Impact of test conditions on *in vitro* cytotoxicity measurements of mainstream cigarette smoke using a whole smoke exposure system

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Abstract

The purpose of this study was to evaluate the impact of several factors on smoke cytotoxicity measurements in an *in vitro* whole smoke exposure system. Effects of incubation time after smoke exposure, cell types used, and smoke regimens on the cytotoxicity of cigarette smoke were investigated. Mainstream cigarette smoke was generated from the 3R4F reference cigarettes using a VC10 smoking robot under the ISO regimen (35/60/2 without blocking of filter ventilation) or the HCI regimen (55/30/2 with complete blocking of filter ventilation). Cells were exposed to fresh whole smoke (WS) in the VITROCELL® system, and cytotoxicity was evaluated using the neutral red uptake (NRU) assay. Results showed 24 hrs after smoke exposure appeared to be an optimal time-point to assess smoke cytotoxicity. CHO cells were more sensitive to smoke-induced cytotoxic effects than A549 cells. Smoke regimen evaluation was conducted in both cigarette smoke condensate (CSC) and WS. For CSC testing, the cytotoxicity decreased going from the ISO regimen to HCI regimen on a per unit of total particulate matter (TPM) basis. For WS exposure testing, cytotoxicity under the ISO regimen was less than that under the HCI regimen when smoke doses were expressed as % of cigarette smoke; notably, when smoke doses were converted to TPM (μg), cytotoxicity under the HCI regimen was less than that under the ISO regimen. A clear dose-response relationship between cell viability and smoke doses was observed under all test conditions. These data indicated that this *in vitro* smoke exposure system can be a useful tool to study the toxicological effects of WS, and the test conditions can have an important impact on the results of cytotoxicity evaluation of cigarette smoke.

Introduction

With the advance of *in vitro* whole smoke (WS) exposure technology, more studies now evaluate the fresh WS for toxicological responses (Aufderheide and Gressmann, 2008; Okuwa et al., 2010). WS might reflect completely the biological effects of native smoke. Cytotoxicity assays are part of a battery of *in vitro* assays for assessing the potential toxicity of tobacco products. Here we evaluated the impact of test conditions on smoke cytotoxicity measurements in an *in vitro* whole smoke exposure system.

Materials and Methods

Cigarettes

3R4F reference cigarettes (Lexington, Kentucky, USA) were conditioned at 22±1 ºC and 60±3% relative humidity for at least 48 hrs before being smoked.

Cell culture

Chinese hamster ovary (CHO) cells and human lung adenocarcinoma epithelial cell line (A549) were purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China).

Approximately 24 hrs before exposure, cells were trypsinized and seeded onto 96 well plates(Corning, NY, USA) at a density of 1×10^4 cells/well for cigarette smoke condensate (CSC) treatment, or seeded onto insert membranes (pore size 0.4 μm, growth area 1.12 cm^2) (Corning) at a density of 3.5×10^4 cells/insert for WS exposure.

Cigarette smoke generation and exposure

WS exposure performed on the VITROCELL® system (VITROCELL, Waldkirch, Germany). Under ISO regimen (35/60/2 without blocking of filter ventilation), the dilution air flow rates were 0, 0.75, 2.25 or 3.75 L/min for 4 smoke concentrations. Under HCI regimen (55/30/2 with complete blocking of filter ventilation), the dilution air flow rates were 0, 1.2, 3.55 or 5.9 L/min. As a negative control, cells were exposed to clean air at a flow rate of 5 ml/min. Smoke doses were expressed as % of cigarette smoke (% of cig.) (Okuwa et al., 2010).

CSC collections were done with a Borgwaldt RM-20H smoking machine (Borgwaldt KC, Hamburg, Germany) under ISO or HCI regimens. Cells were treated with CSC solutions at the following concentrations for 24 hrs: 10, 50, 75, 100, 120, 140, 160, and 200 μg/ml.

NRU cytotoxicity assay

The neutral red uptake (NRU) cytotoxicity assay for smoke was performed according to the method recommended by Health Canada (Health Canada Official Method T-502, 2004) with some modifications. the absorbance was measured at 540 nm with a microplate reader (Model 680, Bio-Rad Laboratories, Tokyo, Japan).

EC_{50} values were estimated using the Origin8.0 software.

Statistical analysis

The data obtained from *in vitro* cytotoxicity assay was expressed as Mean±SD. The differences in the viability of cells exposed to smoke under different test conditions were analyzed by a two tailed t-test as well as the difference in EC_{50} values under different smoking regimens. A *P* value of <0.05 was considered as statistically significant.

Results

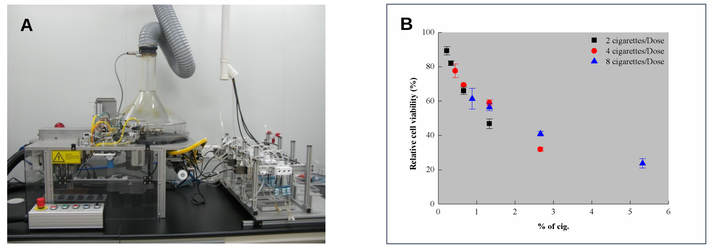

Figure 1. Dose-response relationship between smoke exposure and cell viability.

(A): The VITROCELL® system is composed of a smoking machine (VITROCELL® VC 10 smoking robot), 4 smoke dilution systems, and 5 exposure modules (one for air control exposure).

(B): CHO cells were exposed to the diluted smoke generated under ISO regimen. Four different smoke concentrations were generated simultaneously during one smoke session and 2, 4 or 8 cigarettes were smoked per smoke concentration. A clear dose-response relationship between smoke exposure and cell viability were observed using this whole smoke exposure system. (n=3)

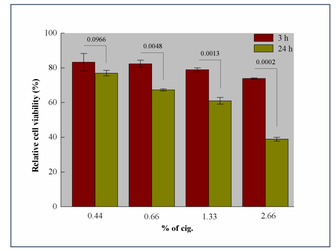

Figure 2. Effect of the period of culture after whole smoke exposure on the cytotoxicity measurment (n=3).

After exposure, CHO cells were cultured for 3 and 24 hrs, respectively. 24 hrs after smoke exposure appeared to be an optimal time-point to assess smoke cytotoxicity.

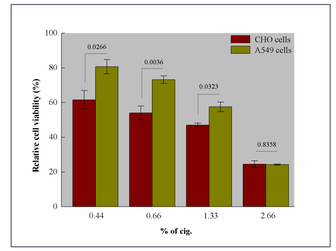

Figure 3. Comparison of the sensitivity to cytotoxicity of WS between CHO cells and A549 cells (n=3).

CHO cells were more sensitive to smoke-induced cytotoxic effects than A549 cells.

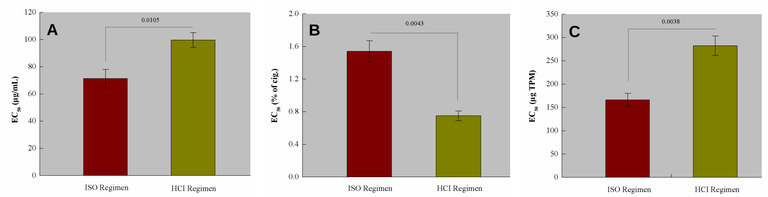

Figure 4. Effect of smoking regimens on the cytotoxicity of cigarette smoke.

(A): For CSC testing, the cytotoxicity decreased going from ISO regimen to HCI regimen on a per unit of total particulate matter (TPM) basis. (n=4)

(B): For WS exposure testing, the cytotoxicity under ISO regimen was less than that under HCI regimen when smoke doses were expressed as % of cig. (n=3)

(C): When smoke doses were converted to TPM (μg), the cytotoxicity under HCI regimen was less than that under ISO regimen. (n=3)

Conclusions

- A clear dose-response relationship between cell viability and smoke doses was observed under all test conditions.
- 24 hrs after smoke exposure appeared to be an optimal time-point to assess smoke cytotoxicity.
- CHO cells were more sensitive to smoke-induced cytotoxic effects than A549 cells.
- For CSC testing, the cytotoxicity decreased going from the ISO regimen to HCI regimen on a per unit of total particulate matter (TPM) basis.
- For WS exposure testing, cytotoxicity under the ISO regimen was less than that under the HCI regimen when smoke doses were expressed as % of cigarette smoke; notably, when smoke doses were converted to TPM (μg), cytotoxicity under the HCI regimen was less than that under the ISO regimen.

Acknowledgements

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