Aerosol from a candidate modified risk tobacco product has reduced effects on chemotaxis and transendothelial migration compared to smoke from conventional cigarettes

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Abstract

Introduction. Cigarette smoking increases the risk of cardiovascular disease. Reduction in the levels of harmful constituents by heating rather than combusting tobacco without modifying the amount of delivered nicotine has the potential to reduce the risk of tobacco related disease.

Aim. In this study, we investigated the effect of extract from a new candidate modified risk tobacco product (MRTP), the tobacco heating system (THS) 2.2, on the migratory behavior of monocytes in comparison with extract of smoke from combustible 3R4F reference cigarettes.

Methods. The extracts were generated by aerosol-bubbling RPMI media from 3R4F and MRTP according to Health Canada standards. Endpoints included cytotoxicity (cell death assessed by 7-AAD staining followed by FACS analysis), inflammation (cytokine measurements), THP-1 cell migration (Boyden chambers), and transendothelial migration (TEM) determined by real-time impedance.

Results. The results show that treatment of THP-1 cells with extract from 3R4F smoke or THS2.2 aerosol induced a concentration-dependent increase in cytotoxicity and inflammation (IL-8 and TNF-α secretion). The inhibitory effects of THS2.2 aerosol extract for chemotaxis and TEM were at least ~18 times less effective compared to 3R4F smoke extract. Furthermore, we found decreases in the integrity of a HCAEC monolayer in a concentration-dependent manner. However, for all other examined endpoints, the extract from 3R4F smoke showed more than one order of magnitude stronger effects than that from THS2.2 aerosol extract.

Conclusion. These data indicate the potential of the Tobacco Heating System to reduce the risk for cardiovascular disease compared to combustible cigarettes.

Introduction

Smoking cigarettes is a major risk factor for the development of cardiovascular diseases (Ambrose and Barua, 2004; Prevention, 2014). A reduction in the exposure to harmful constituents using heated rather than combusted tobacco could be a promising approach to reduce the harmful effects associated with cigarette smoking.

Cigarette smoke (CS) constituents induce endothelial gene expression of cell adhesion molecules, which promote monocyte adhesion (Pousin et al. 2014; Shen et al. 1996). In the present study, the combined use of conventional Boyden chamber migration and impedance-based experimental systems enabled us to investigate whether aqueous extract from a candidate modified risk tobacco product (MRTP) exhibited differential effects on the migratory behavior of monocytes compared with the reference cigarette 3R4F.

Materials & Methods

Cell Culture – Human endothelial coronary cells (HCAECs) were grown on collagen A-coated plates. THP-1 cells were grown in RPMI 1640 medium.

Reference cigarette (3R4F) and the Tobacco-heating system (THS)2.2 – Reference research filtered cigarettes 3R4F were purchased from the University of Kentucky (Lexington, KY, USA). THS2.2 system as well as tobacco heat sticks were provided by Philip Morris Products S.A. (Neuchâtel, Switzerland).

Generation of extract – Extract was generated by bubbling an aerosol from 3R4F or THS2.2 heat sticks through RPMI 1640 medium. Chemical analyses were conducted directly after the generation of the extract. Serum-starved THP-1 cells and HCAECs were stimulated for 4 h with increasing concentrations of freshly prepared extracts from 3R4F and THS2.2 heat sticks.

Monocyte chemotaxis and TEM assays using conventional Boyden chambers – Chemotaxis assays using Boyden chambers were conducted in the absence or presence of a monolayer of HCAECs. THP-1 cells were serum-starved for 18 h and seeded on migration inserts (5 μm pore size). Serum-free medium with or without CXCL12 was added to the bottom chamber. Monocyte chemotaxis was assessed after 4 h of incubation.

Monocyte chemotaxis and TEM assays using impedance-based systems – Chemotaxis assays were conducted using cell invasion/migration (CIM) plates and a real-time cell analyzer dual-plate (RTCA-DP) xCELLigence instrument. HCAECs were seeded on the inserts of collagen-coated CIM plates. After the HCAECs had reached confluency, serum-starved THP-1 cells were seeded on the HCAEC monolayer. CXCL12 was added to the bottom chamber. Cell index (impedance) was used to calculate the TEM rate.

Identification of monocyte-macrophage differentiation – To detect cell surface markers, THP-1 cells were incubated with monoclonal mouse anti-human antibodies CD18-BV421 and CD11b-AF488 or relevant isotypes controls for 15 min at 4°C. After incubation, the cells were washed, resuspended in 1% BSA/ PBS and analyzed using a FACS-Canto™ II and BD FACSDiva™ software.

Cytotoxicity and inflammation assays – THP-1 cells were stimulated for 18 h with various concentrations of freshly prepared extracts from 3R4F and THS2.2 heat sticks. Cells were stained with 7-AAD and analyzed by flow cytometry. THP-1 cell culture supernatants were collected, snap frozen and stored at −80 °C. IL-8 and tumor necrosis factor-α (TNF-α) levels in the supernatants were measured with ELISA kits.

Statistics – Data are expressed as mean ± SEM unless otherwise indicated. The data were evaluated with a one-way ANOVA. When the overall F test of the ANOVA analysis was significant, multiple comparison tests (Dunnet) were applied to determine the sources of differences.

Table 1. Selective chemical analysis of 3R4F and THS2.2 extracts

	3R4F (μg/cig)	THS2.2 (μg/stick)	3R4F/THS2.2
Nicotine	80.2 ± 7.3	76.8 ± 15.9	1
Formaldehyde	37.9 ± 8.6	3.0 ± 0.6 *	12
Acetaldehyde	1089 ± 150	174 ± 44 *	6.2
Acetone	408 ± 80	24.1 ± 4.8 *	17
Acrolein	100 ± 13	5.5 ± 1.0 *	18
Propionaldehyde	65 ± 10	11.5 ± 1.6 *	5.6
Crotonaldehyde	48 ± 6	2.3 ± 0.4 *	21
Methyl ethyl ketone	87 ± 21	4.6 ± 0.9 *	19
Butyraldehyde	19 ± 3.0	9.9 ± 2.0 *	1.9

Eight independent samples were applied to chemical analysis. Data are expressed as the mean ± SD. *P<0.01 vs. 3R4F (reference cigarette). cig: cigarette; THS2.2: Tobacco-heating system 2.2.

Results

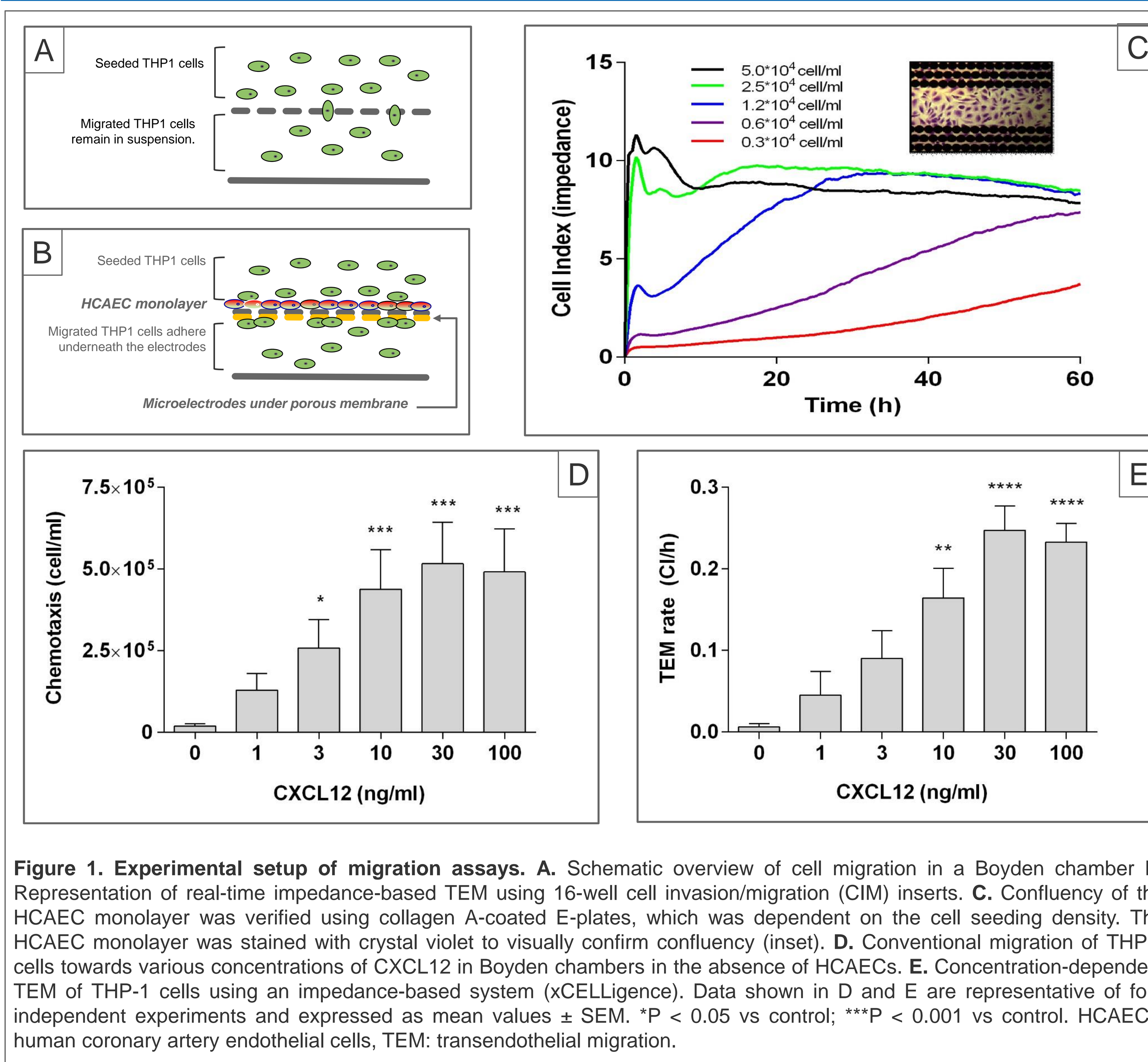


Figure 1. Experimental setup of migration assays. A. Schematic overview of cell migration in a Boyden chamber **B.** Representation of real-time impedance-based TEM using 16-well cell invasion/migration (CIM) inserts. **C.** Confluency of the HCAEC monolayer was verified using collagen A-coated E-plates, which was dependent on the cell seeding density. The HCAEC monolayer was stained with crystal violet to visually confirm confluency (inset). **D.** Conventional migration of THP-1 cells towards various concentrations of CXCL12 in Boyden chambers in the absence of HCAECs. **E.** Concentration-dependent TEM of THP-1 cells using an impedance-based system (xCELLigence). Data shown in D and E are representative of four independent experiments and expressed as mean values ± SEM. *P < 0.05 vs control; ***P < 0.001 vs control. HCAECs: human coronary artery endothelial cells, TEM: transendothelial migration.

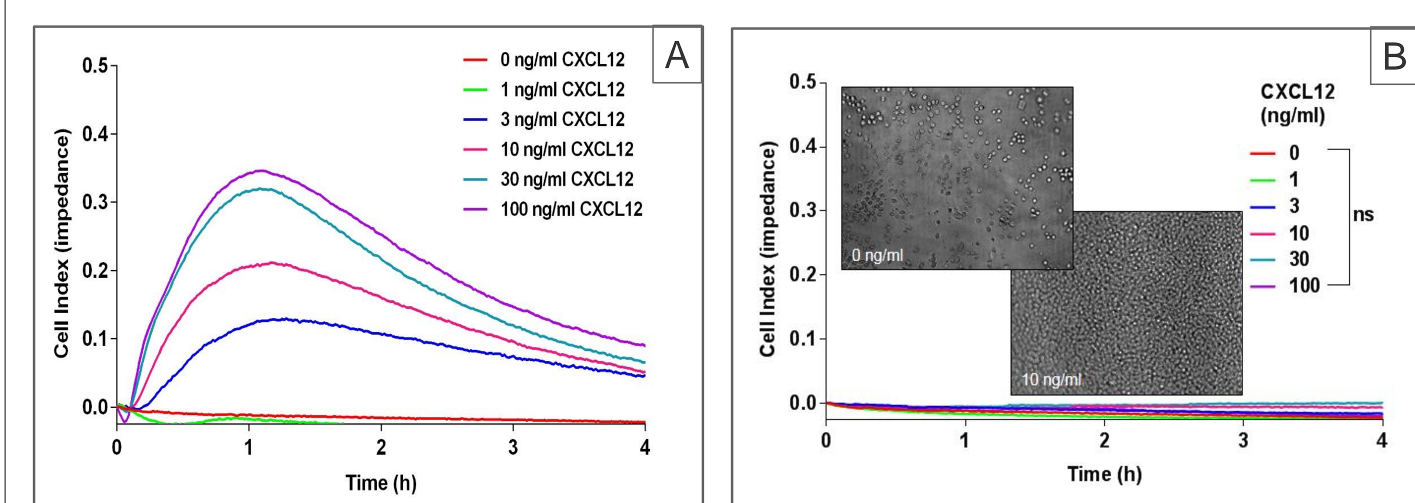
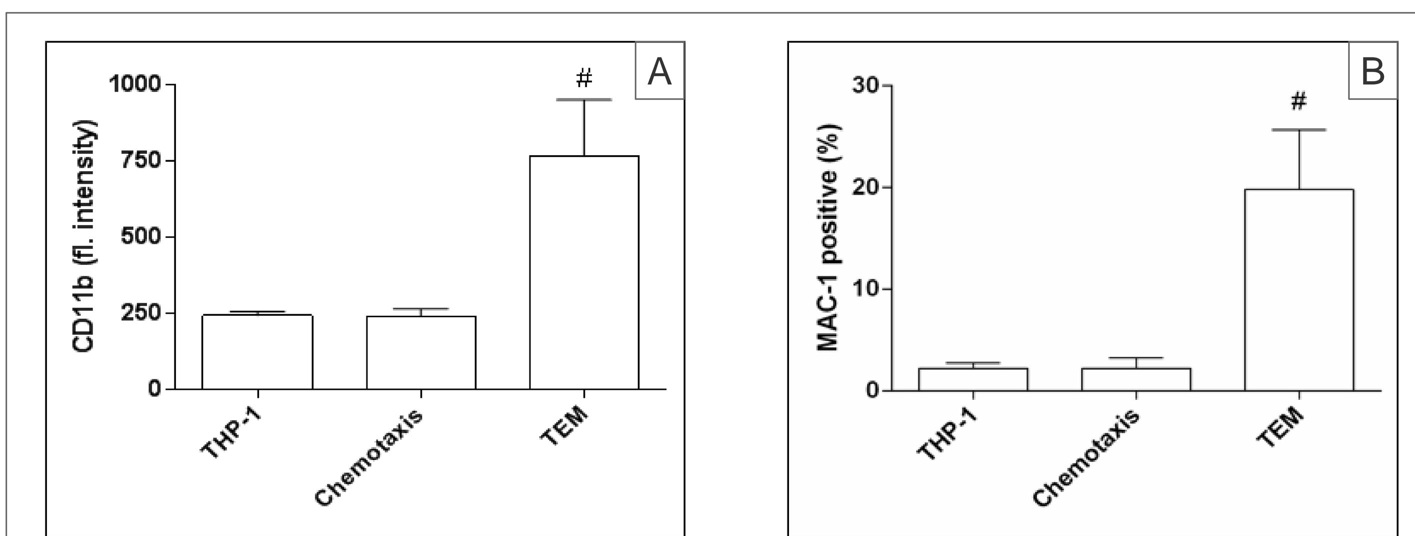


Figure 2. Migration of THP-1 cells through a monolayer of HCAECs promotes acquisition of an adhesive phenotype. A. Induction of concentration-dependent migration towards CXCL12 in the presence of HCAECs. THP-1 cells adhered to the lower side of the porous membrane as revealed by increased impedance (cell index). **B.** Induction of concentration-dependent migration towards CXCL12 in the absence of HCAECs. THP-1 cell adherence to the lower side of the membrane did not occur as evidenced by the lack of changes in impedance. Representative recordings from four independent experiments are shown.



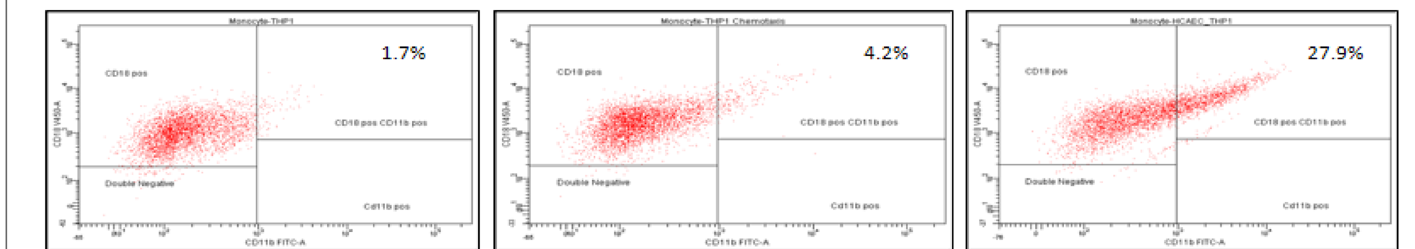


Figure 3. Acquisition of an adhesive phenotype by monocytes in the absence or presence of HCAECs. Acquisition of an adhesive phenotype was defined as positivity for **A.** CD11b or **B.** MAC-1. Representative flow cytometric data from non-migrated (lower left), migrated (lower middle), and transendothelial migrated (lower right) THP-1 cells towards CXCL12 are shown. Data are expressed as the mean ± standard deviation (SD) of three experiments. #P < 0.05 vs THP-1 control by paired t-test. ns: not significant. CXCL12: chemoattractant C-X-C motif ligand 12. MAC-1: membrane-activating complex 1. TEM: transendothelial migration.

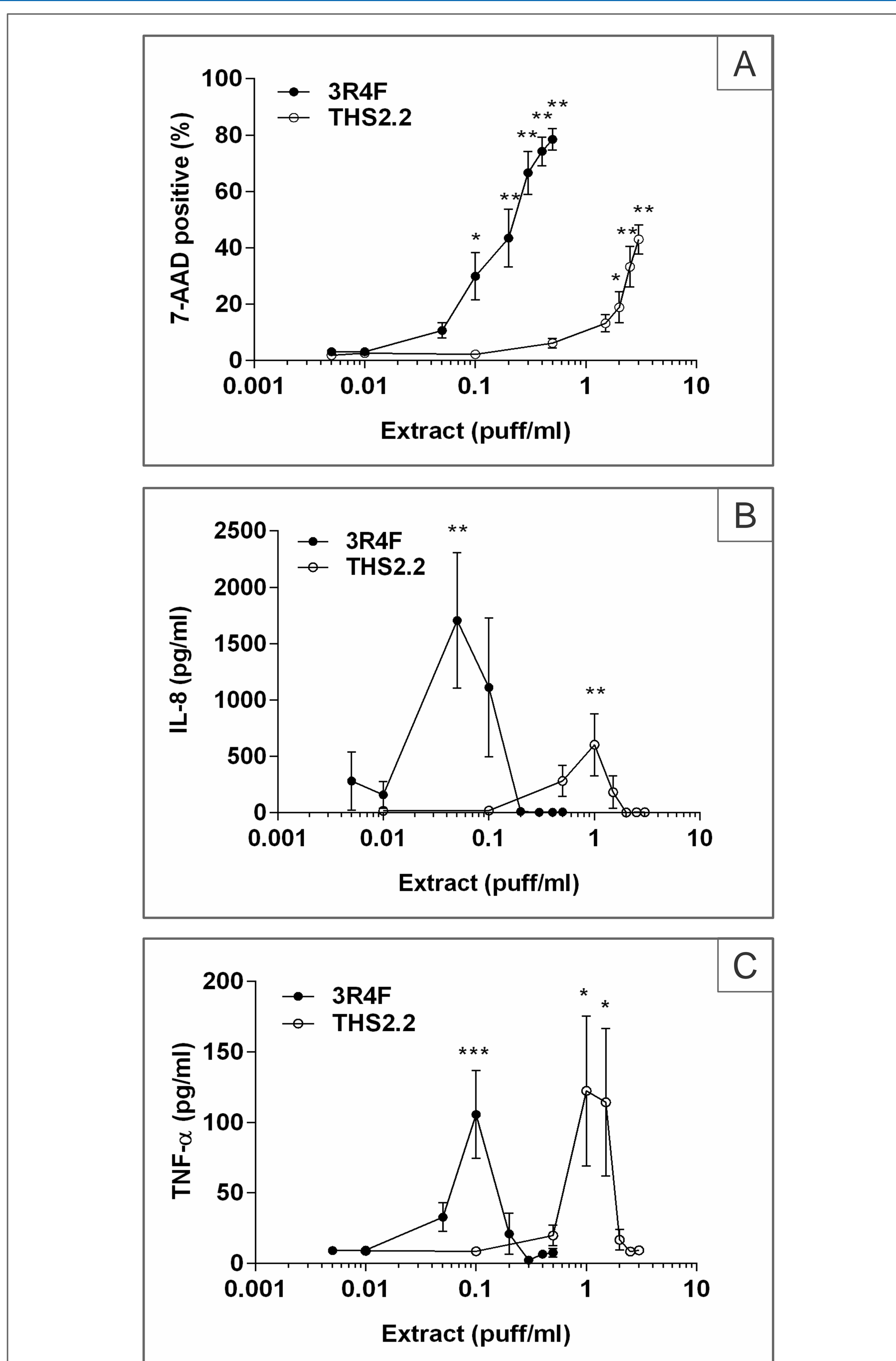


Figure 3. Effects of 3R4F and THS2.2 extracts on THP-1 cell cytotoxicity and inflammation. A. Percentage of THP-1 cells positive for 7-AAD after stimulation with 3R4F (filled circles) or THS2.2 (hollow circles) extract. **B and C.** Mean concentrations of IL-8 and TNF-α in the culture supernatants of THP-1 cells treated with 3R4F (filled circles) or THS2.2 (hollow circles) extract. Data are expressed as the mean ± SEM of four independent experiments. *P < 0.05 vs control; **P < 0.01 and ***P < 0.001 vs control. THS2.2: tobacco heating system 2.2.

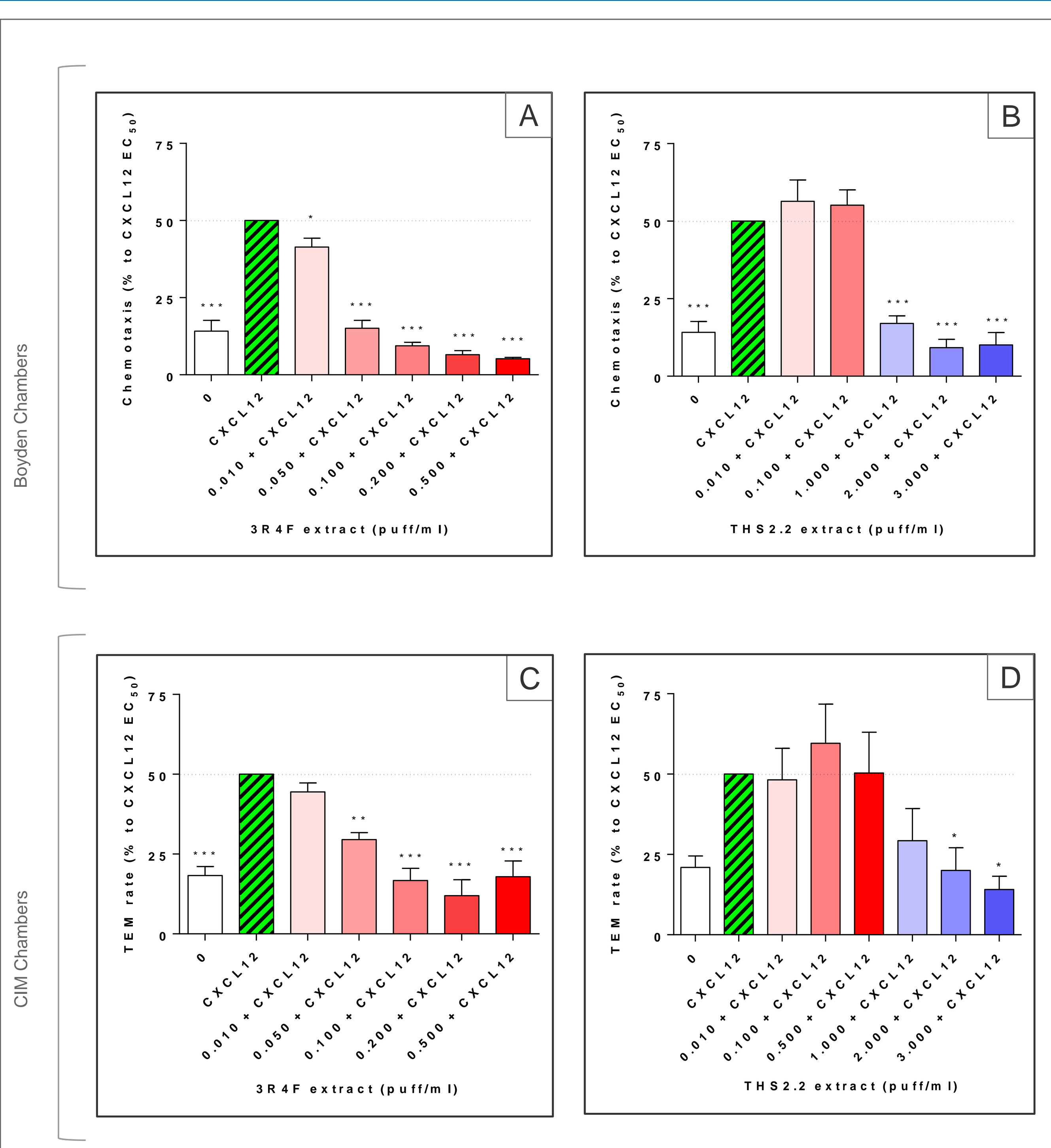


Figure 4. Effect of extracts from 3R4F and THS2.2 on monocytic migration and real-time impedance-based TEM. Migration of THP-1 cells exposed to increasing concentrations of **A.** extracts from 3R4F and **B.** THS2.2 in conventional Boyden chambers. Real-time impedance-based TEM of THP-1 cells exposed to increasing concentrations of extracts from **C.** 3R4F or **D.** THS2.2 in CIM chambers. Extracts from 3R4F and THS2.2 decreased the CXCL12-mediated migration of THP-1 cells in a concentration-dependent manner. Data are expressed as the mean ± SEM of four independent experiments. *P < 0.05 vs control; **P < 0.01 vs control; ***P<0.001 vs control.

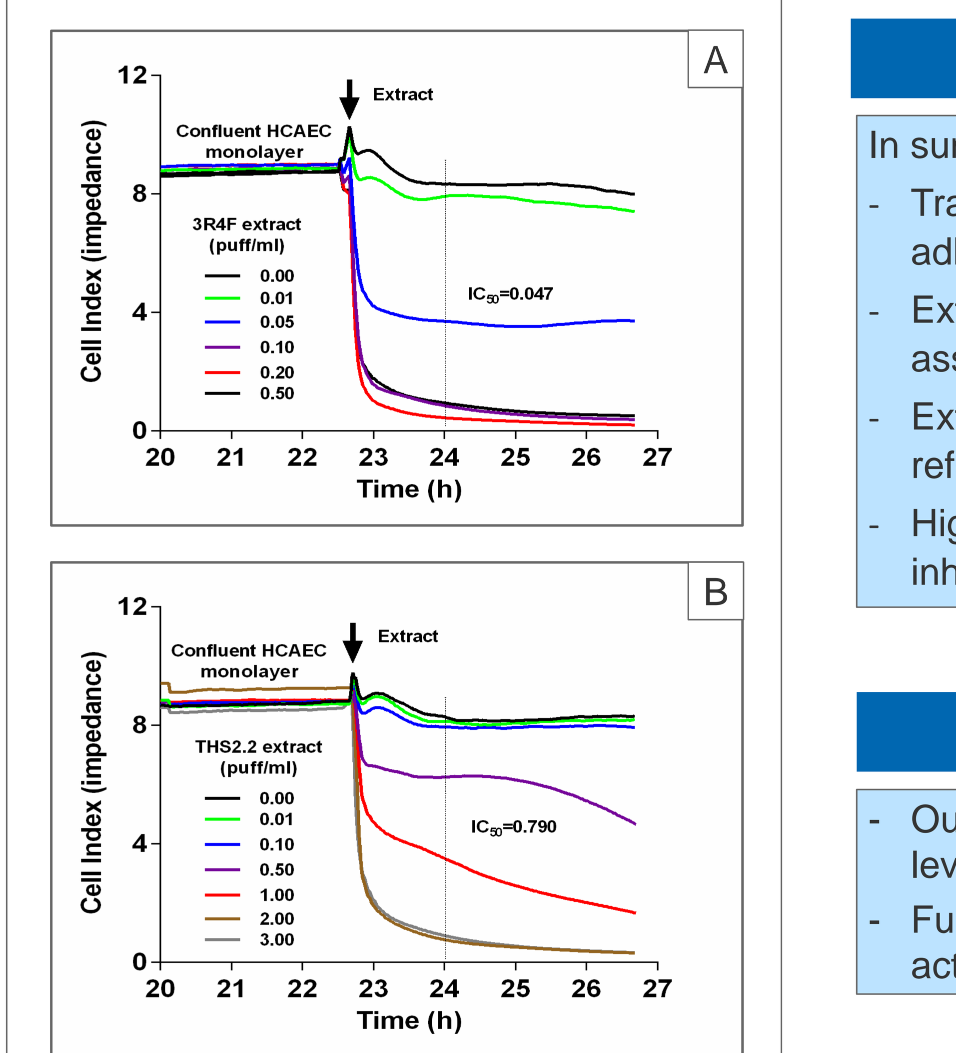


Figure 6. Effect of extracts from 3R4F and THS2.2 on HCAEC adhesion. HCAECs seeded in E-plates were stimulated with increasing concentrations of extracts from **A.** the reference cigarette 3R4F and the **B.** THS2.2. Extracts from 3R4F and THS2.2 induced a concentration-dependent decrease in impedance. The THS2.2 IC_{50} was ~17 times higher than that for 3R4F. Representative recordings from three independent experiments are shown.

Summary

In summary, our results indicate that:

- Transendothelial migration of monocytes is accompanied by significant changes in adhesion and expression of MAC-1.
- Extracts from 3R4F or the THS2.2 induces concentration-dependent responses in assays of inflammation, cytotoxicity, chemotaxis, and TEM.
- Extract from the THS2.2 is less cytotoxic and less proinflammatory than that from the reference tobacco product, 3R4F.
- Higher concentrations of extract from the THS2.2 compared to 3R4F are required to inhibit chemotaxis and TEM of monocytes.

Conclusion / Outlook

- Our data demonstrate that heating tobacco rather than combusting can reduce the levels of harmful constituents.
- Further functional and molecular studies are required to better understand the actions of cigarettes and MRTPs at all stages of vascular lesion development.

References

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