Experimental Studies on the Pro-thrombotic Effect of Inhaled Particles

Peter HOET
Unit of Lung Toxicology
Occupational & Environmental Medicine
K.U.Leuven, Belgium
peter.hoet@med.kuleuven.be
Episodes of increased PM$_{10}$ cause increase in respiratory morbidity such as:

- Respiratory symptoms
- Decrease in lung function
- Exacerbations of asthma in adults and children
- Hospitalization for bronchitis and pneumonia
Cardiovascular effects of particle exposure

• epidemiology & “clinical” studies:
  – increased particulate pollution associated with:
    • Heart rate variability ↑, arrhythmias without hypoxia or respiratory distress
    • Plasma viscosity ↑, C reactive protein ↑, fibrinogen ↑, factor VII ↑
    • PMN ↑, platelets ↑, mast cells ↑, endothelial adhesion molecules ↑
Particles are significant contributors to morbidity and mortality not only with regard to the respiratory tract, but also the cardiovascular system.
Mechanisms?

- experimental toxicology:
  - which constituents of the particles?
  - by what mechanisms?

→ “biological plausibility”?
Brook RD et al. Air pollution and cardiovascular disease. A statement for health-care professionals from the expert panel on population and prevention science of the American Heart Association. Circulation 2004 (June 1); 109: 2655-71
Translocation: Radioactivity in blood

Inhalation of $^{99m}$Tc-carbon particles ("Technegas")

In the graph, the CPM (counts per minute) per gram of blood is plotted against time (in minutes). The data shows a largely particle-bound distribution with a peak around 30 minutes.

Controversy going on …


• Mills et al., Do inhaled carbon nanoparticles translocate directly into the circulation in man? *Am J Respir Crit Care Med* Articles in Press. Dec 9, 2005.
Brook RD et al. Air pollution and cardiovascular disease. A statement for health-care professionals from the expert panel on population and prevention science of the American Heart Association. *Circulation* 2004 (June 1); 109: 2655-71
Methods

Pulmonary inflammation

- DEP 5 - 500 µg/animal or vehicle were i.t. instilled to hamster

1 hour

Bronchoalveolar lavage (BAL)

- Saline

- Cells
- Proteins
- Histamine

Results

Pulmonary inflammation (BAL)

**Neutrophils**

<table>
<thead>
<tr>
<th>i. t. DEP (µg/animal)</th>
<th>0</th>
<th>5</th>
<th>50</th>
<th>500</th>
</tr>
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<tbody>
<tr>
<td>0</td>
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**Proteins**

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<thead>
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<td>500</td>
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**Histamine**

<table>
<thead>
<tr>
<th>i. t. DEP (µg/animal)</th>
<th>0</th>
<th>5</th>
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<th>500</th>
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<tbody>
<tr>
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<td>500</td>
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*p < 0.05, **p < 0.01
Thrombus Formation: Methods

1. inject particles (or vehicle) i.v. or i.t.
2. 10 min later, inject Rose Bengal i.v.
3. 2 min transillumination (540 nm) → oxidative damage to endothelium
4. follow thrombus formation during 40 min
5. BAL (protein, LDH, cells) (only after i.t.)
Thrombus Formation: Materials & animals

• Polystyrene microspheres of 60 nm Ø
  – unmodified: neutral
  – carboxylate-modified: negatively charged
  – amine-modified: positively charged

suspended in NaCl 0.9%
sonicated + vortexed immediately before administration

• Hamsters (100-150g), anaesthetized
n=3-4 per day, including 1 vehicle control
Thrombus Formation:
Results
particles i.v.

AUC (arbitrary units)

mg/kg bw
Thrombus Formation: Results

Particles i.t.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose</th>
<th>Light Intensity (A.U.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>(n=6)</td>
</tr>
<tr>
<td>Neutral</td>
<td>5 mg/kg</td>
<td>(n=6)</td>
</tr>
<tr>
<td>Negative</td>
<td>5 mg/kg</td>
<td>(n=6)</td>
</tr>
<tr>
<td>Positive</td>
<td>0.5 mg/kg</td>
<td>(n=6)</td>
</tr>
<tr>
<td>Positive 5 mg/kg</td>
<td></td>
<td>(n=6)</td>
</tr>
</tbody>
</table>

Statistical significance:
- p < 0.05
- p < 0.01

(n=6)

**Thrombus Inducing Time (TIT)**
Aminated (+) Polystyrene UFPs (60 nm) injected iv or it 10-15 min after baseline (without Rose Bengal)
Thrombus Formation: Results

Peripheral venous thrombosis

Light Intensity (A.U. x 1000)

i. t. DEP (µg/animal)
Thrombus Formation: Results

Peripheral arterial thrombosis

![Image](image.png)

<table>
<thead>
<tr>
<th>i. t. DEP (µg/animal)</th>
<th>P=0.01</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
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<tr>
<td>50</td>
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Light Intensity (A.U. x 1000)
Within 1 hour after their deposition in the lungs, (Polystyrene - DEP)

- cause pulmonary inflammation
- aggravate thrombosis
Additional questions

The kinetics of

• Pulmonary inflammation?
• Platelet function following exposure to particles?

• And how is thrombogenicity affected by pulmonary inflammation?
Methods

- **Time effects:** 1, 6 and 24h after DEP exposure
- **Hamsters i.t. instilled with DEP**
  - (50 µg/animal, n= 4-6)
- **Endpoints assessed**
  - Lung inflammation (PMN)
  - Thrombosis (+ Platelet activation)
  - **Histamine concentrations** in BAL & in plasma
Methods

Platelet activation

Platelet function analysis
PFA-100®

800 µl blood

- 40 mbar

Opening: Ø 150 µm

Membrane coated with collagen & epinephrine

von Willebrand Factor

FLOW

capillary 200 µm

red blood cells

platelet
Methods

Platelet activation

Platelet function analysis
PFA-100®

Opening: Ø 150 µm

- 40 mbar

Membrane coated with collagen & epinephrine

von Willebrand Factor

FLOW

capillary 200 µm

red blood cells

platelet

closure time

t₀ t closure

closure time
**Methods**

Platelet function analysis

**ex vivo**
- i.t. DEP (50 µg/animal) or saline
- blood
- blood collection
- 5, 15, 30 and 60 min

**in vitro**
- DEP (0.1 - 5 µg)
- + 1ml hamster
- 5 min incubation
Time effect of i.t. DEP

Thrombosis in vivo

Time (h)

Light intensity (A.U.)

- DEP (i.t. 50 µg/animal)
- Control

p<0.005
p<0.0005
p<0.001
Time effect of i.t. DEP

Platelet aggregation (PFA) ex vivo

Control
DEP

Closure time (s)

Time (h)

p<0.05
p<0.001
p<0.05
Time effect of i.t. DEP

Platelet aggregation (PFA) ex vivo

Platelet number (10^3/µl blood)

Closure time (s)

Time (h)

Control

DEP

p<0.001

p<0.05

p<0.05
Time effect of i.t. DEP

Pulmonary inflammation (BAL)

PMN (%)

Time (h)

Control

DEP (50 µg/animal)

p<0.05

p<0.01

p=0.01
Time effect of i.t. DEP *in vivo*

**Histamine measurements**

- Control
- DEP

Histamine in BAL (nM)

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Histamine (nM)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>24</td>
<td></td>
<td>p&lt;0.05</td>
</tr>
</tbody>
</table>
Time effect of i.t. DEP in vivo

**Histamine measurements**

- **Histamine in BAL (nM)**
  - Time (h): 1, 6, 24
  - DEP: p<0.01, p<0.05
  - Control: p<0.05

- **Histamine in plasma (nM)**
  - Time (h): 1, 6, 24
  - DEP: p<0.05
  - Control: p<0.05
Time effect of i.t. DEP:

Summary

- I.t. instillation of DEP leads to a significant prothrombotic effect and lung inflammation, which persist up to 24 h.

- Histamine concentrations were increased in BAL at all time points but in plasma, histamine levels were only increased at 6 and 24h and **not at 1h**.

- Effects of i.t. DEP on pulmonary inflammation and peripheral thrombosis (at 6 & 24 h) can be blocked by pretreatment with diphenhydramine, dexamethasone or cromoglycate.
Interpretation

• We conclude that:
  - Pulmonary inflammation and peripheral thrombosis are correlated at 6 and 24h.
  - At 1h, the prothrombotic effect does not appear to result from pulmonary inflammation.

  This is compatible with direct platelet activation by “particles” (or its constituents) having penetrated into the circulation
Brook RD et al. Air pollution and cardiovascular disease. A statement for health-care professionals from the expert panel on population and prevention science of the American Heart Association. *Circulation* 2004 (June 1); 109: 2655-71
Acknowledgements

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- OT-KULeuven
Thank you for your attention
Some recent data - unpublished

- Pro-thrombotic effect of carbon nanotubes (24 hr after dosing)
- Thrombotic effect of quantum dots (iv dosing)
Pro-thrombotic effect of carbon nanotubes

![Graph showing thrombus size (A.U.) with different CNT (µg/mouse) concentrations.](image)

- Control
- 200 µg/mouse
- 400 µg/mouse

*Significance levels:*
- p < 0.05
- p < 0.01
Mouse dosed iv with:
- Biocompatible carboxylated quantum dots
- 4 µg/animal

Non stained - fluorescence
Mechanisms of particle-induced lung inflammation and vascular thrombosis?

Silica particles

**Pulmonary inflammation**

- **24 h**

<table>
<thead>
<tr>
<th>Silica particles (µg/hamster)</th>
<th>BAL-MACROPHAGES (x10⁴/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>0</td>
</tr>
<tr>
<td>2 µg/hamster</td>
<td>2.5 (p&lt;0.05)</td>
</tr>
<tr>
<td>20 µg/hamster</td>
<td>7.5 (p&lt;0.05)</td>
</tr>
<tr>
<td>200 µg/hamster</td>
<td>15.0 (p&lt;.01)</td>
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**Peripheral thrombosis**

<table>
<thead>
<tr>
<th>Silica particles (µg/hamster)</th>
<th>Thrombus size (A.U.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>0</td>
</tr>
<tr>
<td>2 µg/hamster</td>
<td>200000 (p&lt;0.05)</td>
</tr>
<tr>
<td>20 µg/hamster</td>
<td>400000 (p&lt;0.05)</td>
</tr>
<tr>
<td>200 µg/hamster</td>
<td>600000 (p&lt;0.001)</td>
</tr>
</tbody>
</table>

- Pulmonary inflammation
  - Saline, 2, 20, 200 µg/hamster
  - 24 h
  - BAL-MACROPHAGES (x10⁴/ml)
  - Saline: 0
  - 2 µg/hamster: 2.5 (p<0.05)
  - 20 µg/hamster: 7.5 (p<0.05)
  - 200 µg/hamster: 15.0 (p<.01)

- Peripheral thrombosis
  - Saline, 2, 20, 200 µg/hamster
  - Thrombus size (A.U.)
  - Saline: 0
  - 2 µg/hamster: 200000 (p<0.05)
  - 20 µg/hamster: 400000 (p<0.05)
  - 200 µg/hamster: 600000 (p<0.001)
Silica particles

- **Strategy of depletion:**
  - i.t. clodronate liposomes: pulmonary macrophages (↓ 70 %)
  - i.p. cyclophosphamide: PMN depletion (↓ 80 %)

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Lung inflammation</th>
<th>Peripheral thrombosis</th>
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</thead>
<tbody>
<tr>
<td>Clodronate</td>
<td>inhibition</td>
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</tr>
<tr>
<td>Cyclophosphamide</td>
<td>inhibition</td>
<td>inhibition</td>
</tr>
</tbody>
</table>

- **Additional results:**
  - Elastase increases in BAL and plasma (+ partial but significant reduction of thrombosis after i.t. pretreatment with MeOSuc-AAPV-CMK, a specific neutrophil elastase inhibitor)
  - Critical role of pulmonary macrophage-neutrophil cross-talk releasing neutrophil elastase into the blood circulation.
  - Elastase, triggering activation of circulating platelets, may then predispose platelets to initiate thrombotic events on mildly damaged vasculature.
Translocation: Radioactivity in blood

intratracheal instillation of $^{99m}$Tc-albumin nanocolloid particles (80 nm) in hamsters

from Brook et al. Circulation 2004, 109, 2655-71)