

RESOLUTION OF MICRONUCLEUS INDUCTION GENOTOXICITY IN ENDS: IN VITRO AND IN VIVO TESTING

Manoj Misra¹, Ed Carmines¹, Mariano Scian², David L. McCormick³

Chemular Inc¹, Hudson, MI USA, Enthelphy Analytical², Richmond, VA, USA and IIT Research Institute³ (IITRI), Chicago, IL USA

TSRC Poster #61

ABSTRACT

One of the regulatory toxicology assays used to assess the genotoxic potential of ENDS products is the in vitro micronucleus (ivtMN) test. The hazard assessment of aerosol generated from various E-Liquids was assessed by in vitro genotoxicity testing. None of the test articles were mutagenic in Ames assay. The ivtMN assay was conducted according to OECD guidelines Test No. 487 in human lymphoblast TK6 cells. Three E-Liquid test articles induced a weak but statistically significant increase in micronuclei, suggesting positive or equivocal result. According to ICH guidance S2 (R1), to determine the biological relevance of ivtMN results, a follow-up in vivo micronucleus (ivoMN) test should be conducted. Following OECD Guidelines Test No. 474 male CrI:CD(SD) rats were exposed nose-only to a Limit dose of 5 mg/L aerosol for 4 hours per day for three consecutive days. Negative and positive control groups were included. The aerosol chemistry confirmed that the aerosol mass concentration and the particle size distribution data were within the respirable size range for rats. There was no evidence of bone marrow cytotoxicity for the test article-treated groups. There were no statistically significant increases in micronucleated polychromatic erythrocytes for any of the test article-treated groups compared to control group. Therefore, under the tested in vivo condition, the tested E-Liquids were negative for genotoxicity, implying no biological relevance of the weak in vitro genotoxicity results. In summary, the absence of mutagenicity in Ames test and negative for genotoxicity in the in vivo MN assays demonstrated absence of significant genotoxic risk for E-Liquids.

OBJECTIVE

Access the potential genotoxicity of an aerosol generated from the Liquid Labs 6 mg/mL nicotine E-Liquids.

METHODS

TEST ARTICLES: Keep It 100 brand E-Liquids Bacco 6 mg/mL Nicotine, OG Blue 6 mg/mL Nicotine, OG Tropical Blue 6 mg/mL Nicotine manufactured by Liquid Labs LLC.

GENERATION DEVICE: The aerosol was generated with an Aspire Nautilus Clearomizer (Aspire BVC General Coils 1.6 ohm). The air vent setting on the air control ring of the tank was set to 1.1 mm.

PUFFING PROFILE: CORESTA CRM NO. 81 (Puff duration: 3 seconds, Puff frequency: 30 seconds, Puff profile: Square-wave, Vent blocking: None).

IN VITRO MICRONUCLEUS ASSAY (ivtMN)

SAMPLE PREPARATION: E-vapor condensate was collected on a pre-weighed 55 mm Cambridge filter pad followed in series by an impinger filled with 20 mL of USP ethanol. The ethanol from the impinger was used to extract the pad to produce the e-vapor condensate solution. An appropriate number of devices was vaped per sample to generate an adequate amount of e-vapor condensate concentration (60-80 mg/mL).

CELL LINE: Human lymphoblast TK6 cells (TK6, ATCC® CRL-8015™).

METHOD: OECD Guideline Test No.487 (In vitro Mammalian Cell Micronucleus Test)

EXPOSURE: The ivtMN assay of the E-Liquids were assayed using TK6 cells both in the presence and absence of metabolic activation. Three treatment schedules were performed; Short-term (4 hours) without (S9-) metabolic activation; Short-term (4 hours) with (S9+) metabolic activation; and Long-term (27 hours) without (S9-) metabolic activation. Ethanol condensate was prepared at the concentration of 100 mg Aerosol Collected Mass (ACM)/mL of ethanol for each E-Liquid and five concentrations of ACM were tested (0, 125, 250, 500, 750, and 1000 µg ACM/plate). Cytotoxicity was evaluated using the method of Rapid Population Doubling (RPD). The highest concentration should aim to achieve 55±5% cytotoxicity using RPD. Only dose levels with less than 60% cytotoxicity by RPD was scored for micronuclei.

IN VIVO MICRONUCLEUS ASSAY IN MALE RATS

The objective of this study was to determine potential clastogenic effects in the bone marrow of male rats receiving nose-only inhalation exposure to test articles (Bacco 6 mg/mL, OG Blue 6 mg/mL, or OG Tropical Blue 6 mg/mL) at the limit dose of 5 mg/L for three consecutive days (4 hours per day).

METHOD: OECD Guidelines Test No. 474 (Mammalian Erythrocyte Micronucleus Test).

LABORATORY ANIMAL: CD [CrI:CD(SD)] male rats

EXPERIMENTAL DESIGN

Group	Number of Male Rats	Dose Group/ Treatment	Number of Exposure Days	Target Test Atmosphere Concentration (mg/L)	Target Exposure Duration (Minutes)	Group
1	6	Negative Control	3	0 (Filtered Air)	240	1
2	6	Bacco 6 mg/mL	3	5	240	2
3	6	OG Blue 6 mg/mL	3	5	240	3
4	6	OG Tropical Blue 6 mg/mL	3	5	240	4
5	6	Positive Control	1	Cyclophosphamide (30 mg/kg, i.p.)		5

TEST ATMOSPHERE GENERATION

Test atmospheres were generated in the nose-only inhalation exposure chambers (Lab Products, Seaford, DE) located inside acrylic enclosures to isolate the exposure chamber and protect laboratory personnel. Aspire Nautilus devices were used for test atmosphere generation. Each of the devices consisted of either a Nautilus 2S or a Nautilus Mini tank with a 1.6 Ω Nautilus replacement atomizer. Each exposure generation system used six Aspire Nautilus devices for test atmosphere generation, with a timing system set to have each device produce 2 puffs per minute, with a puff duration of 5 seconds.

TEST ATMOSPHERE MONITORING

Aerosol Mass Concentration: During each day of exposure, total particulate matter (TPM) concentration was determined in each chamber using a gravimetric filter- collection method.

Aerosol Particle Size: The aerosol particle size distribution in each chamber used for test article exposures was determined on the second day of exposure using a 10-stage quartz crystal microbalance (QCM) cascade impactor (California Measurements, Sierra Madre, CA). The Mass Median Aerodynamic Diameter (MMAD) and Geometric Standard Deviation (GSD) of the test aerosol were calculated from the mass accumulated on each stage of the QCM.

MICRONUCLEUS ASSAY : The MN was determined in the bone marrow of femur bone. The bone marrow was flushed with calf serum and the suspension containing the marrow cells was then centrifuged. The pellets were re-suspended in serum, and thin wedge smears were made on clean microscope slides. The slides were then fixed in absolute methanol for five minutes and air dried before being stained with acridine orange according to the method described by Hayashi et al. (1983). To determine whether the test substance was cytotoxic to bone marrow cells, 1000 erythrocytes per slide (TE; total erythrocytes) were examined to determine the proportion of polychromatic erythrocytes (PCE) relative to TE. At least 4000 PCE per slide were screened for the presence of micronuclei using a fluorescent microscope. The frequencies of micronucleated polychromatic erythrocytes (MN-PCEs) were determined from the counts

STATISTICAL ANALYSIS : Raw counts of MN-PCE were transformed by adding 1 to each count and taking the log of the adjusted number. Transformed MN-PCE data from the treated groups were compared to the negative control group using a t-test. Probability values of p < 0.05 were considered significant, with the criterion for a positive response being a statistically significant elevation of the percentage of MN-PCEs in the treated group. Statistical comparisons were performed using SigmaPlot software (version 13.0; Systat Software, Inc.; San Jose, CA).

IN VITRO MICRONUCLEUS

BACCO 6MG (Short-term)

Short-term	Metabolic Activation	Cytotoxicity	MN count Average	Fold Increase	t-test	Chi² test for trend
VC (1%)		51	4.5	1.0	N/A	N/A
50 ug/ml	-S9	6.6	5.0	1.1	ns	ns
125 ug/ml		18	5.5	1.2	ns	
200 ug/ml		23	6.0	1.3	ns	
300 ug/ml		31	9.5	2.1	ns	
600 ug/ml		36	11.0	2.3	0.0127	
VC (1%)		6.4	4.5	1.0	N/A	N/A
50 ug/ml	+S9	6.8	5.5	1.2	ns	ns
125 ug/ml		13	5.5	1.2	ns	
200 ug/ml		15	7.0	1.6	ns	
300 ug/ml		34	9.0	2.0	ns	
600 ug/ml		43	11.0	2.4	0.0338	

BACCO 6MG (Long-term)

Long-term	Metabolic Activation	Cytotoxicity	MN count Average	Fold Increase	t-test	Chi² test for trend
VC (1%)		9	5.5	1.0	N/A	N/A
20 ug/ml	-S9	16	6.5	1.2	ns	ns
40 ug/ml		23	7.5	1.4	ns	
80 ug/ml		36	8.0	1.5	ns	
160 ug/ml		34	10.0	1.8	ns	
200 ug/ml		43	11.0	2.0	0.0127	

OG BLUE 6MG (Short-term)

Short-term	Metabolic Activation	Cytotoxicity	MN count Average	Fold Increase	t-test	Chi² test for trend
VC (1%)		4.7	4.0	1.0	N/A	N/A
50 ug/ml	-S9	3.38	5.0	1.2	ns	0.0410
125 ug/ml		17.7	5.5	1.4	ns	
200 ug/ml		21.5	6.0	1.5	ns	
300 ug/ml		31.1	7.5	1.9	ns	
600 ug/ml		36	10.5	2.6	ns	
VC (1%)		-1.7	5.0	1.0	N/A	N/A
50 ug/ml	+S9	6.47	6.0	1.2	ns	ns
125 ug/ml		26.7	7.5	1.5	ns	
200 ug/ml		27.7	7.5	1.5	ns	
300 ug/ml		35.2	10.0	2.0	ns	
600 ug/ml		37.5	9.5	1.9	ns	

OG BLUE 6MG (Long-term)

Long-term	Metabolic Activation	Cytotoxicity	MN count Average	Fold Increase	t-test	Chi² test for trend
VC (1%)		4.7	5.5	1.0	N/A	N/A
20 ug/ml	-S9	7.2	6.5	1.2	ns	0.0183
40 ug/ml		18.8	8.0	1.5	ns	
80 ug/ml		31.4	12.0	2.2	ns	
160 ug/ml		41.3	15.0	2.7	ns	
200 ug/ml		52.4*	N/A	N/A	N/A	

OG TROPICAL BLUE 6MG (Short-term)

Short-term	Metabolic Activation	Cytotoxicity	MN count Average	Fold Increase	t-test	Chi² test for trend
VC (1%)		3.0	4.0	1.0	N/A	N/A
50 ug/ml	-S9	4.6	5.0	1.2	ns	0.0223
125 ug/ml		-2.8	5.5	1.4	ns	
200 ug/ml		-5.0	6.5	1.6	ns	
300 ug/ml		4.5	10.0	2.5	ns	
600 ug/ml		6.8	11.0	2.6	ns	
VC (1%)		-4.1	3.5	1.0	N/A	N/A
50 ug/ml	+S9	3.8	6.0	1.7	ns	ns
125 ug/ml		7.2	6.5	1.9	ns	
200 ug/ml		7.0	7.0	2.0	ns	
300 ug/ml		10.6	8.5	2.4	ns	
600 ug/ml		8.5	8.0	2.3	ns	

OG TROPICAL BLUE 6MG (Long-term)

Long-term	Metabolic Activation	Cytotoxicity	MN count Average	Fold Increase	t-test	Chi² test for trend
VC (1%)		9.7	5.0	1	N/A	N/A
20 ug/ml	-S9	-12	4.5	0.9	ns	ns
40 ug/ml		-13	6.0	1.2	ns	
80 ug/ml		-9	6.0	1.2	ns	
160 ug/ml		-2	6.5	1.3	ns	
200 ug/ml		-9	8.0	1.6	ns	

IN VIVO TEST ATMOSPHERE

Summary of Aerosol Mass Concentration and Particle Size Distribution

Group	Dose Group/Treatment	Target Aerosol Concentration (mg/L)	Aerosol TPM Concentration (mg/L) (Avg ± SD) ^a	MMAD (µm) ^b	GSD ^b
1	Negative Control (Filtered Air)	0	0.00 ± 0.004	----	----
2	Bacco 6 mg/mL	5	5.40 ± 0.313	1.37	1.34
3	OG Blue 6 mg/mL	5	5.32 ± 0.250	1.21	1.53
4	OG Tropical Blue 6 mg/mL	5	5.35 ± 0.250	1.13	1.64
a N = 24 (8 per exposure day); bN = 1					

PLASMA NICOTINE LEVEL

Summary of Nicotine Levels in Plasma

Group	Dose Group/ Treatment	Concentration (ng/mL) (Avg ± SD)
1	Negative Control (Filtered Air)	BQL --
2	Bacco 6 mg/mL	358 ± 38.8
3	OG Blue 6 mg/mL	459 ± 54.3
4	OG Tropical Blue 6 mg/mL	349 ± 52.5
a N = 6		
b BQL = below quantifiable limits (5 ng/mL)		

IN VIVO MICRONUCLEUS

Summary of Bone Marrow Micronucleated Polychromatic Erythrocyte Data					
Group	Dose Group/ Treatment	Number of Male Rats	%PCE/TE ± SD (Avg ± SD)	MN-PCE /4000 PCE (Avg ± SD)	% MN-PCE ± SD (Avg ± SD)
1	Negative Control (Filtered Air)	6	91.6 ± 2.01	8.8 ± 2.64	0.22 ± 0.068
2	Bacco 6 mg/mL	6	87.3 ± 4.02	10.0 ± 5.83	0.25 ± 0.145
3	OG Blue 6 mg/mL	6	89.6 ± 2.91	10.3 ± 4.41	0.26 ± 0.111
4	OG Tropical Blue 6 mg/mL	6	89.4 ± 4.01	10.7 ± 2.80	0.27 ± 0.070
5	Positive Control ^a	6	74.6 ± 7.57	140.8 ± 33.67	3.53 ± 0.843

CONCLUSION

- ✓ In the in vitro MN assay, three Liquid Labs E-Liquid test articles induced a weak but statistically significant increase in micronuclei, suggesting positive or equivocal findings.
- ✓ In in vivo MN assay, no statistically significant increases in micronucleated polychromatic erythrocytes were observed for any of the test article-treated groups compared to negative control group. The positive control group responded as expected.
- ✓ In summary, the absence of mutagenicity in Ames test (data not shown) and negative for genotoxicity in the in vivo MN assays demonstrated absence of significant genotoxic risk for Liquid Labs E-Liquids.

REFERENCES

- OECD(2016), Test No. 487: In Vitro Mammalian Cell Micronucleus Test, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris.
- OECD(2016), Test No. 474: Mammalian Erythrocyte Micronucleus Test, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris