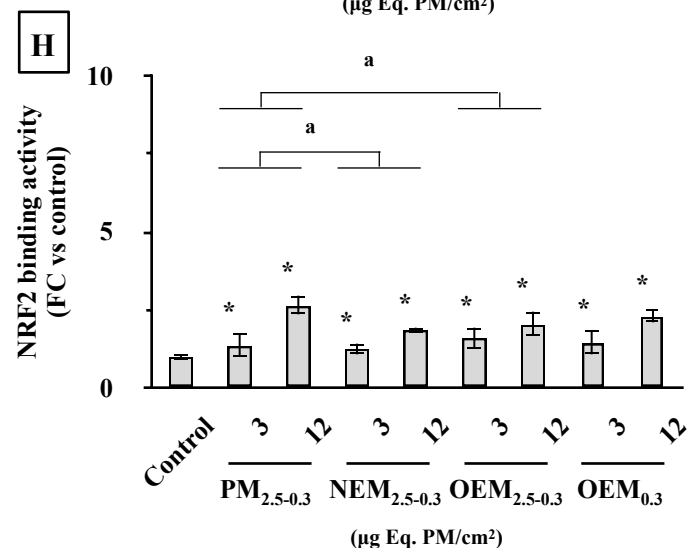
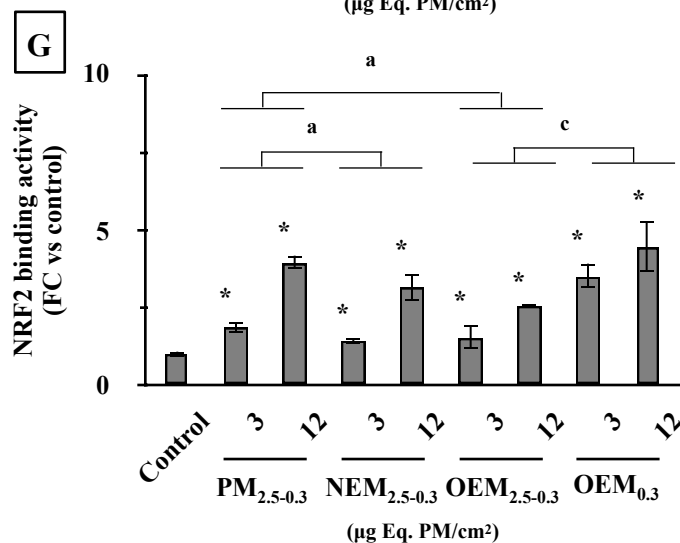
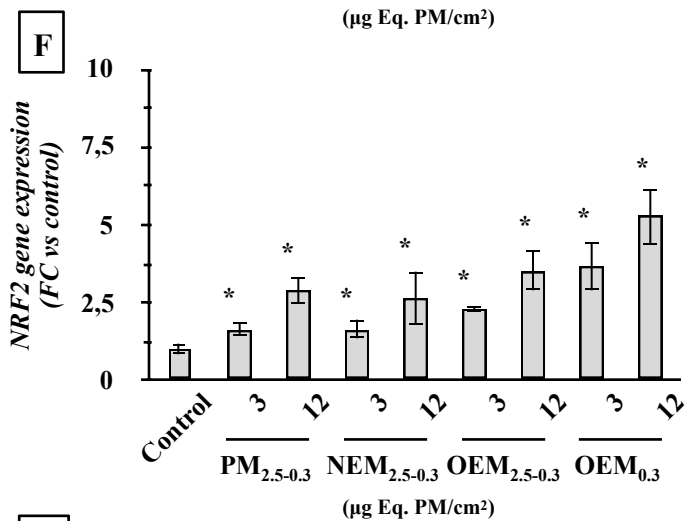
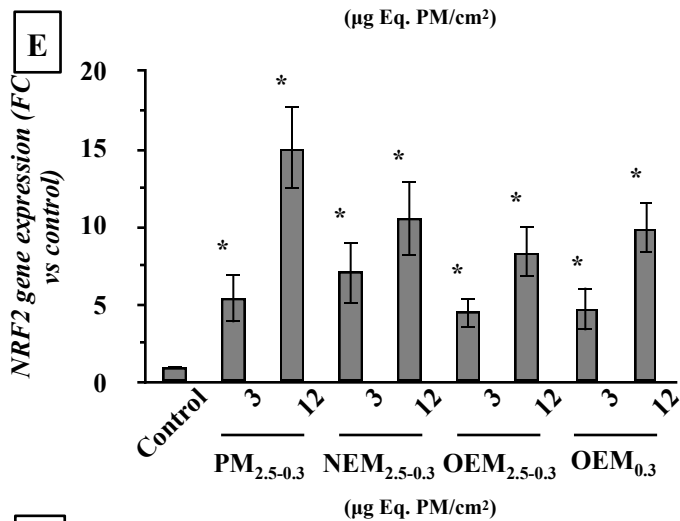
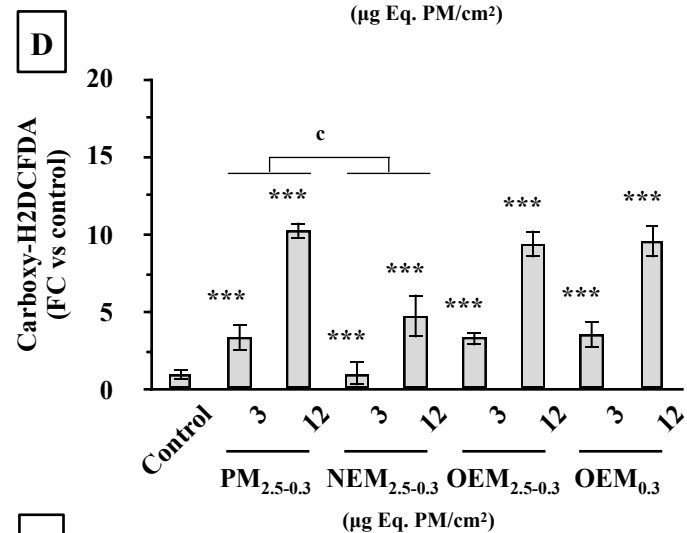
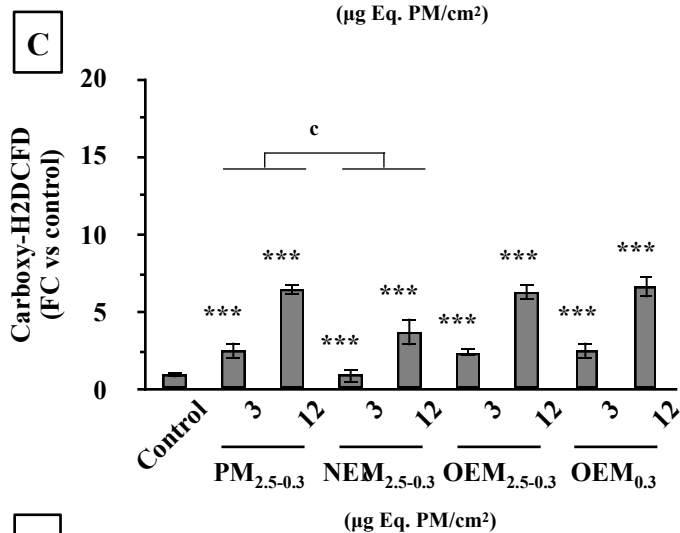
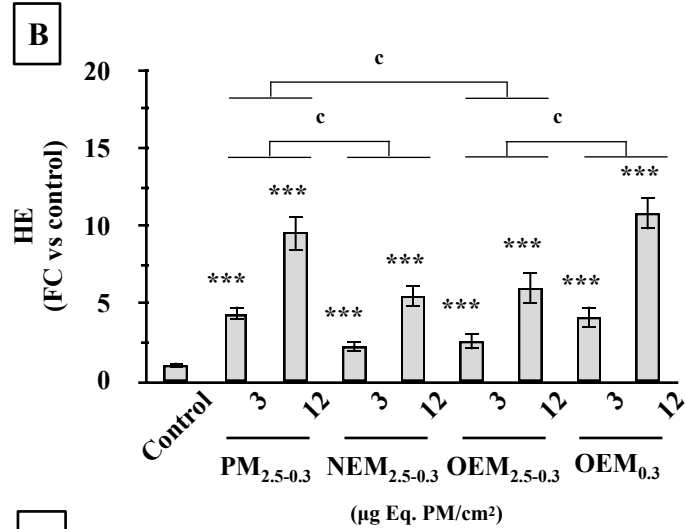
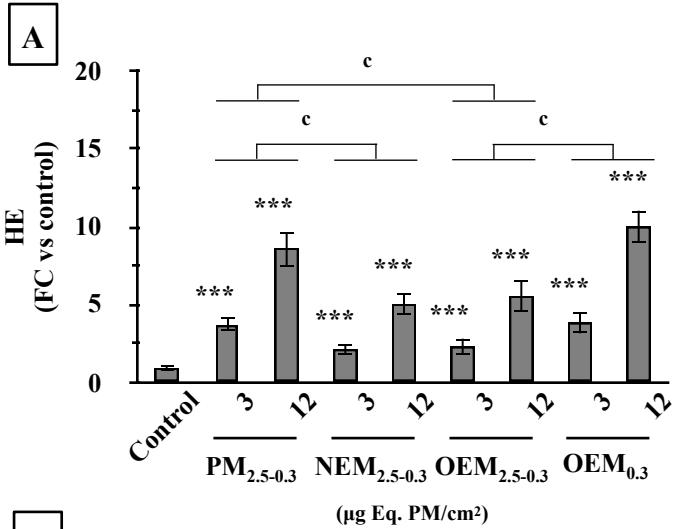
595 **Figure 4:** Concentrations of tumor necrosis factor (TNF- α) (Figure 4-A, B), interleukin-6 (IL-6)
596 (Figure 4-C, D), interleukin-8 (IL-8) (Figure 4-E, F), and monocyte chemoattractant protein-1
597 (MCP-1) (Figure 4-G, H), in BEAS-2B cells, 6 (Figure 4-A, C, E, G) and 24 h (Figure 4-B, D, F, H)
598 H) after BEAS-2B cell exposure to fine particles ($PM_{2.5-0.3}$) and their extractable ($OEM_{2.5-0.3}$) and
599 non-extractable ($NEM_{2.5-0.3}$) fractions, and to the extractable fraction ($OEM_{0.3}$) of the ultrafine
600 particles ($PM_{0.3}$). Results are normalized to controls and presented as means and standard
601 deviations of 3 replicates from three independent experiments. Baseline cytokine secretion of
602 BEAS-2B cells for 6 and 24 h were respectively: 36 ± 14 pg/mL and 48 ± 16 pg/mL for TNF- α ,
603 66 ± 20 pg/mL and 78 ± 21 pg/mL for IL-6, 410 ± 49 pg/mL and 648 ± 74 pg/mL for IL-8, and
604 973 ± 94 pg/mL and 1390 ± 173 pg/mL for MCP-1. (Mann-Whitney U-test; vs. controls: $* = p <$
605 0.05 ; vs. $PM_{2.5-0.3}$ or vs $OEM_{0.3}$: $c = p < 0.05$).

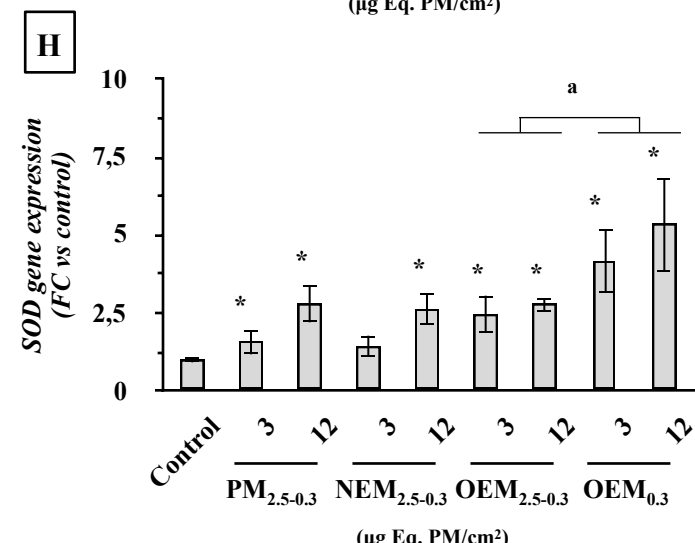
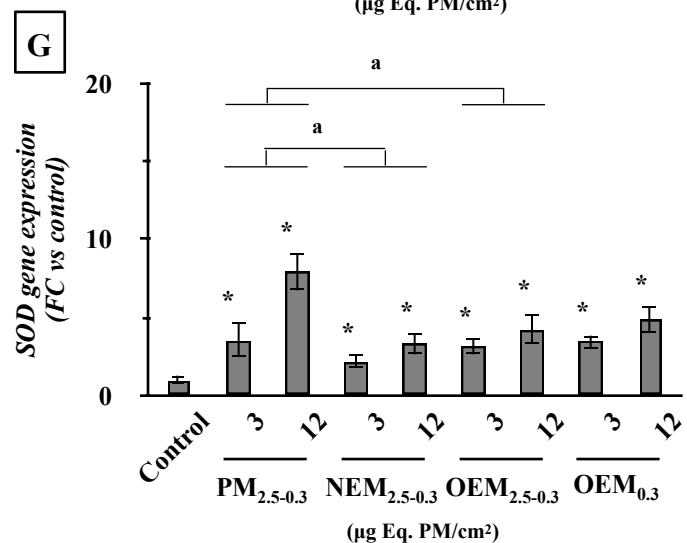
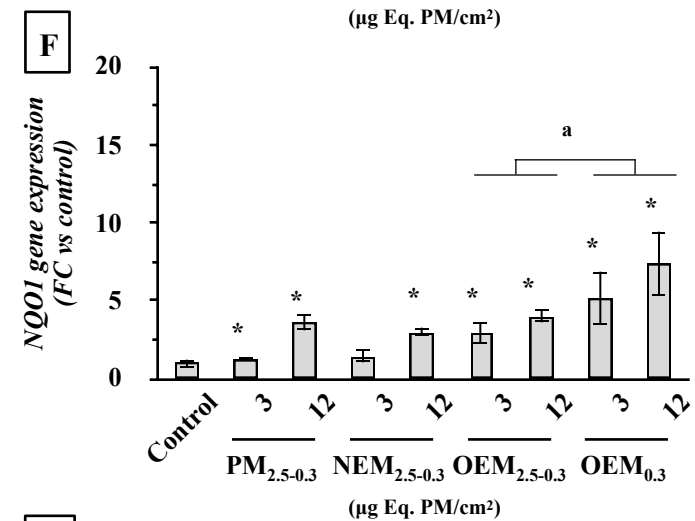
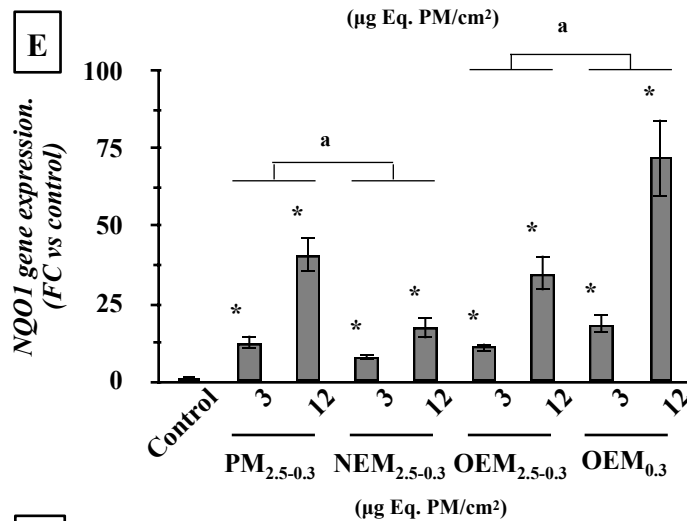
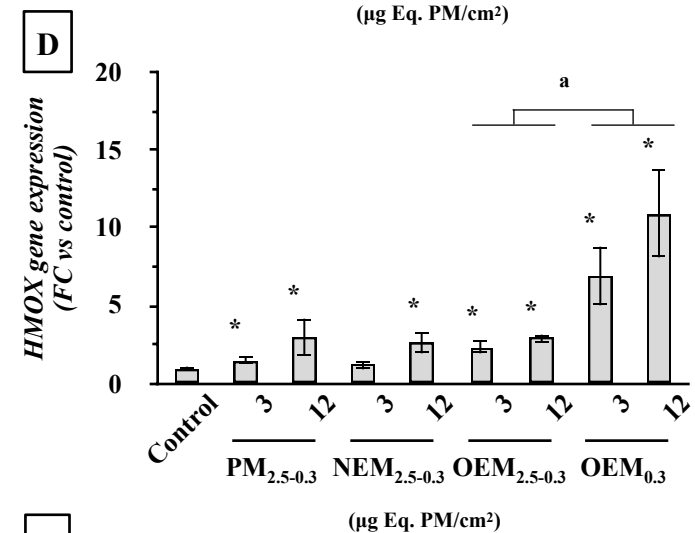
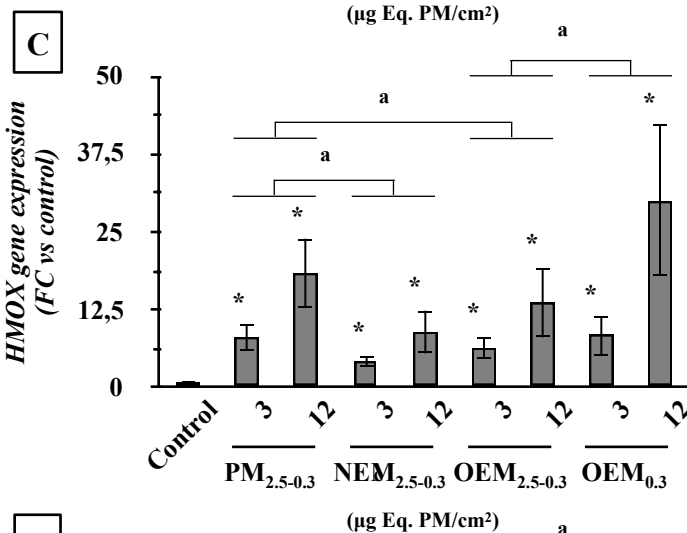
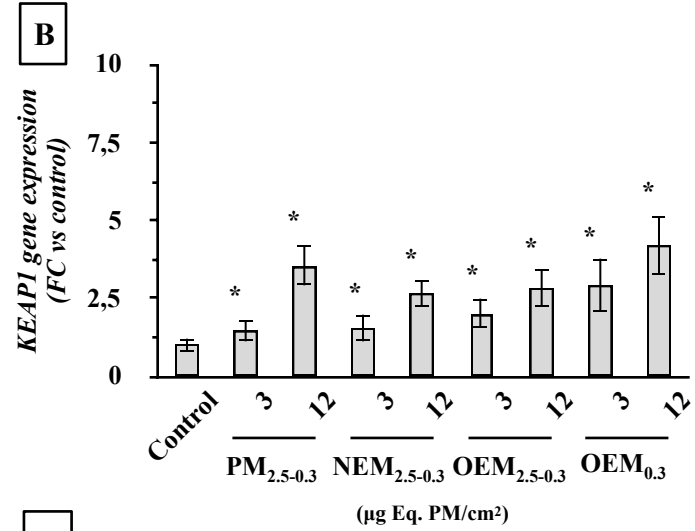
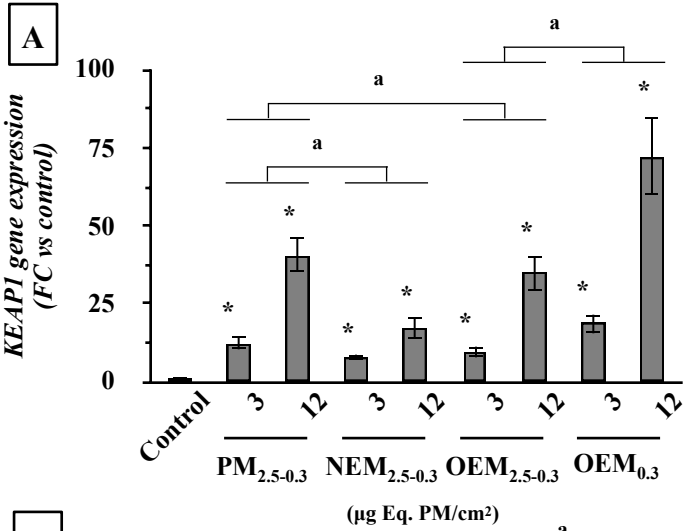
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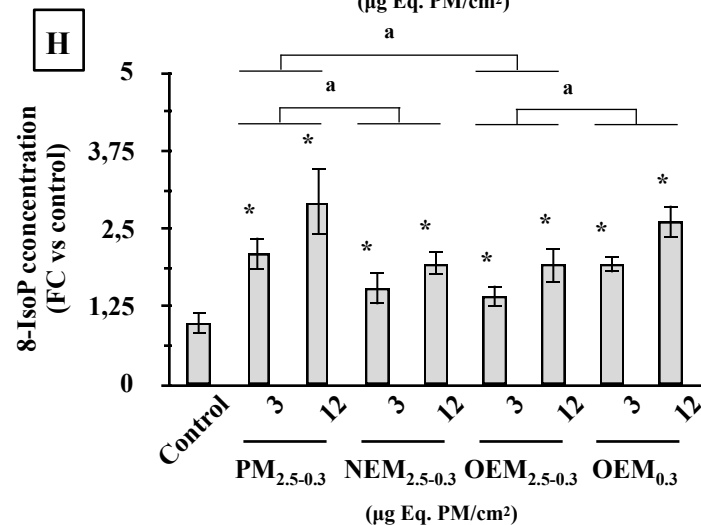
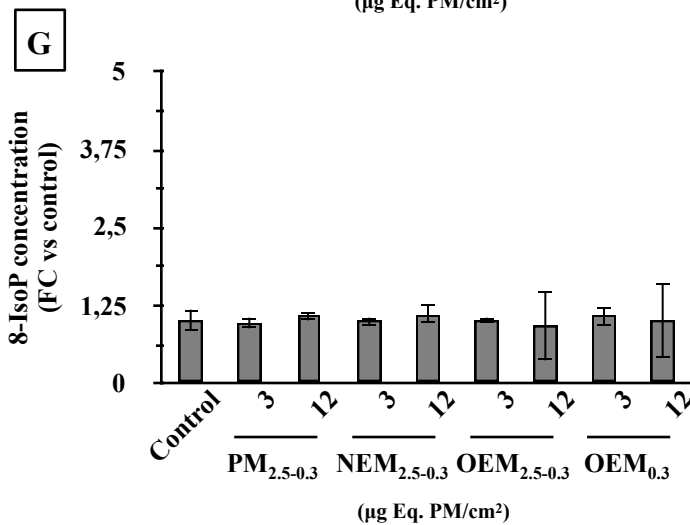
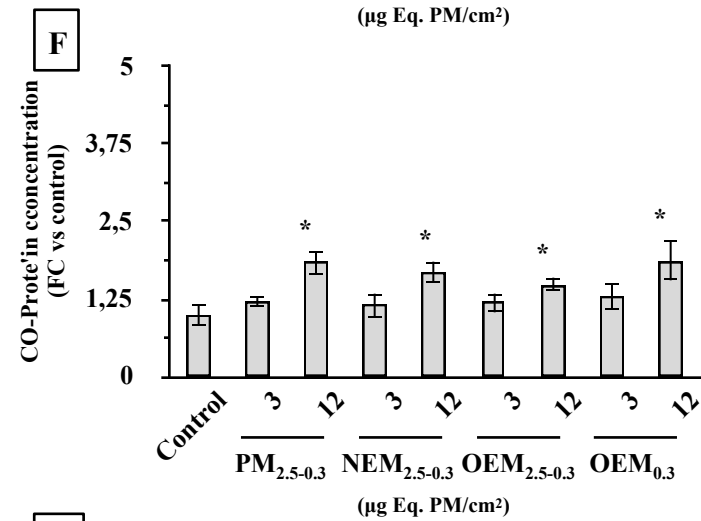
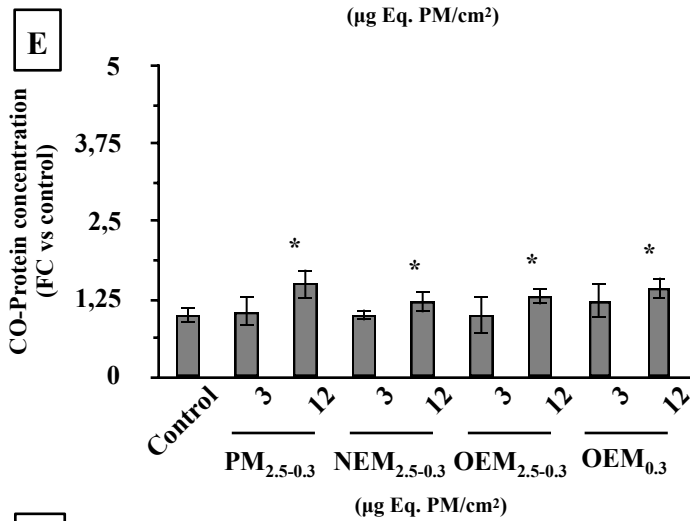
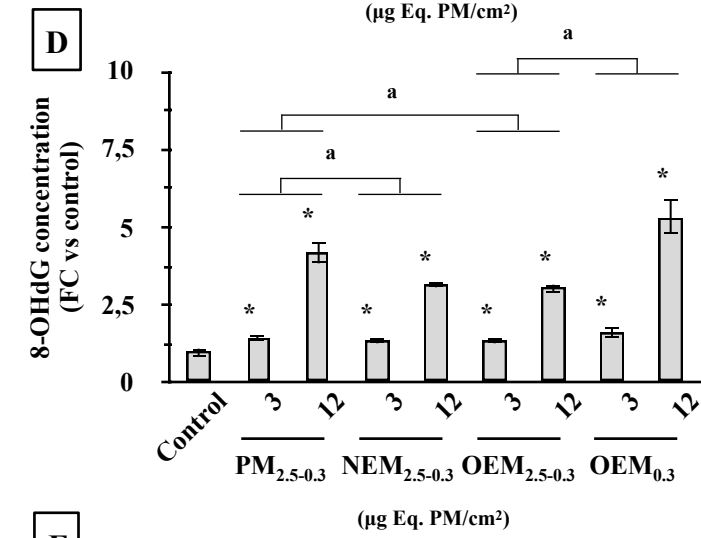
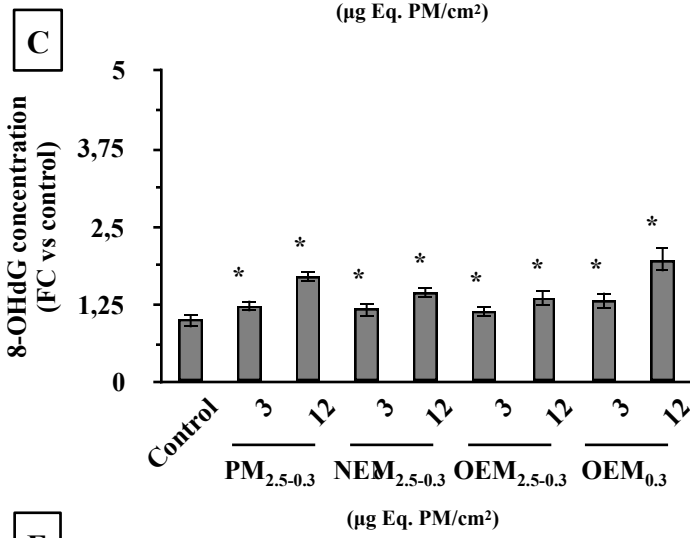
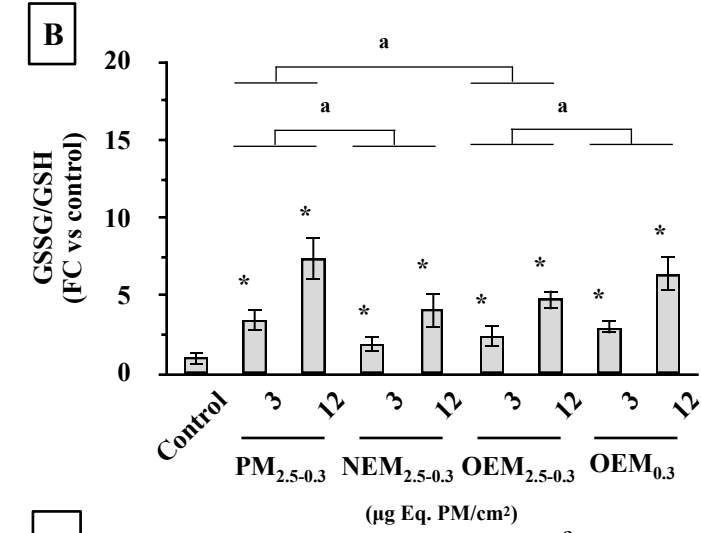
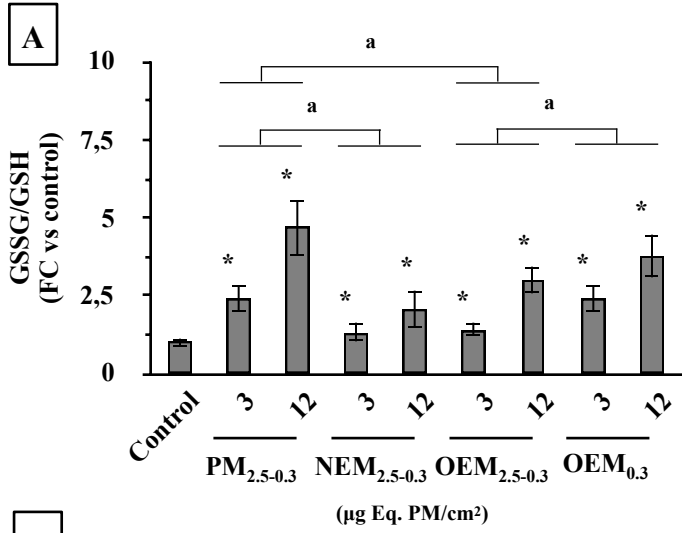
607 **Figure 5:** Protein expression of autophagy-related 5 (Figure 5-A), BECN1/Beclin 1 (Figure 5-B),
608 SQSTM1/p62 protein (Figure 5-C), and microtubule-associated protein 1 light chain 3 b
609 (MAP1LC3B/LC3B) (Figure 5-D) in BEAS-2B cells 24 and 48 h after BEAS-2B cell exposure to
610 fine particles ($PM_{2.5-0.3}$) and their extractable ($OEM_{2.5-0.3}$) and non-extractable ($NEM_{2.5-0.3}$)

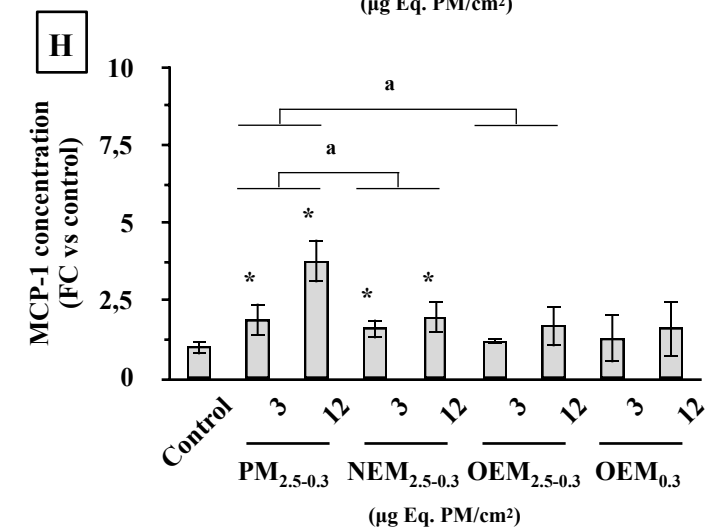
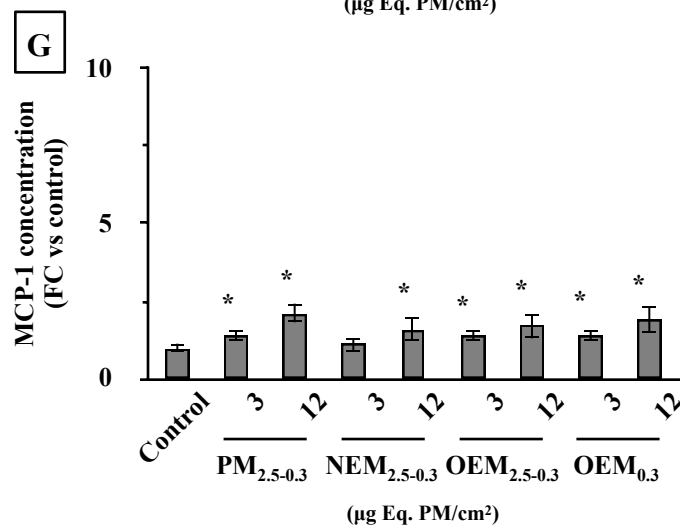
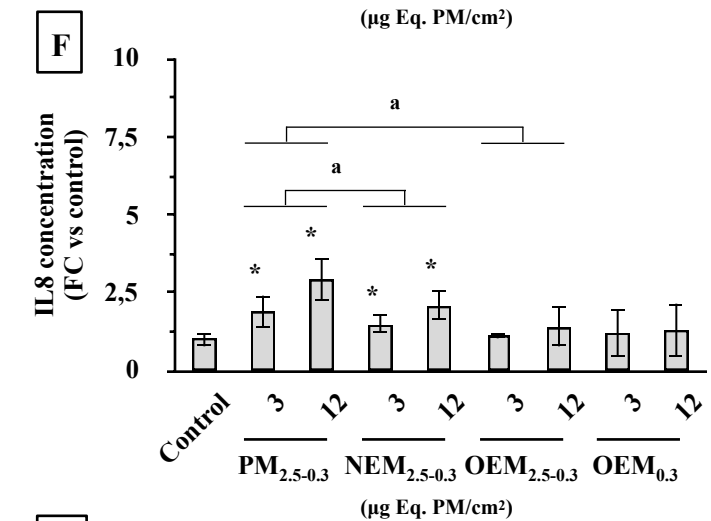
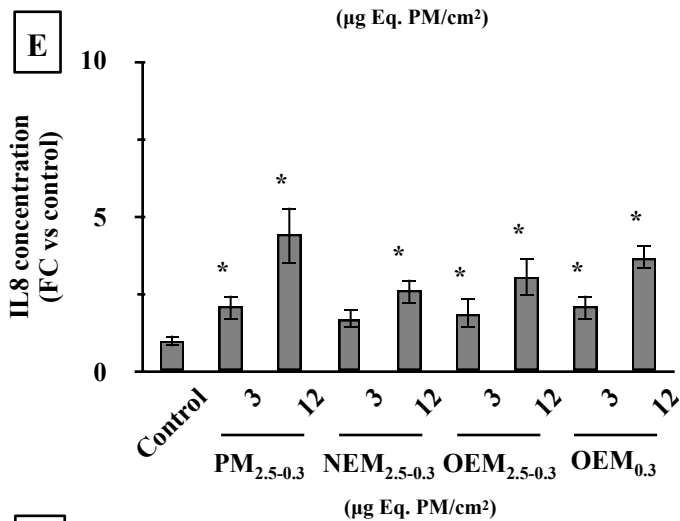
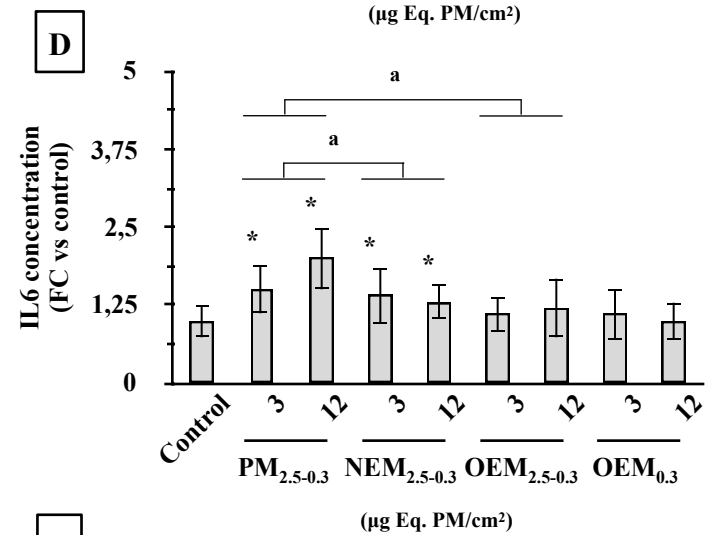
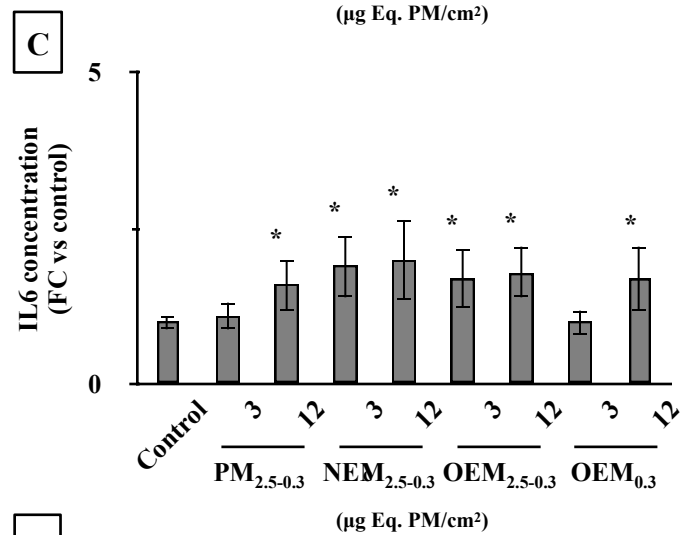
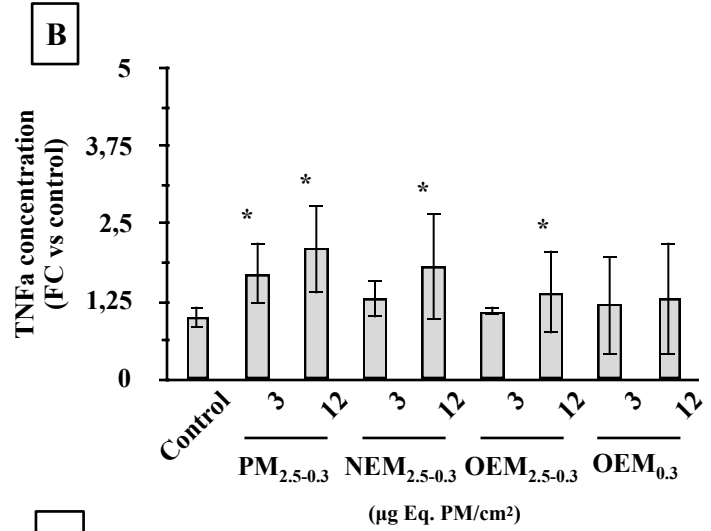
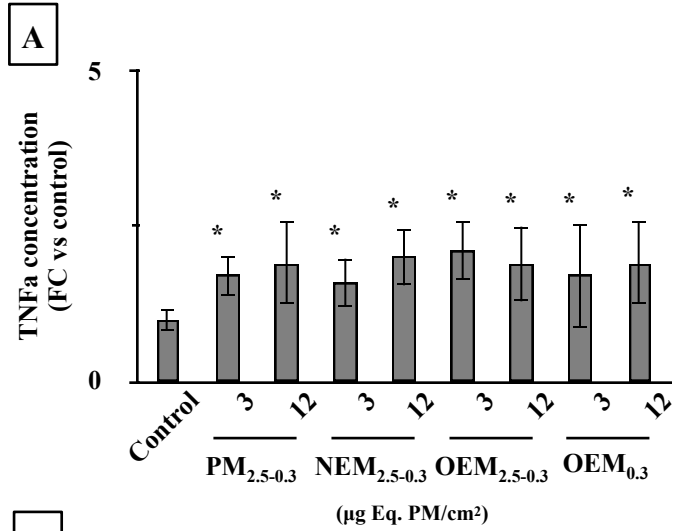
611 fractions, and to the extractable fraction (OEM_{0.3}) of the ultrafine particles (PM_{0.3}) at 12 µg Eq.
612 PM/cm². Results are normalized to controls and presented as means and standard deviations of 3
613 replicates from three independent experiments. (Mann-Whitney U-test; vs. controls: * = $p < 0.05$;
614 vs. PM_{2.5-0.3} or vs OEM_{0.3}: $c = p < 0.05$).

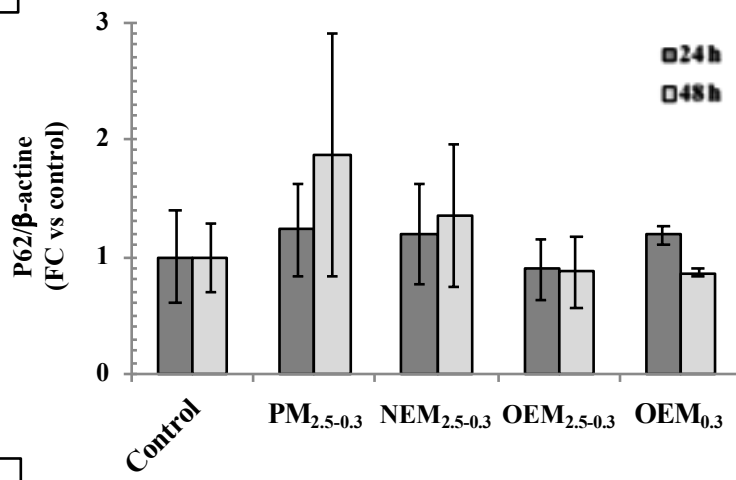
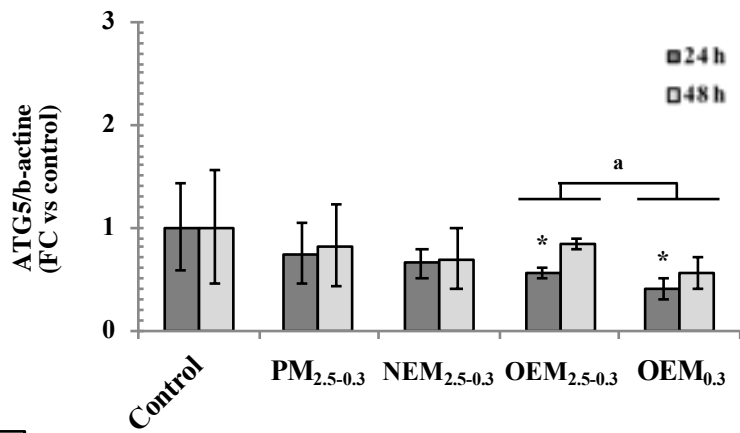
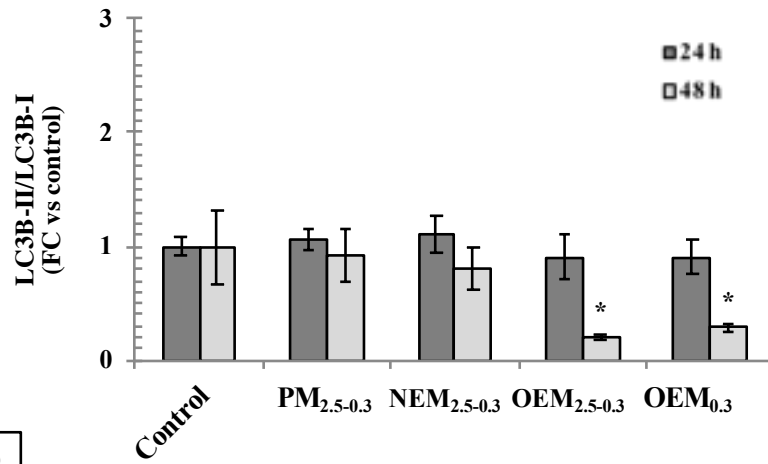
615
616 **Figure 6:** Activities of caspases 3/7, 8, and 9 (Figure 6-A, B, C, respectively),
617 immunofluorescence labelling of apoptotic cells using the TdT-mediated dUTP Nick-End
618 Labeling (TUNEL) method (Figure 6-D), detection of apoptotic cells using the Single-channel
619 dead cell apoptosis kit with Annexin V Alexa Fluor™ 488 and SYTOX™ green dyes (Figure 6-
620 F) and cell cycle analysis using Click-iT™ Plus EdU Alexa Fluor™ 647 flow cytometry assay kit
621 (Figure 6-G) in BEAS-2B cells, 24 and 48 h after BEAS-2B cell exposure to fine particles (PM<sub>2.5-
622 0.3</sub>) and their extractable (OEM_{2.5-0.3}) and non-extractable (NEM_{2.5-0.3}) fractions, and to the
623 extractable fraction (OEM_{0.3}) of the ultrafine particles (PM_{0.3}) at 12 µg Eq. PM/cm². Staurosporine
624 (2.5 µM, 24 h) has been used as positive control. Results are normalized to controls and presented
625 as means and standard deviations of 3 replicates from three independent experiments. (Mann-
626 Whitney U-test; vs. controls; * = $p < 0.05$).









A**B****C****D**