Juul Labs Science

In Vivo Genotoxicity Testing of Aerosols Generated from Commercial JUUL ENDS Products

Guy Lalonde[1], Sridhar Jaligama[2], Amit Gupta[2], Monica Lee[3], Utkarsh Doshi[3], Jenny Yao[1], Jingjie Zhang[3]

[1] JUUL Labs Inc., San Francisco, CA, USA

[2] Battelle Memorial Institute, West Jefferson, OH, USA

[3] Altria Client Services LLC, Richmond, VA, USA, a Service Provider to Juul Labs, Inc.

Background and Rationale

- JUUL ENDS products J1 and J2 were found to be positive and equivocal, respectively, for the induction of micronucleated cells in the *in vitro* micronucleus (MN) assay using the TK6 cell line.
- The ICHS2(R1) recommends an *in vivo* genotoxicity follow-up to determine the biological significance of *in vitro* genotoxicity signals:

  "...recommended follow-up for positive mammalian cell assays would be to provide experimental evidence... by carrying out two appropriate in vivo assays... usually with different tissues, and with supporting demonstration of exposure."

Outline of In Vivo Genotoxicity Study

- Aerosols generated from J1 and J2 were administered to male Sprague Dawley (SD) rats by nose-only inhalation for 4 consecutive days and up to 6 hours/day.
  - Negative control group received filtered air for the same exposure duration
  - Positive control group received ethyl methane sulfonate and cyclophosphamide
- J1 and J2 aerosols were characterized for particle size, primary constituents, carbonyls and glycidol.
- J1 and J2 aerosol exposure was confirmed post-exposure by measuring plasma nicotine, cotinine and PG concentrations.
- Following the last exposure, SD rats were sacrificed and samples were collected per OECD TG 474 and 489.
  - Bone marrow was harvested for MN enumeration.
  - Nasal, lung and liver tissues were harvested for Comet assays.

Experimental Design

Group Number	Group Description	Nicotine Concentration in E-liquid (%w/w)	Target Exposure Duration[a] (hr)	Total Particulate Mass Target Exposure Concentration (µg/L)	Number of Male Rats
1	Filtered Air	Not Applicable	6	0	5
2	J1-Low	3	1.5	4000	5
3	J1-Mid	3	3	4000	5
4	J1-High	3	6	4000	5
5	J2-Low	5	1.5	3000	5
6	J2-Mid	5	3	3000	5
7	J2-High	5	6	3000	5
8	Positive Control	Not Applicable (NA)	NA[b]	NA[b]	5

[a] On Day 1, nose-only inhalation animals (Groups 1 through 7) were exposed for half the target duration for acclimation, followed by 3 days of exposure at the target duration.
[b] Ethyl methane sulfonate was given by oral gavage at 200mg/kg on Day 4. Cyclophosphamide was given via oral gavage at a dose of 10 mg/kg/day on Days 2 & 3.

Aerosol Characterization

Test Article [Group #(s)]		Total Particulate Mass (TPM) Exposure Concentrations				Temporal Stability	Nicotine Conc.	Propylene Glycol (PG) Conc.	PSD[c]
		Target (µg/L)	Measured Mean[a] (µg/L)	% of Target[a]	N[a]	Mean %RSD[b]	Mean ± SD (ug/L)	Mean ± SD (ug/L)	Mean MMAD[c] (µm) [GSD[c]]
Filtered Air	[1]	0	0	-	8	-	BLOQ[c]	NP[c]	NA
J1	[2-4]	4000	4066	102	12	20.0	116.8 ± 13	1117 ± 111	1.0 [1.6]
J2	[5-7]	3000	3129	104	12	27.8	121.8 ± 7.2	646 ± 68	0.9 [1.6]

[a] Based on daily mean concentrations, N values are total number of data points collected
[b] Calculated as a mean of daily %RSDs
[c] PSD = Particle size distribution; MMAD = Mass Median Aerodynamic Diameter; GSD = Geometric Standard Deviation; NA = Not Applicable; BLOQ = Below Limit of Quantitation; NP = Not Performed

Systemic Exposure: Plasma Concentrations of Nicotine, Cotinine and Propylene Glycol (PG)

Group	Target Exposure Concentration (µg/L)	Target Exposure Duration[a] (hr)	Nicotine (ng/mL) Mean ± SD	Cotinine (ng/mL) Mean ± SD	PG (ng/mL) Mean ± SD
Filtered Air	0	6	BLOQ[b]	BLOQ	BLOQ
J1-Low	4000	1.5	625 ± 62.6	655 ± 68.1	20400 ± 1700
J1-Mid	4000	3	776 ± 99.2	1210 ± 104	24600 ± 3480
J1-High	4000	6	1260 ± 265	2800 ± 493	48000 ± 8050
J2-Low	3000	1.5	822 ± 110	857 ± 105	12100 ± 1340
J2-Mid	3000	3	1090 ± 107	1720 ± 91.3	17400 ± 1530
J2-High	3000	6	1240 ± 158	2860 ± 336	22100 ± 3090

[a] On Day 1, exposure was for half the target duration for acclimation, followed by 3 days of exposure at the target duration.
[b] BLOQ = Below Limit of Quantitation

Comet Assay Procedure

- Four hours after end of dosing, 5 rats/group were euthanized with CO_2/O_2, exsanguinated and nasal, lung and liver tissues harvested.
- Tissues were minced (in cold HBSS with EDTA and DMSO) to release cells and sieved through a cell strainer.
- Cell suspensions in agarose were deposited on slides and solidified. Slides were treated in succession for cell lysis, DNA unwinding, electrophoresis, neutralization, drying and staining (Sybr-gold).
- % Tail DNA, defined as the percentage of DNA fragments present in the tail, was measured with an automated scoring system (Perceptive Instruments Ltd., UK) in a total of 150 cells/rat.

Comet Assay Results: No Evidence of DNA Damage in J1 and J2 Aerosol Treated Animals

Group	Lung (% Tail DNA)	Liver (% Tail DNA)	Nasal (% Tail DNA)
Filtered Air	0.64 ± 0.25[a]	1.59 ± 1.08	0.92 ± 0.24
J1 (6 hr/day)	0.81 ± 0.22	2.24 ± 2.39	1.17 ± 0.50
J2 (6 hr/day)	0.62 ± 0.23	1.07 ± 0.43	0.67 ± 0.21
Positive Control[b]	38.74 ± 6.53*	41.53 ± 7.05*	39.76 ± 8.16*

*Significantly different from air control ($p < 0.05$).
[a] Mean ± SD, n = 5.
[b] EMS (200 mg/kg/day on Days 3 and 4)

Micronucleus Assay Procedure

- Four hours after end of dosing, 5 rats/group were euthanized with CO_2/O_2 and femoral bone marrow was harvested and smear slides prepared.
- Slides were stained with Acridine Orange for 1-2 minutes and rinsed in PBS. Slides were scored manually.
- 4000 polychromatic erythrocytes (PCEs)/animal were scored for the presence of micronuclei (MnPCEs). In addition, at least 500 total erythrocytes were scored per animal to determine the proportion of PCEs as an index of bone marrow cytotoxicity. PCE proportions <20% of the Filtered Air Control value were considered excessively cytotoxic, and the animal data were excluded from evaluation. (OECD 474, 2016)

Micronucleus Assay Results: No Evidence of Increased Micronucleation in J1 and J2 Aerosol Treated Animals

Group	% PCE[c]	% MNPCE[c]
Filtered Air	53.6 ± 1.9[a]	0.08 ± 0.05
J1 (6 hr/day)	54.4 ± 3.6	0.08 ± 0.06
J2 (6 hr/day)	55.8 ± 4.6	0.08 ± 0.02
Positive Control[b]	52.7 ± 1.7	2.17 ± 0.47*

* Significantly different from air control ($p < 0.05$).
[a] Mean ± SD, n = 5.
[b] Cyclophosphamide (10 mg/kg/day on Days 2 and 3).
[c] PCE = polychromatic erythrocytes, MNPCE = Micronucleated polychromatic erythrocytes

Summary and Conclusion

- The inhalation exposure data demonstrated that test article exposure concentrations observed at the nose port were concordant with targeted total particulate concentrations for both J1 and J2 exposure systems.
- Adequate systemic exposure was demonstrated with measured plasma nicotine, cotinine, and PG concentrations.
- Data from the micronucleus and Comet assays indicated that under the conditions of the study, there was no increase in the induction of micronuclei formation in the bone marrow and there was no induction of DNA damage in nasal, lung, and liver tissues when rats were exposed to either J1 or J2 test article atmosphere.
- **In conclusion, under the testing conditions of the assay, the ENDS products J1 and J2 did not cause genotoxicity *in vivo*.**

Contact email: guy.lalonde@juul.com