MEDETOX

Innovative Methods of Monitoring of Diesel Engine Exhaust Toxicity in Real Urban Traffic

(LIFE10 ENV/CZ/651)

OXIDATIVE DAMAGE ANALYSIS – OXIDATION OF DNA, PROTEINS AND LIPIDS AFTER THE *IN VITRO* TREATMENT OF HEL AND A549 CELL LINE

SUMMARY

1. Treatment of cells and preparation of cell pellets

2. Oxidative DNA damage by ELISA

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4. Oxidative damage of proteins and lipids

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- **4.2.** Determination of total protein concentration
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- **4.4.** 15-F_{2t}-isoP concentration in cell lysates by ELISA (Cayman, cat.no. 516351)

1. Treatment of cells and preparation of cell pellets

1. Grow the cells in 75 cm^2 flasks with 15-30 ml culture medium (with 10% FCS) to 80-90% confluency.
2. Replace culture medium with 10 ml of fresh medium containing 1% FCS, add tested chemicals and incubate for 24 h (or other appropriate time) at 37°C (in a thermostat).
3. Scrape the cells with a scraper, transfer into a 15-ml tube, wash a flask with 5 ml of PBS (pH 7.4).
4. Centrifuge 3000 rpm, 5 min, 4 °C.
5. Aspirate the supernatant, resuspend in 13 ml of PBS, pH 7.4, repeat the step 4.
6. Aspirate the supernatant, resuspend in 4 ml of PBS, pH 7.4, pipette 1 ml into an 1.5 ml microtube marked with the name of the project Medetox (cells will be used for a carbonyl and 15-F_{2t}-isoP analysis), keep remaining suspension in a 15 ml tube.
7. Repeat step 4, aspirate the supernatants and keep pellets at -70 °C until analysis.

2. Oxidative DNA damage by ELISA

Solutions

Deferoxamine mesylate (DFA) (100 ml)

0.1 mM deferoxamine mesylate (MW=656.8)	6.6 mg

Store at 4 ºC.

50 mM Tris

Trizma-base	1.21 g
0.1 mM DFA	200 ml

Adjust pH to 7.4, store at 4 ºC.

Extraction buffer

Trizma-base (MW 121.1)	2.42 g
EDTA (MW 372.3)	3.72 g
SDS-Sodiumdodecylsulphate (MW 268.4)	10.0 g
Deferoxamine mesylate	66 mg
dH_2O	1.0 liter

Store at 4 °C.

RNase mix

Ribonuclease A (Sigma, R-5125)	10 mg
50 mM Tris	1 ml

Mix and incubate 10 min at 80 °C. Cool down, pipette into a vial of Ribonuclease T1 (5000 U/ml, Sigma, R-1003) and mix gently. Aliquot and store at -20 °C.

Proteinase K

10 mg/ml 0.1 mM DFA (for 40U/mg)

Prepare fresh before use.

5M NaCl

NaCl	29.23g
0.1 mM DFA	100 ml

Store at 4 °C.

CI (24 : 1 ratio)

Chloroform	240 ml
3-methyl-1-butanol	10 ml

Mix and add 80 ml of 50 mM Tris, store at 4 °C.

Phosphate-buffered saline (1x PBS)

NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.42 g
KH_2PO_4	0.24 g
Adjust pH to 7.4	
Add dH_2O to	1 liter

PBS-DFA (0.1 mM DFA in 1x PBS)

DFA	6.6 mg
1xPBS	100 ml

DNA extraction for 8-oxodG

1. Remove tubes from -70 °C, add 1 ml of Extraction buffer, vortex, add 20 µl of RNAse mix, incubate 1 h at 37 °C.
2. Add 20 µl of proteinase K (5 mg/500 µl dH_2O), incubate 1.5 h at 37 °C.
3. Transfer supernatant to a 15 ml tube containing Phase Lock Gel, add the same volume (1 ml) of CI with antioxidant and shake vigorously.
4. Centrifuge 5 min, 3000 rpm.
5. DNA precipitation: remove the upper phase, add 5M NaCl (100 µl; 1/10 volume) and ice-cold absolute ethanol (2.2 ml; 2 volume), mix well.
6. Centrifuge (5 min, 4000 rpm) and remove supernatant, alternatively remove DNA using a pipette tip.
7. Transfer each sample into a separate 1.5 ml microtube, add 1 ml of 70% ethanol and spin 5 min, 6000 rpm.
8. Vacuum-dry DNA pellet (20 - 25 min) and store at -70 °C until use, or dissolve immediately in PBS-DFA as described further.
9. To dissolve DNA pellet, add PBS-DFA (50-100 µl for DNA extracted from a 75 cm^2 flask) and keep for 30-60 min at 4 °C.

10. Break the pellet with the homogenizer (Pellet Pestle Motor, Sigma) and incubate for 40 min at 55 °C; vortex several times to dissolve the pellet as much as possible.
11. To remove undissolved fragments of the pellet, centrifuge 2 min, 6 000 rpm and transfer supernatant to another 1.5 ml microtube.
12. Denature DNA 5 min at 100 °C, let stand for 5 min at room temperature to cool the DNA solution and fragment using a syringe and a 22G needle.
13. Dilute 20x (2 µl + 38 µl PBS) and measure absorbance at 260 nm and 280 nm. Adjust concentration of the stock solution to 0.75 – 1.25 µg/µl by adding PBS-DFA.
14. Store at -70 °C until analysis.

3. Detection of 8-oxodG in DNA

Solutions

Phosphate-buffered saline (PBS) (1000 ml)

NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.42 g
KH_2PO_4	0.24 g
pH 7.4	

Wash buffer

1x PBS	997.5 ml
10% NaN_3	2 ml
Tween	500 µl

PBS/Tween

1x PBS	499.75 ml
Tween	250 µl

Blocking buffer (1% FCS in PBS/Tween)

PBS/Tween	49.5 ml
FCS	500 µl

1M diethanolamine

52.5 g in 500 ml dH_2O
pH 8.6 with conc. HCl

0.01M diethanolamine

1M diethanolamine	1 ml
dH_2O	99 ml

PBS-DFA (0.1 mM DFA in 1x PBS)

DFA	6.6 mg
1xPBS	100 ml

Detection of 8-oxodG in DNA

1. Coating the plates (Corning Costar, cat. # 3690, Half Area Plates): use 5 ng of 8-oxoG-BSA/well/25 µl of PBS, preferably coat 60 inner wells only, incubate at 37 °C, overnight, do not cover the plate.
2. Wash the plate, block with 100 µl of blocking buffer/well, 1 hour at room temperature.
3. Dilute primary antibody N45 (JaICA, Japan; 1:500 (final conc. 0.2 µg/ml) in blocking buffer). Dilute 8-oxodG standard (Sigma, cat. # H5653) in PBS to prepare the standard curve (the concentration of 8-oxodG stock solution is 1 mg/ml; 5 mg in 5 ml PBS):

8-oxodG (final conc.)	8-oxodG (stock sol., or previous dil., µl)	PBS (µl)
10 µg/ml	10 (1 mg/ml)	990
100 ng/ml	10 (10 µg/ml)	990
80 ng/ml	800 (100 ng/ml)	200
60 ng/ml	600 (80 ng/ml)	200
40 ng/ml	480 (60 ng/ml)	240
20 ng/ml	400 (40 ng/ml)	400
10 ng/ml	400 (20 ng/ml)	400
5 ng/ml	200 (10 ng/ml)	200
2.5 ng/ml	200 (5 ng/ml)	200
1.25 ng/ml	200 (2.5 ng/ml)	200

   Prepare samples without competitor (no competitor samples, determine total binding; 25 µl of PBS + 25 µl antibody solution/well) and samples without antibody (no antibody or blank samples; 25 µl of PBS + 25 µl of blocking buffer/well).

4. Remove the blocking solution, add 25 µl of DNA samples (conc. 0.75–1.25 mg/ml) and standards, 50 µl of no competitor and no antibody samples; after adding samples and standards, add 25 µl of primary antibody to all wells except of no competitor and no antibody wells; incubate 90 minutes at room temperature.
5. Wash the plate, add secondary antibody (anti-mouse, alkaline phosphatase conjugated, Sigma, cat. # A-4656) (50 µl/well) diluted 1:750 in blocking buffer; incubate 90 minutes at room temperature.

6. Wash the plate, rinse once with 0.01M diethanolamine, add substrate (p-Nitrophenyl Phosphate, Disodium, 5 mg/tablet, Sigma) 50 μl of substrate/well (2 tablets/10 ml of 1M diethanolamine); incubate 20-60 minutes at room temperature, read absorbance at 405 nm (absorbance of wells without competitor should be between 0.5 – 1.0).
7. Calculate the 8-oxodG concentration using the prepared standard curve.

4. Oxidative damage of proteins and lipids

4.1. Preparation of cell lysates for protein carbonyl and 15-F_{2t}-isoP

1. Mix the sample (minimum: 0.5-1x10^6 cells) with 50-100 µl of CelLytic reagent (Sigma), incubate for 15 min at room temperature, vortex repeatedly; centrifuge 15 min at 12,000 – 20,000 rpm, 4 °C.
2. Carefully remove the supernatant (cell lysate), store at -70 °C; use 5 µl of cell lysate + 20 µl water (5x dilution) to determine protein concentration.

4.2. Determination of total protein concentration

1. Dissolve BSA in water (stock solution, conc. 30 mg/ml). Make the standard curve by diluting the stock solution of BSA in water:

BSA (final conc. mg/ml)	BSA (stock sol., or previous dil.,µl)	Water (µl)
3.0	100	900
2.5	500	100
2.0	400	200
1.5	300	100
1.0	200	100
0.5	150	150
0.0	0	300

2. Dilute the analyzed samples 5x in water (5 µl + 20 µl water).
3. Mix bicinchoninic acid with $CuSO_4$ (i.e. 20 ml + 400 µl/96-well plate)
4. Add 10 µl of diluted samples and standards (in duplicates) to 96-well plate
5. Add 200 µl of bicinchoninic acid + $CuSO_4$ mixture to each well and incubate 30 min at 37 °C

6. Read the absorbance at 562 nm and calculate the total protein concentration using the standard curve and the dilution factor 5.

4.3. Carbonyl ELISA assay

Solutions for carbonyl ELISA assay

PBS - 10mM sodium phosphate buffer in 140 mM NaCl - pH 7.0 (Coating buffer)
10mM Na_2HPO_4 (M.W. 141.96) = 1.4196 g/l
140mM NaCl (M.W. 58.44) = 8.1816 g/l
pH adjusted to 7.0 with HCl

0.1% BSA v PBS
100 mg BSA in 100 ml PBS, pH 7.0

0.1% BSA, 0.1% Tween 20 v PBS
100 mg BSA, 100 μl Tween 20 in 100 ml PBS, pH 7.0

Derivatization Solution (100 ml) -10mM 2,4 DNPH, 6.0M Guanidine Hydrochloride, 0.5M Potassium Phosphate, pH 2.5
198 mg 2,4-DNPH in 3.33 ml conc. Phosphoric acid (85%)
57.318 g guanidine hydrochloride in 25 ml water (heat to dissolve completely)
Add 2,4- DNPH solution drop-wise while stirring
Use 10M KOH to bring pH 2.5.
Adjust volume to 100ml with distilled water.

10x PBS for Wash buffer (1000 ml)

NaCl	80 g
KCl	2 g
Na_2HPO_4	14.2 g
KH_2PO_4	2.4 g

Dissolve in 800 ml of distilled water, adjust pH to 7.4, bring to 1000 ml.

Wash buffer (5000 ml)

10x PBS for Wash buffer	500 ml
dH_2O	4500 ml
10% NaN_3	10 ml

Tween-20 2.5 ml

2M TRIS
24.2 g in 100 ml dH_2O

2.5 M Sulphuric acid
2.68 ml H_2SO_4 (98%)/100ml

Protein derivatization

1. Using the standards prepared previously make the standard curve. Dilute the highest standard (contains about 1.9-2.0 nmol carbonyl/mg protein) with PBS:

Carbonyl conc. (nmol/mg protein)	Standard BSA (µl)	PBS (µl)
0.612	30	170
0.510	25	175
0.408	20	180
0.306	15	185
0.204	10	190
0.102	5	195
0	0	100

2. Dilute the analyzed samples with PBS so that the total protein concentration is 2 mg/ml (for 10 µl of sample, the calculation is: (10* prot. conc. in mg/ml)/2)-10)); alternatively, if the amount of total proteins is too low, samples can be diluted up to 0.4 mg/ml (for 10 µl of sample, the calculation is (10* prot. conc. in mg/ml)/0.4)-10).
3. In 1.5 ml reaction microtubes, or 96-well plates mix 10 µl of DNPH solution (derivatization solution) and 10 µl of sample diluted in step 2 (protein conc. 2 mg/ml, 0.4 mg/ml) to give the final protein conc. 1 mg/ml (0.2 mg/ml).
4. Incubate the samples at the room temperature in dark for 45 min, vortex every 10-15 min (if incubating in a plate vortex on the Illumina vortex at lowest speed).

5. Stop the derivatization by adding 30 µl (1.5 volume) of 2M Tris, vortex, or mix.
6. Add 5 µl of derivatized samples to 1 ml of the Coating buffer (if the amount of total proteins is too low: 25 µl of derivatized samples to 1 ml of the coating buffer); total amount of proteins in samples – 2 µg; dilute 12.5 µl of standard + 1 ml of coating buffer; vortex all samples and use for coating the Elisa plate.

Carbonyl ELISA protocol

1. Add 200 µl of each sample and standard to the plate (in duplicates or triplicates) and incubate the plate overnight at 4 °C; use derivatized PBS as the blank.
2. Wash the plate with 350 µl/well of Wash buffer and block with 0.1% BSA in PBS (250 µl/well) at room temperature for 1.5 h.
3. Wash the plate and add primary biotinylated anti-DNP antibody (1:1500 dilution in 0.1% BSA, 0.1% Tween 20 in PBS solution; i.e. 16.6 µl/25 ml for 96-well plate) (200 µl/well); incubate at 37 °C for 1 h.
4. Wash the plate and add secondary streptavidin-biotinylated horseradish peroxidase conjugate (1:4000 in 0.1% BSA, 0.1% Tween 20 in PBS solution; i.e. 6.25 µl/25 ml for 96-well plate) (200 µl/well); incubate at room temperature for 1 h.
5. Wash the plate, add TMB substrate (200 µl/well) and incubate at room temperature in dark for 15-25 min.
6. Stop the reaction by adding 2.5M H_2SO_4 (100 µl/well).
7. Read the absorbance at 450 nm, subtract the blank absorbance and using the standard curve calculate the carbonyl concentration in samples.

2.3. 15-F_{2t}-isoP concentration in cell lysates by ELISA (Cayman, cat.no. 516351)

Sample purification and ELISA assay

1. Pipette cell lysates containing minimum 50 µg of proteins into a 1.5 ml microtube, dilute with water to 100 µl, add 100 µl of 15% KOH, incubate 60 min at 40 °C.
2. Adjust pH by 300 µl 1M KH_2PO_4 (resulting pH ~ 7.3), add 100 µl Column buffer (total volume 600 µl; original sample diluted 5x), mix.
3. Add 50 µl Isoprostane Affinity Sorbent (cat. # 416359), incubate 60 min at room temperature on a shaker.
4. Centrifuge 1 min at 4000 rpm, remove the supernatant very carefully by decanting.
5. Add 1 ml of water, vortex, centrifuge 1 min at 4000 rpm and carefully remove supernatant.
6. Resuspend the pellet in 0.5 ml of Elution Solution (95% ethanol in water), vortex and centrifuge 1 min at 4000 rpm.
7. Remove the ethanol wash, store in a 1.5 ml tube; repeat the wash and combine solutions.
8. Centrifuge 1 min at 4000 rpm, transfer combined ethanol washes to a new 1.5 ml microtube.
9. Store at -70 °C until analysis, or vacuum dry (takes about 2h), resuspend in 110 µl of EIA buffer and use immediately for the assay.
10. Perform the ELISA according to the manufacturer's recommendations (Cayman, 8-Isoprostane EIA Kit, cat. # 516351).