# Cigarette Mainstream Smoke Affects Arterial Thrombosis and Vessel Remodeling after Vascular Injury in Apolipoprotein E-Deficient Mice

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## Background

Cigarette smoking has long been recognized as a major risk factor for enhanced atherosclerosis progression and acute arterial thrombosis (Surgeon General's Report, 2004). Epidemiological studies have shown that the risk of death from coronary heart disease is elevated at least 2-fold among smokers compared to non-smokers (Ockene and Miller, 1997). Moreover, two-thirds of sudden cardiac deaths due to acute coronary thrombosis have been reported to occur in cigarette smokers (Burke et al., 1997). In contrast, the association between cigarette smoking and vessel wall remodeling in response to vascular injury is less clear, and clinical studies have reported conflicting results regarding the association between smoking and intimal hyperplasia (Galan et al., 1988; Macdonald et al., 1990; Hasdai et al., 1997).

Data supporting the procoagulant and atherogenic consequences of cigarette smoke or its components have so far been obtained mainly through *in vitro* studies, clinical observations, and the histological analysis of atherectomy and/or autopsy specimens in humans (Burke et al., 1997; Ruengsakulrach et al., 1999). Currently, little is known about the underlying pathomechanisms of cigarette smoke-induced thrombosis and the subsequent vessel wall remodeling, partly due to a lack of adequate *in vivo* models. Only a few short-term experimental studies have systematically investigated the effects of cigarette smoke exposure on intimal hyperplasia in animal models of endothelial injury (Petrik et al., 1995; Anazawa et al., 2004; Davis et al., 2004; Tani et al., 2004). Appropriate animal models are needed to evaluate the underlying pathomechanisms of thrombosis and intimal hyperplasia.

## Objective

To investigate the biological effects of sub-chronic exposure to cigarette mainstream smoke (MS) on arterial thrombosis and neointima formation using the ferric chloride ($FeCl_3$) model of vascular injury in the apolipoprotein E knockout ($ApoE^{-/-}$) mouse.

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## Materials and Methods

**Cigarette Mainstream Smoke Generation**

- 2R4F (University of Kentucky Reference Cigarette)
- MS generated on a 30-port smoking machine (SM85i) in basic conformity with ISO standards 3402 (1999) and 3308 (2000)
- Dilution to 600 µg total particulate matter (TPM)/l with conditioned fresh air

**Animal and MS Exposure**

- Young adult, male $ApoE^{-/-}$ mice from Taconic (Denmark)
- Whole-body exposure 5 days/week for 90 days
  - 4 hours/day to conditioned fresh air (Sham, N = 60)
  - 1 hour/day to 600 µg TPM/l (MS 600, N = 20)
  - 2 hours/day to 600 µg TPM/l (MS 1200, N = 20)
  - 3 hours/day to 600 µg TPM/l (MS 1800, N = 58)
  - 4 hours/day to 600 µg TPM/l (MS 2400, N = 23)

**$FeCl_3$-Vascular Injury Protocol and Determinations**

- Induction of carotid injury with 10% $FeCl_3$ after 70 days of exposure according to Konstantinides et al. (2001)
- Determination of thrombotic occlusion (blood flow rate <0.2 ml/min)
- Determination of patency rate
- Determination of intimal hyperplasia (sham, MS 1800, and MS 2400 groups only) after an additional 20 days of exposure
  - immunohistochemical analysis of the injured carotid artery
  - morphometric analysis of the injured carotid artery

**Statistics**

- Data are presented as mean ± SEM unless stated otherwise.
- One-way analysis of variance (ANOVA) was performed followed by Dunnett's multiple comparison test (for parametric values) or Kruskal-Wallis test followed by Dunn's multiple comparison test (for non-parametric values).
- For comparison of categorical parameters (thrombotic occlusion, vascular patency rate), chi-square analysis was employed.
- Statistical significance was assumed at p <0.05, *; p <0.01, **; p <0.001, ***.

Care and use of the mice was in accordance with the American Association for Laboratory Animal Science Policy on the Humane Care and Use of Laboratory Animals (www.aalas.org). The animal experiments were previously approved by the Institutional Animal Care and Use Committee (IACUC).

**Induction of Carotid Injury with 10% $FeCl_3$**

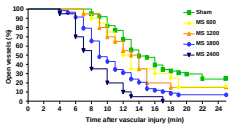
Picture from p. 577 of Konstantinides, S. et al., Circulation 103 (2001).
Image used on original poster with permission of Lippincott, Williams & Wilkins

**A:** Left carotid artery (arrowhead) was exposed and elevated by 2 threads, and an ultrasound flow probe was placed around vessel.
**B:** A strip of filter paper soaked in 10% $FeCl_3$ was then applied to the surface of adventitia for 3 min.
**C:** Thrombus formation (arrowheads)

## Results

### Time to Thrombotic Occlusion

**Measured after $FeCl_3$-Induced Vascular Injury**




| Group | Median time to occlusion (min) | p value |
|---|---|---|
| Sham | 14.9 | - |
| MS 600 | 13.0 | 0.138 vs. sham, n.s. |
| MS 1200 | 13.9 | 0.122 vs. sham, n.s. |
| MS 1800 | 9.7 | <0.001 vs. sham, *** |
| MS 2400 | 8.0 | <0.001 vs. sham, *** |

**Vascular Occlusion in Response to $FeCl_3$ Injury**

| Group | Occluded vessels (n) | % | p value |
|---|---|---|---|
| Sham | 45 | 75 | - |
| MS 600 | 17 | 85 | 0.538 vs. sham, n.s. |
| MS 1200 | 17 | 85 | 0.538 vs. sham, n.s. |
| MS 1800 | 54 | 93 | 0.015 vs. sham, * |
| MS 2400 | 23 | 100 | 0.008 vs. sham, ** |

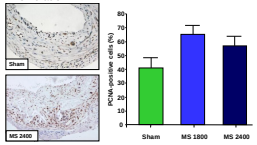
**Vascular Patency Rates at the End of 25-min Observation Period**

| Group | No occlusion (n) | % | p value |
|---|---|---|---|
| Sham | 24 | 40 | - |
| MS 600 | 4 | 20 | 0.175 vs. sham, n.s. |
| MS 1200 | 7 | 35 | 0.794 vs. sham, n.s. |
| MS 1800 | 6 | 10 | <0.001 vs. sham, *** |
| MS 2400 | 0 | 0 | <0.001 vs. sham, *** |

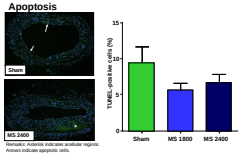
### Intimal Hyperplasia

**Cellular Proliferation and Apoptosis within the Vascular Lesions**

**Proliferation**



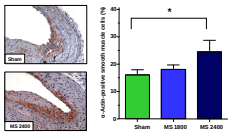

**Apoptosis**



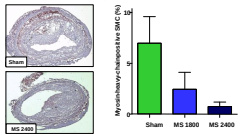

Remarks: Asterisk indicates acellular regions. Arrows indicate apoptotic cells.

**Smooth Muscle Cells (SMC) within the Vascular Lesions**

**α-Actin-positive SMC**

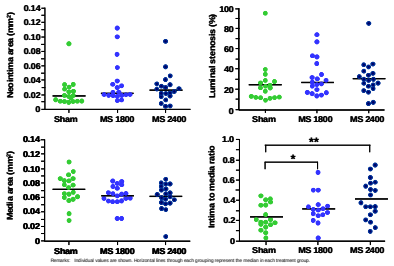



**Myosin-heavy-chain-positive SMC**




### Intimal Hyperplasia

**Neointima Formation 20 Days after Injury**




Remarks: Individual values are shown. Horizontal lines through each grouping represent the median in each treatment group.

## Summary

- **Arterial Thrombosis (all groups)**
  - reduced time to thrombotic vessel occlusion in a dose-dependent manner.
  - increased number of occluded vessels in response to $FeCl_3$ injury
  - reduced number of vessels open 25 min after the $FeCl_3$ injury
- **Intimal Hyperplasia (MS 1800 and MS 2400)**
  - altered composition of the neointima (cellular proliferation, apoptosis, SMC)
  - increased neointima to media ratio

## Conclusion

Our findings indicate that sub-chronic exposure of $ApoE^{-/-}$ mice to MS promotes arterial thrombosis and modulates the size and composition of vascular lesions after $FeCl_3$-induced vascular injury.

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