Rat Erythrocyte Micronucleus Test - Role of Erythropoiesis and Effect of Cigarette Mainstream Smoke

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Introduction and Objective

- The genotoxicity of cigarette mainstream smoke (MS) has been investigated in the in vivo micronucleus test using rats and mice (e.g., Coggins et al., 1990; Izotti et al., 2001).
- According to OECD guideline 474 (1997), the dose of any chemical tested in the micronucleus test should be relatively high, i.e.: "...The highest dose is defined as the dose producing signs of toxicity such that higher dose levels, based on the same dosing regimen, would be expected to produce lethality....".
- MS from the Kentucky Reference Cigarette 2R4F contains carbon monoxide (CO), i.e., ~1400 ppm at a total particulate matter (TPM) concentration of 1200 µg/l.
- CO has been reported to stimulate erythropoiesis (Sherpa et al., 1989).

Investigate the influence of enhanced erythropoiesis on the formation of micronuclei in rats exposed to MS.

Materials and Methods

Smoke Generation

- Generation according to ISO protocol (35 ml/puff in 2 s, each cigarette puffed once every minute, butt length 35 mm, Vanscheeuwijck et al., 2002)

Test Atmosphere Characterization

- Determination of concentrations of TPM, CO, nicotine, and selected aldehydes (formaldehyde, acetaldehyde, acrolein) at the breathing zone of the animals

Rats and Treatment

- Female Sprague-Dawley rats, 200 ±10 g (mean ± SD) at start of inhalation, 8 rats/group
- Nose-only exposure, 2 x 1 h with a 30-min fresh air exposure break for 4 consecutive days
- Exposure to fresh air (sham), to 250-1250 µg TPM/l (MS from the Kentucky Reference Cigarette 2R4F), or to 1250 µg TPM/l (MS from the Kentucky Reference Cigarette 2R1)
- Exposure to CO: in combination with fresh air or with 2R1 MS
- Mouse recombinant erythropoietin (EPO). (Roche cat. no. 11 276 964 001) treatment, 30 or 100 U/kg, i.p. daily for 4 days
- Positive control substance: cyclophosphamide (CPA) (Sigma, C-0768), 1.5 or 15 mg/kg, single i.p. injection 48 h before sacrifice

Sample Collection and Determinations

- Retro-orbital blood sample collection, 24 h after last exposure (or 48 h after single CPA treatment)
- Processing of samples: according to MicroFlow® -Rat Kit (Litron Laboratories, Rochester, NY)
- Cell enumeration done using flow cytometry analysis by Litron Laboratories
  - quantitation of CD-71 (transferrin receptor)-positive reticulocytes (proportion of reticulocytes among total erythrocytes: RET)
  - quantitation of micronucleated reticulocytes among 20,000 reticulocytes (proportion of micronucleated reticulocytes among total reticulocytes: MnRET)

Statistics

- Results expressed as mean ± SD
- One-way ANOVA followed by Dunnett post-hoc test for comparisons with the corresponding sham group
- One-way ANOVA followed by Tukey test for pairwise comparisons between treatment groups
- Differences considered statistically significant at p <0.05; asterisks in graphs indicate statistical differences compared to sham group

Results

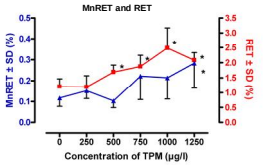
MS Concentration Response

Test Atmosphere Characterization

Group	TPM (µg/l)	CO (ppm)
sham	<QL	<QL
MS250	237 ± 8	259 ± 10
MS500	503 ± 24	543 ± 29
MS750	740 ± 9	795 ± 33
MS1000	1011 ± 18	1099 ± 15
MS1250	1262 ± 67	1347 ± 147

Remarks: MS from Kentucky Reference Cigarette 2R4F
QL: quantitation limit
for TPM: QL = 12 µg/l; for CO: QL = 5 ppm; means ± SD, N = 4

- MS induces an increase in the proportion of MnRET and RET.



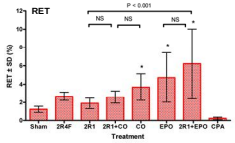
Effect of CO and EPO on MS Response

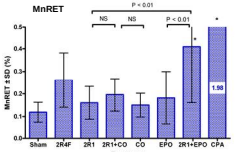
Test Atmosphere Characterization

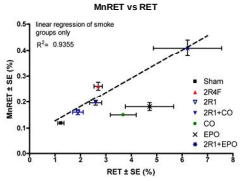
Group	TPM (µg/l)	CO (ppm)
sham	<QL	<QL
2R4F	1285 ± 29	1417 ± 37
2R1	1237 ± 9	703 ± 18
2R1 + CO	1255 ± 9	1315 ± 72
CO	<QL	1331 ± 18
EPO (sham)	<QL	<QL
2R1 + EPO	1237 ± 9	703 ± 18

Remarks: QL: quantitation limit
for TPM: QL= 12 µg/l; for CO: QL= 5 ppm; means ± SD, N = 4

- Exposure to CO or treatment with EPO, alone or in combination with MS, increases RET.
- Exposure to CO or treatment with EPO in combination with MS increases MnRET.
- There is an influence of RET on MnRET.







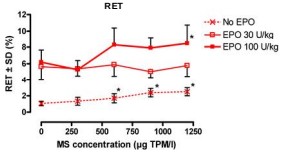
Effect of EPO on MS Response

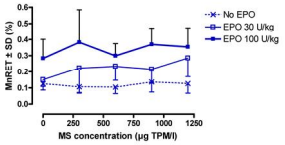
Test Atmosphere

Group	TPM (µg/l)	CO (ppm)
sham	<QL	<QL
MS300	304 ± 13	319 ± 14
MS600	594 ± 11	608 ± 7
MS900	906 ± 2	900 ± 4
MS1200	1177 ± 34	1123 ± 51

Remarks: MS from Kentucky Reference Cigarette 2R4F
QL: quantitation limit
for TPM: QL = 12 µg/l; for CO: QL = 5 ppm; means ± SD, N = 4

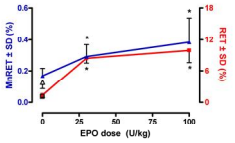
- Treatment with EPO increases RET and MnRET.
- No effect of MS inhalation on micronucleus formation.





Proof of Concept

- EPO in combination with a non-cytotoxic dose of CPA (1.5 mg/kg) increases MnRET and RET dose dependently.



open symbols represent no CPA and no EPO treatment

Summary

- Erythropoiesis is stimulated after:
  - exposure to MS (probably due to CO)
  - exposure to CO
  - treatment with EPO
- An increased proportion of RET results in an increased proportion of MnRET after:
  - exposure to MS
  - administration of the positive control CPA

Conclusion

- We have demonstrated that the proportion of micronucleated reticulocytes correlates with the proportion of reticulocytes in rat blood.

  ⇨ Enhanced erythropoiesis influences the proportion of micronucleated reticulocytes after exposure to MS.
- Further studies are needed to determine how much of the MS-induced increase in MnRET is due to MS constituents other than CO.

References

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