Supplemmentary materials

**The effects of nano-sized PbO on biomarkers of membrane disruption and DNA damage in a sub-chronic inhalation study on mice.**

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# *Supplementary information S1 - List of chemicals and reagents*

4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (HEPES, ≥99.5%); sodium chloride (NaCl); calcium chloride (CaCl2); 4-(1,1,3,3-Tetramethylbutyl)phenyl-polyethylene glycol (Triton™ X-100), glycerol (≥99%); bovine serum albumin (BSA); phospholipase A2 from honey bee venom (PLA2); acetone (≥99.9%); chloroform; hexane (≥95%); ethanol (>98 %), n-butanol; ribonuclease A from bovine pancreas (RNase A); butylated hydroxytoluene (BHT); hydrochloric acid (HCl); tris(hydroxymethyl)aminomethane (TRIS, ≥99.9%); thiobarbituric acid (TBA); phosphate buffer saline (PBS); trichloroacetic acid (TCA); lead wire (≥99.99 %) were purchased from Sigma-Aldrich (Merck). MS grade acetonitrile, methanol and formic acid (FA, 99%) were purchased from BIOSOLVE BV (Netherlands).

Standard 8-hydroxy-2’-deoxyguanosine (8-OHdG, 98 %), standard 2’-deoxyguanosine monohydrate (dG, (≥99%) and standard 1,1,3,3-tetraethoxypropane (MDA, ≥96%) were obtained from Sigma-Aldrich (Merck) and standard 15N5-8-OHdG Cambridge Isotope Laboratories, Inc. Standards of isoprostane: 8-iso prostaglandin E2 (15-E2–IsoP; 8-isoE, 0.5 mg in 100 μl of methanol), 8-iso prostaglandin F2α (15-F2t–IsoP; 8-isoF, 1 mg in 100 μl of methyl acetate) and internal standards of 8-iso prostaglandin E2–d4 (25 μg in 250 μl of methyl acetate), and iso prostaglandin F2α–d4 (25 μg ve 250 μl of methyl acetate) were purchased from Cayman chemical company.

For isolation of oxidized guanosine DNeasy Blood and Tissue kit (DNeasy® Blood & Tissue Kit; QIAGEN) and 8-OHdG Assay Preparation Reagent Set (WAKO Chemicals GmbH, Germany) were used. For decomposition of organ samples, concentrated subboil grade nitric acid was prepared in a quartz distillation system (model MSBQ 2, Maasen, Eningen, Germany). High purity ammonium phosphate and magnesium nitrate (Merck, Darmstadt, Germany) were used as chemical modifiers in determination of Pb.

# *Supplementary information S2 - Validation of isoprostanes measurement*

Linearity of the assay was assessed by repeat analysis of the calibration solutions (six points) independently prepared on 3 different days. The calibration curves together with representative chromatogram of 8-isoF2in extract of liver tissue are shown in Figure S1.The coefficients of variance (CV, in %) for 8-isoF2 and 8-isoE2 of the independent calibration solutions did not exceed 24.3% and 25.2%, respectively, except the concentration 0.05 ng/mL with CV lower than 50%. Linear regression was used to determine the slope, intercept, and correlation coefficient. Variability in the extraction process was characterized by study of replicates. For 8-isoPGF2, the highest 75th percentile of duplicates (N= 12) had a coefficient of variation less than 25%, with a maximum observed value of 33 % for liver; and further, lower values of 75th percentile with maximum CV was observed for brain, lung and kidney 16% (max 28%), 14% (max 21%) and 11% (max 26%), respectively. The 75th percentile and maximum CV was also determined for 8-isoE2, observed values were 16% (max 34%), 24% (max 23%), 17% (max 27%) and 23% (max 34%) for liver, brain, lung and kidney, respectively. The retention time repeatability was also evaluated, the relative standard deviations were always lower than 4 % for 8-isoPGF2and 8-isoPGE2. The LOQ as the lowest amount of analytes in a sample that can be quantitatively determined was 0.05 ng/mL and corresponds to the lowest level of the calibration curve.

Supplementary Table S1: Parameters of HPLC and MS in analysis of isoprostanes

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| compound | retention time (min ± SD)  N=30 | LOQ  ng/mL | MRM  transitions | collision energy (V) |
| 8-isoF2 | 2.52±0.09 | 0.05 | 353.2 → 193.1 | 24 |
|  |  |  | 353.2 → 291.2 | 21 |
|  |  |  | 353.2 → 309.2 | 20 |
| 8-isoE2 | 2.77±0.08 | 0.05 | 351.2 → 271.2 | 17 |
|  |  |  | 351.2 → 315.2 | 12 |
| d4-8-isoF2 |  |  | 357.2 → 197.1 | 24 |
|  |  |  | 357.2 → 295.2 | 21 |
|  |  |  | 357.2 → 313.2 | 20 |
| d4-8-isoE2 |  |  | 355.2 → 275.2 | 17 |
|  |  |  | 355.2 → 319.2 | 12 |

Supplementary Figure 1S: Validation of analytical method for analyses of isoprostanes.

Upper panel – the calibration curves of 8-isoPGF2and8-isoPGE2, each data point represents the mean area ± SD from three different calibration curves; typical chromatogram produced by the LC-MS/MS of 8-IsoF2: internal standard (middle panel) of 8-IsoF2 in liver sample (bottom panel)



Supplementary Table S2: Pathological changes in lung, liver and kidney after 13 week of exposure to PbONP. Increased level of phenotype is labelled by increased number of + symbols, where "+" means mild phenotype and "++" moderate phenotype in relevant type of alteration in organ; co\_1 - co\_5 control animals, Pb\_1 – Pb\_5 lead exposed animals

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Lung | control/13w | | | | | PbO/13w | | | | |
| co\_1 | co\_2 | co\_3 | co\_4 | co\_5 | Pb\_1 | Pb\_2 | Pb\_3 | Pb\_4 | Pb\_5 |
| infl. cell infiltrate peribronchiolar |  |  |  |  |  | + | + | + | + |  |
| infl. cell infiltrate perivascular |  |  |  |  |  |  |  | + | + |  |
| atelectasis | + |  |  |  |  | + | + | + |  |  |
| bronchiolitis | + |  | + |  |  | + |  | + |  | + |
| hyperemia, congested capillaries | + |  | + |  |  | + | + | + | + | + |
| alveolar emphysema |  |  |  |  |  |  | + |  |  |  |
| hemostase with siderophages |  |  |  |  |  |  |  |  |  |  |
| foamy macrophages |  |  |  |  |  | + |  |  |  | ++ |
| hemorrhage (artificial) | + |  | ++ | ++ |  |  |  |  | ++ | + |

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Liver | control/13w | | | | | PbO/13w | | | | |
| co\_1 | co\_2 | co\_3 | co\_4 | co\_5 | Pb\_1 | Pb\_2 | Pb\_3 | Pb\_4 | Pb\_5 |
| mononuclear cell infiltrate | + | + |  |  | + | + | + |  | + | + |
| focal necrosis |  |  |  |  |  |  |  | + | + |  |
| polynuclear hepatocytes |  |  |  |  |  |  |  |  |  |  |
| macrovesicularis steatosis |  |  |  |  |  |  |  | + |  | + |
| hemostase, affected sinusoids |  |  |  |  |  | + | + | + | ++ | + |
| hepatic remodeling |  | + |  |  |  | + | ++ | + | + |  |
| hypertrophic hepatocytes |  |  |  |  |  |  |  | + |  | + |
| infiltrate in portal area | + |  |  |  | + |  |  | + | + | + |

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Kidney | control/13w | | | | | PbO/13w | | | | |
| co\_1 | co\_2 | co\_3 | co\_4 | co\_5 | Pb\_1 | Pb\_2 | Pb\_3 | Pb\_4 | Pb\_5 |
| infl. cell infiltrate perivascularis |  |  | + |  | + |  |  | ++ | + | + |
| infl. cell infiltrate peritubularis | + |  | + |  | + |  | + | + |  | + |
| obliteration of vessels |  |  |  |  |  |  |  |  | + |  |
| glomerular metaplasia |  |  |  |  |  | + |  | + | + |  |
| higher cellularity in glomeruli |  |  |  |  |  | + | + | + | + | + |
| dilatation of proximal tubules |  |  |  |  |  | + |  |  |  |  |

# *Supplementary information S3 - Validation of 8-OHdG and dG analyses by LC-MS/MS*

Linearity of the assay was assessed by repeat analysis of the calibration solutions (six points in duplicates) independently prepared on 3 different days. The coefficients of variance (CV, in %) for 8-OHdG and dG of the 5 independent calibration solutions did not exceed 24% and 22%, respectively. The extraction method provided sufficient amounts of DNA for the assessment of 8-OHdG by LC-MS/MS. Representative chromatograms of the standard solution and mice tissues extract are shown in Figure S2.Both analytes were eluted from the column close to each other, but no interference among the peaks was observed (the mean retention times for 8-OHdG and dG were 2.96±0.10 and 2.29±0.08 min, respectively). The retention time of 8-OHdG and dG in the complex matrix of target organs did not differ from retention time of standard solution.

For 8-OHdG, the ratio of the signal of quantification transition (284.1 > 168.1) and the one used for confirmation - i.e. qualification ion (284.1 > 140.1) remained stable in standard solutions (5.1±0.6) as well as during analyses of selected tissues (4.8±0.8) and did not exceed 18 % RSD. The precision of the method was also evaluated according to the retention time repeatability, where the relative standard deviations were lower than 3.3 % and 3.0 % for 8-OHdG and dG, respectively.

Variability in the method of extraction and determination was characterized on the basis of the results performed in duplicates. For 8-OHdG, 75th percentile of all samples analyzed (i.e. 12 duplicates) had a degree of variability for liver of less than 14%, with a maximum observed coefficient of variance (CV) of 23 %, and further, 75th percentile with maximum observed CV were 18% (max 25%), 7% (max 19%) and 7% (max 20%) for brain, lung and kidney respectively. The LOQ as the lowest amount of analyte in a sample that can be quantitatively determined were 0.01 ng/mL for 8-OHdG and corresponds to the lowest level of the calibration curve.

Supplementary Figure 2S: Validation of analytical method for analyses of 8-OHdG and dG.

Upper panel – the example of calibration curves of 8-OHdGanddG,. Typical chromatogram produced by the LC-MS/MS of 8-OHdG in analytical standard (middle panels); chromatogram of 8-OHdG in the sample (bottom panels). Shown are examples of chromatogram in brain (A), kidney (B), liver (C) and lung (D) extracts



