In Vitro Cytotoxicity Evaluation of Commercial JUUL® Product E-Liquids and Aerosol Condensates

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Abstract

To help understand the health risks associated with the JUUL Electronic Nicotine Delivery System (ENDS) products, four JUUL ENDS products and the reference 3R4F cigarette were tested for cytotoxicity in the *in vitro* neutral red uptake (NRU) assay. Each JUUL ENDS product was tested as an e-liquid and as aerosol condensates, collected on a Cambridge filter pad followed by an impinger filled with ethanol at 0°C, using intense and non-intense puffing regimens. Cigarette smoke was collected with a similar apparatus and tested as smoke condensate using the ISO 20778:2018 puffing regimen. The NRU assay was conducted in accordance with OECD TG129 using two cell lines: the murine fibroblast BALB/c 3T3 cell line and the human lung adenocarcinoma A549 cell line. No cytotoxicity was observed with the JUUL ENDS e-liquids or condensates (viability for both 3T3 and A549 cells >80% at all tested concentrations, up to 0.5% v/v or 17 µg nicotine/mL). In contrast, the reference cigarette 3R4F condensate was cytotoxic and at much lower concentration ranges compared to ENDS condensates (IC_{50}s: 3T3: 3.00 ± 0.14 µg/mL nicotine, A549: 4.98 ±1.57 µg/mL nicotine). The results demonstrated that the four tested JUUL products were significantly less cytotoxic than the combustible (3R4F) cigarette under the tested conditions.

Introduction

In "Guidance for Industry: Premarket Tobacco Product Applications for Electronic Nicotine Delivery Systems" (FDA 2019), the Food and Drug Administration requires applicants to provide information regarding studies assessing toxicology and, in Section IV(H)(2)(A), "recommends providing a full assessment of the toxicological and pharmacological profile associated with the new tobacco product." In an effort to address the toxicological profile, the JUUL Labs Inc. nonclinical toxicology program included *in vitro* cytotoxicity (Neutral Red Uptake assay [NRU]) evaluation of product- specific e-liquids and aerosol condensates*. Results from these *in vitro* studies are compared to those from the 3R4F Kentucky reference combustible cigarette.

The NRU assay was used to assess the *in vitro* cytotoxicity in accordance with OECD TG129 (OECD 2010), using two cell lines: the murine fibroblast BALB/c 3T3 cell line and the human lung adenocarcinoma A549 cell line. The JUUL System ENDS condensates were characterized for primary constituents (propylene glycol, vegetable glycerin, menthol, nicotine, and benzoic acid), whereas 3R4F condensates were characterized for nicotine, select volatile organic compounds (VOCs), and carbonyls. Analysis of primary constituents, VOCs, and carbonyls over a period of eight weeks after collection indicated that the condensates were stable for the duration of the *in vitro* toxicity testing performed*.

* Please also see the poster by Yao J. et al., titled "Evaluation of Commercial JUUL Product E-Liquid and Aerosol Condensate Using AMES Methodology"

Methods

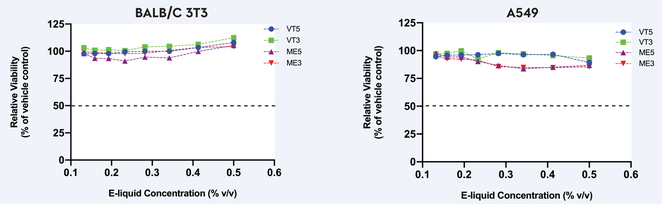
In vitro toxicological studies were conducted on three types of samples: 1. The e-liquid from JUULpods ENDS; 2. The condensate of the aerosol produced by the JUUL System ENDS; 3. The condensate of mainstream smoke (hereafter referred to as smoke) from the 3R4F reference cigarette. The four JUUL ENDS products tested were Virginia Tobacco (5.0%, 3.0%) and Menthol (5.0%, 3.0%). The ENDS e-liquid samples were obtained by partially disassembling pods and collecting the fluid by centrifugation. The condensate samples were prepared by capturing aerosols or smoke through a filter pad in series with an ethanol containing impinger maintained in an ice bath. The aerosol or smoke captured on the filter pad was then extracted in the ethanol from the impinger, thus creating a liquid solution of aerosol or smoke and is referred to as condensate. Two types of condensates were prepared for each JUUL System product tested: one using a "non-intense" puffing regimen based on ISO 20768 and consisting of a 55 mL puff volume over 3 seconds with a 30 second interval between puffs, and the other using an "intense" puffing regimen which is defined by the longest puff duration possible given the design of the JUUL Device and consisting of 110 mL puff volume over 6 seconds with a 30 second interval between puffs. The 3R4F reference cigarette condensate had a single condensate prepared, using the the ISO 20778:2018 puffing regimen, which consists of a 55 mL puff volume over 2 seconds with a 30 second interval between puffs.

The NRU *in vitro* cytotoxicity assay is based on the ability of viable cells to incorporate and bind neutral red (NR), a supravital dye. Toxic substances can alter the cell or lysosomal membranes to cause lysosomal fragility that can decrease the amount of NR retained by the culture. Cytotoxicity is expressed as a concentration-dependent reduction of the uptake of NR after chemical exposure. Two mammalian cell types were used for the evaluation, the murine fibroblast BALB/c 3T3 cell line, and the human lung adenocarcinoma A549 cell line. The human A549 cell was included because the lung is the main site of exposure to cigarette smoke and ENDS aerosol. In addition, A549 may provide metabolic activation of inhaled compounds relevant to the human lung. Dimethylsulfoxide and ethanol were used as vehicle controls for e-liquids and condensates, respectively.

Results

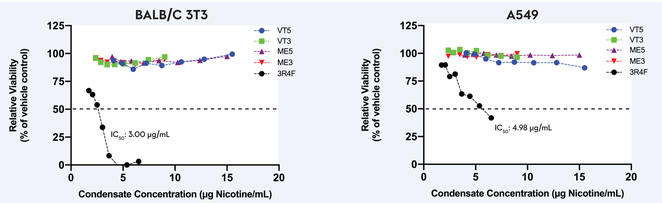
E-liquid

Fig.1 Comparison of Relative Viability Between the JUUL System E-Liquids in the NRU Assay Using BALB/c 3T3 Cells and A549 Cells



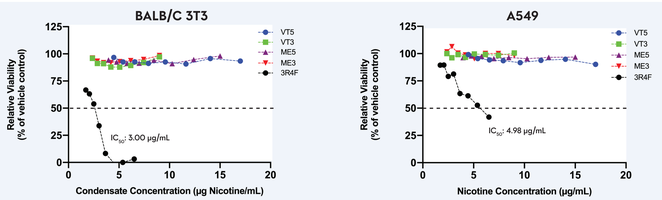
Non-Intense Condensate

Fig.2 Comparison of Relative Viability Between the Non-Intense JUUL System Condensates and Intense 3R4F Smoke Condensate in the NRU Assay Using BALB/c 3T3 Cells and A549 Cells



Intense Condensate

Fig.3 Comparison of Relative Viability Between the Intense JUUL System Condensates and Intense 3R4F Smoke Condensate in the NRU Assay Using BALB/c 3T3 Cells and A549 Cells



ME3= Menthol 3.0%; ME5=Menthol 5.0%; VT3=Virginia Tobacco 3.0%; VT5=Virginia Tobacco 5.0%.
Note: Relative viability of less than 70% of vehicle control indicates cytotoxicity (ISO 10993-5:2009, Biological Evaluation of Medical Devices: Tests for *in vitro* cytotoxicity).

Discussion

None of the JUUL System e-liquids caused cytotoxicity (defined as a viability < 70% relative to vehicle control; ISO 10993-5:2009 (ISO 2009) up to the highest tested concentration (0.5% v/v in either the BALB/c 3T3 or A549 cells (**Figure 1**).

None of the JUUL System ENDS aerosol condensates demonstrated cytotoxicity up to the highest concentration (17 µg nicotine/mL) tested in either of the cell lines. In contrast, the 3R4F smoke condensate caused a dose-dependent increase in cytotoxicity (a decrease in viability) in both BALB/c 3T3 and A549 cell lines (IC_{50}s: 3T3: 3.00 ± 0.14 µg/mL nicotine, A549: 4.98 ±1.57 µg/mL nicotine). The effects from the 3R4F condensate exposure were observed at lower nicotine-normalized concentrations compared to the JUUL System ENDs non-intense and intense condensates (**Figures 2 and 3**).

These results support the conclusion that the JUUL System is less cytotoxic than combustible cigarettes.

References

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