

NEUTRAL RED UPTAKE (NRU) CYTOTOXICITY ANALYSIS OF AEROSOL GENERATED FROM A TEMPERATURE-REGULATED NICOTINE-SALT BASED ENDS PRODUCT

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ABSTRACT #53

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JUUL ENDS is a temperature-regulated product which was designed to minimize the generation of combustion related degradation byproducts in the aerosol. The overall purpose of this study was to test the cytotoxicity of the CHO-K1 cells in response to 24h exposure to various concentrations of ENDS aerosol using the NRU in vitro cytotoxicity assay using CHO-K1 cells according to OECD guidelines (TG 129).

Methods: CHO-K1 cells were treated with ENDS aerosol, positive control, or vehicle control. JUUL ENDS aerosol was tested for three different flavor profiles. The cells were exposed to the different test articles; ENDS aerosol at the concentration of (0- 300 µg/mL) and compared with the mainstream smoke component (WTPM) from 3R4F, at dose range of (0-150 µg/mL). Additionally, the cells were exposed to the positive control, SLS at concentrations 40 and 80 µg/mL.

Results: Amongst all of the different samples tested, no aerosol mediated significant toxicity was observed at any of the concentrations tested for the test articles, Classic Menthol, Cool Mint, Cool Cucumber, as well as for Carrier Control without flavor.

At the doses tested, EC50 for the e-cig aerosol and carrier control aerosol could not be calculated because cell viability was greater than 70% at all concentrations tested. Additionally, WTPM from the 3R4F cigarette which showed the expected toxicity with a calculated EC50 of 59.46 µg/mL with r2 = 0.98.

In summary, under the experimental conditions and based on the criteria for Evaluation of Cytotoxic Response (ISO 10993-5), the test articles Classic Menthol, Cool Mint, Cool Cucumber, as well as for Carrier Control are considered non-cytotoxic.

MATERIALS & METHODS

TEST ARTICLE

- Tobacco burning cigarettes (3R4F, Kentucky Reference Cigarette) conditioned (60% relative humidity, ~23°C) at least 18 hours prior to smoking.
- Electronic Cigarettes (E-cig): JUUL E-cigarette. Batteries fully charged prior to vaping.
- JUUL Flavors: Classic Menthol, Cool Mint and Cool Cucumber at Nicotine 59 mg/mL (5% by weight) and Sample Control without any flavoring.

VEHICLE CONTROL SAMPLES

- Tobacco cigarette: Dimethylsulfoxide (DMSO)
- e-Cigarette: Ethanol (USP)

POSITIVE CONTROL

Sodium Lauryl Sulfate (SLS) [CAS No. 151-21-3]. The SLS stock solution was freshly prepared in water at 5 mg/mL, and diluted as appropriate to give the concentrations used in the assay.

SAMPLE PREPARATION

- Tobacco cigarette smoked under Canadian Intense (CI) puff profile: 55 mL puff volume, 2 sec draw, 30 sec puff interval, 100% blocked air dilution.
- E-cigs vaped with puffing profile: 70 mL Puff volume, 3 second Puff duration, 30 second Puff frequency, square-wave Puff profile, no Vent blocking.
- E-Cigarette Aerosol was collected on a Cambridge filter pad followed in series by an impinger with USP ethanol. Cambridge filter pad collected aerosol and impinger content was extracted together to a final concentration of approximately 60 mg e-Cig aerosol/mL.
- 3R4F Cigarette smoke WTPM (Wet Total Particulate Matter) collected on Cambridge filter pad, extracted in DMSO to a final concentration of 30 mg/mL.

CELLULAR EXPOSURE

- E-cigarette aerosol: Dose range of 0-300 µg/mL
- 3R4F mainstream WTPM: Dose range of 0-150 µg/mL.
- Positive Control (SLS): At two concentrations 40 and 80 µg/mL.

OSMOLARITY AND pH TESTING

Osmolarity and pH were determined for each test article prior to use in the NRU assay. Osmolarity and pH were also measured for the tissue culture media, tissue culture media + vehicle control, and tissue culture media + test sample. If osmolarity and pH changes were noticed (e.g., change in osmolarity by 20% or pH by 1 unit from the media + vehicle control) during the analysis, and there is concurrent cytotoxicity (> 70%) associated from that dose; the test article will not be considered cytotoxic.

NEUTRAL RED UPTAKE (NRU) ASSAY

- The cytotoxicity as measured by neutral red dye uptake method was determined according to OECD guideline (TG 129).
- Chinese Hamster Ovarian Kidney (CHO-K1, ATCC 061417C4) cells grown (complete media: F-12K + 10% FBS) in 96-well plates overnight (37°C, 5% CO2) prior to exposures.
- The cellular exposure concentrations of ethanol and DMSO in the culture medium for the samples was 0.5% v/v for e-cigarette aerosol and 3R4F smoke samples, respectively.
- Cells exposed to increasing doses of samples and incubated for 20 – 24 hrs @ 37°C, 5% CO2 followed by NRU analyses.
- NRU assay was performed by established method.

DATA PRESENTATION

The results are presented as the average % cell viability and SD of the test articles. Figures are also included with bar graphs representing the cytotoxicity of each E-cig aerosol product in comparison to the lab reference control 3R4F and carrier control. Tables for the Osmolarity and pH data are included for the top two doses tested for the E-cig aerosol.

CRITERIA AND INTERPRETATION OF RESULTS

- EC50 (NRU) values were calculated and compared using GraphPad Prism v. 5.04 (two tailed; for comparisons, statistical significance @ p < 0.05).
- Criteria for Valid NRU Assay was followed to OECD guidelines.
- Evaluation of Cytotoxic Response was followed to ISO 10993-5 standard.

Figure 1: Comparison Of Aerosol Mediated Cytotoxicity Of Different e-Liquid Flavors And Mainstream Smoke Component (WTPM) From 3R4F In CHO-K1 Cells

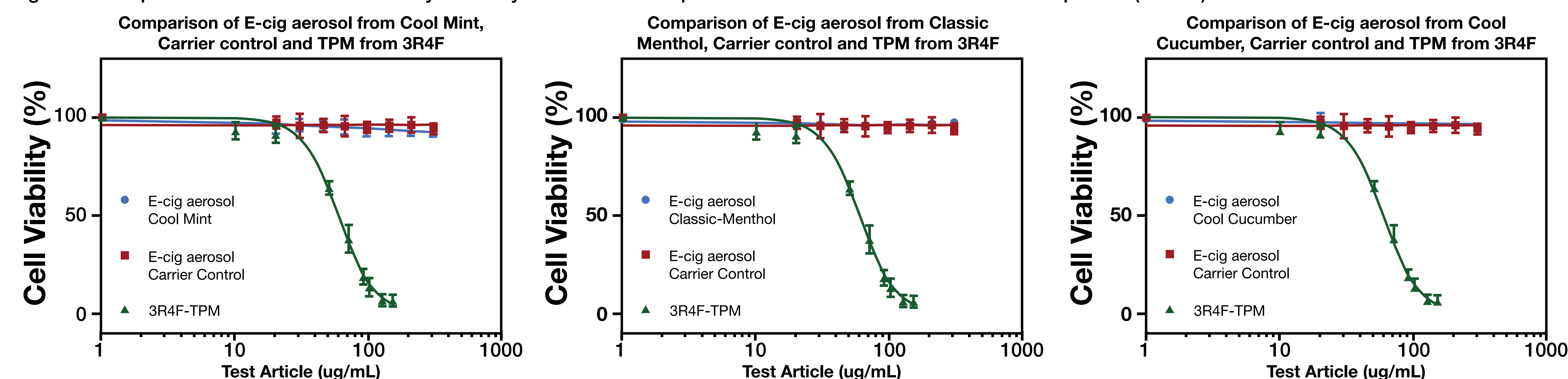


Figure 2 Aerosol Mediated Relative Cellular Viability compared to vehicle control:

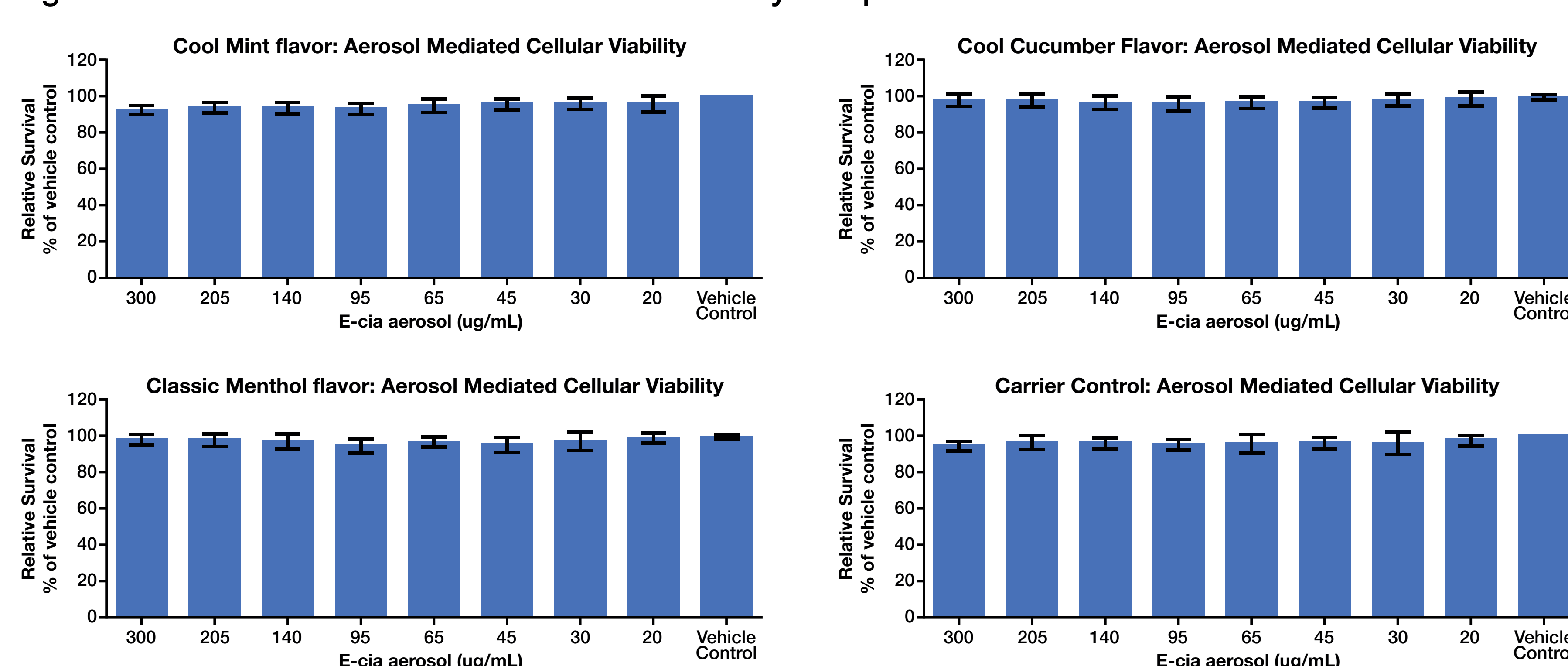


Table 1: Osmolarity and pH of the e-Liquid doses.

	Doses (µg/ml)	Osmolarity (mOsm/L)			pH		
		Mean (n=3)	SD	% RSD	Mean	SD	% RSD
Media with 5 % serum	NA	348.5	10.55	3.03	7.63	0.24	3.12
Vehicle Control (0.5 % DMSO)	NA	432.61	12.45	2.88	7.64	0.24	3.17
Cool Mint	300.0	436.44	5.06	1.16	7.5	0.09	1.19
	205.0	432.78	1.07	0.25	7.49	0.07	0.93
Classic Menthol	300.0	427.56	6.27	1.47	7.71	0.32	4.16
	205.0	425	19.1	4.49	7.73	0.31	4.07
Cool cucumber	300.0	425.22	4.74	1.12	7.73	0.31	3.96
	205.0	426.56	12.74	2.99	7.72	0.31	3.97
Carrier Control	300.0	431.44	2.5	0.58	7.5	0.1	1.27
	205	430.89	2.99	0.69	7.5	0.09	1.2

SUMMARY

- This study present results of Neutral Red Uptake (NRU) in vitro cytotoxicity assay using Chinese Hamster Ovarian Kidney (CHO-K1) cells to determine the cytotoxicity of E-Cigarette Aerosol and compared with 3R4F tobacco cigarette smoke aerosol.
- The cells were exposed to the different test articles; E-cigarette aerosol at the concentration of (0-300 µg/mL) and compared with the mainstream smoke component (WTPM) from 3R4F, at dose range of (0-150 µg/mL).
- No significant aerosol toxicity was observed at any of the concentrations tested for all E-Cig, Classic Menthol, Cool Mint, Cool Cucumber, as well as for Carrier Control.
- At the doses tested, EC50 for the e-cig aerosol and carrier control aerosol could not be calculated because cell viability was greater than 70% at all concentrations tested.
- At the doses tested for 3R4F cigarette smoke aerosol WTPM, significant toxicity was observed with a calculated EC50 of 59.46 µg/mL with r2 = 0.98.
- Thus, based on the criteria for Evaluation of Cytotoxic Response (ISO 10993-5), the aerosol from test articles Classic Menthol, Cool Mint, Cool Cucumber, and Carrier Control are considered non-cytotoxic.

Table 2: Aerosol Mediated Relative Cellular Viability compared to vehicle control.

Treatment	Number of Replicates	Concentration (µg/ml)	Average % Cell Viability	Standard Deviation
Cool Mint	3	300	92.9	2.4
		205	94	3.1
		140	93.8	3.1
		95	93.6	3
		65	95.2	3.8
		45	96	3
		30	96.2	3.1
		20	96.1	4.4
		Vehicle Control		
Classic Menthol	3	300	97.8	3
		205	97.4	3.6
		140	96.7	4.3
		95	94.3	4
		65	96.5	2.8
		45	94.9	4.3
		30	96.9	5.2
		20	98.7	2.8
		Vehicle Control		
Cool cucumber	3	300	97.5	3.4
		205	97.6	3.7
		140	96.1	3.6
		95	95.4	4.2
		65	96.3	3.1
		45	96.3	2.7
		30	97.7	3.2
		20	98.4	3.9
		Vehicle Control		
Carrier Control	3	300	94.3	2.7
		205	96.2	3.9
		140	95.8	3.1
		95	95	2.8
		65	95.6	5.2
		45	96	3.3
		30	95.8	6.1
		20	97.5	3
		Vehicle Control		
3R4F (WTPM)	3	150	6.1	3.1
		125	6.9	2.9
		100	13.9	4.3
		90	19.3	3.9
		70	39	7
		50	64.7	3.6
		20	91.6	4
		10	93.6	4.2
		Vehicle Control		

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