



CORESTA

***In Vitro* Toxicity Testing Sub-Group (IVT SG)**

2016 Annual Report

Berlin, Germany

October 13, 2016



IVT SG Membership

❖ SG Coordinator

➤ Kei Yoshino (JT)

❖ SG Secretary

➤ David Thorne (BAT)

❖ SC Liaison

➤ Paul Harp (RAI)

❖ SG Membership

➤ BAT, Battelle, CNTC, Covance, Enthalpy, ITL, JTI, JTI/Oekolab, KT&G, Labstat, PMI, RAI, Vitrocell



Objectives

- ❖ **Objective 1: To compile and review information on *in vitro* toxicity testing and apply learnings to further biological research.**
- ❖ **Objective 2: To organize and conduct periodically proficiency testing of tobacco and tobacco related products.**



Accomplishments

❖ Technical Reports

➤ **Neutral Red Uptake Assay Proficiency Study**

- Author & Trial Coordinator: Betsy Bombick, Co-author: Alexander Hauleithner
- Published on CORESTA Website in November 2015

➤ **Ames Assay Proficiency Study**

- Author: Wendy Wagstaff, Trial Coordinator: Betsy Bombick, T.S. Kumaravel
- Published on CORESTA Website in March 2016

➤ ***In Vitro* Micronucleus Proficiency Study**

- Author: Kei Yoshino, , Trial Coordinator: Betsy Bombick, Study Statistician: Wendy Wagstaff
- Published on CORESTA Website in March 2016



❖ Recent Two Meetings

➤ **March 12, 2016: New Orleans, US**

- 22 delegates (+1 guest) attended the meeting
- Meeting was hosted by Battelle Institute, and supported by RAI & JT

➤ **October 9, 2016: Berlin, Germany**

- 26 delegates (+3 guests) attended the meeting

❖ Upcoming Meetings

➤ **March 2017: Baltimore, US**

- Meeting will be hosted by ALCS

➤ **September/October 2017**

- CORESTA Joint Meeting



Proficiency Studies

❖ Objectives

- Evaluation of the proficiency of the participating laboratories
- Assessment of the discriminatory power of the test towards different tobacco products

❖ Responsibilities

- Coordinator: Elisabeth Weber (JTI/Oekolab)
- Co-Coordinator: Toshiro Fukushima (JT)
- Statistical analysis: Alexander Hauleithner (JTI/Oekolab)

❖ Basic agreement

- Test cigarettes: 3R4F, 100% FCV and BLY
- Study procedure: Follow lab's own protocol
- Lab Performance: Coefficient of Variance (Standard Deviation in percent of Mean)

❖ Assay Conditions

	LAB 1	LAB 2	LAB 3	LAB 4	LAB 5	LAB 6	LAB 7	LAB 8	LAB 9
Cell Line	V79	CHO WBL	CHO WBL (IVGT)	V79	CHL/IU	CHO-K1	TK6	V79	CHO-K1
CytB	YES	NO	NO	YES	NO	YES	NO	NO	NO
Scoring	Automated Microscope	Flow Cytometry	Manual	Manual	Automated Microscope	Manual	Manual	Automated Microscope	Manual
Staining	DAPI		Acridine Orange	Acridine Orange	DAPI - Cell Mask Orange	DAPI	Acridine Orange	DAPI	Acridine Orange

❖ Treatments

	LAB 1	LAB 2	LAB 3	LAB 4	LAB 5	LAB 6	LAB 7	LAB 8	LAB 9
SHORT -S9	X	X	X	X	X	X	X		X
SHORT +S9	X	X	X		X	X	X	X	X
LONG		X	X			X	X		

❖ Results (Draft)

- Almost no difference in "Nicotine/TPM" in TPM extracts among laboratories.
- A Generalized Linear Model (Logistic Regression) was applied to the data.
- Goodness of Fit statistics show that the model is suitable for the reported data.
- In SHORT –S9, SHORT +S9 and LONG the mutagenicity ranking of test items was mainly 100 FC > KR 3R4F > 100 BLY.
- The Coefficient of Variation (CoV) of the mutagenic rates is used to assess Lab performance. It is mainly below/around 30% for all test items and schedules.
- The median of the Minimal Detectable Difference between the slopes of two test items tested in three replicates is 60-70%.



Upcoming Proficiency Studies

Assay	Schedule	Organization	Study Contributors
NRU	2016 start	ITL, PMI, JT, JTI/Oekolab, KT&G, ZTRI, Covance, Labstat, Enthalpy, ALCS, CNTQSTC [11 labs]	Coordinator: K. Yoshino Co-coordinator: R. Wieczorek Statistical analysis: A. Hauleithner Nicotine analysis: JT
MLA	2017 start	PMI, JTI/Oekolab, ZTRI, Labstat, Covance [5 labs]	Coordinator: D. Smart (TBC) Co-coordinator: E. Weber Statistical analysis: A. Hauleithner Nicotine analysis: JT
Ames	2018 (TBC)	ITL, PMI, KT&G, JT, JTI/Oekolab, Labstat, Covance, CNTQSTC [8 labs]	Coordinator: (TBD) Co-coordinator: (TBD) Statistical analysis: A. Hauleithner Nicotine analysis: JT



Whole Smoke Exposure



Whole Smoke Exposure

❖ Publication Plan:

➤ **Journal: Toxicology In Vitro (or similar)**

➤ **Key Publication Messages:**

- The survey results emphasize the diversity of *in vitro* exposure parameters and methodologies employed across the *in vitro* Sub Group and tobacco industry.
- Pockets of harmonization already exist. For example, many of the biological protocol parameters are consistent across the Sub Group.
- However, variables such as cell type and exposure time remain largely inconsistent.
- The key next steps for this work will be to map parameter and system data against biological findings and investigate whether the observed commonalities and inconsistencies translate into biological variability.
- Analysing data will give a better understanding of how data is presented and interpreted and how data may be more accurately aligned between laboratories irrespective of the lack of harmonized protocols.
- Finally, this survey was conducted across one biological end-point, cytotoxicity. In order to understand the environment in its completeness, other biological end-points and parameters should also be assessed



Whole Smoke Exposure

Item	Status	Action	Date
Survey	Complete		2015
Poster	Complete		Oct 2016
Publication Plan	Ongoing	Agree	Oct 2016
Compile data	Ongoing	Collect remaining data	Nov 2016
Analyse/Review data	Ongoing	Compare data against parameters	Dec 2016
Present data	Ongoing	Circulate data amongst group	Dec 2016
Draft publication	Ongoing	Drafting	Jan 2017
Review publication	Ongoing	SG	Feb 2017



Review information on *in vitro* toxicity testing



Information on *in vitro* toxicity testing

❖ Two external presentations shared

- IIVS Respiratory Toxicology Program Update: Dr. Holger P. Behrsing (Institute of *in vitro* Sciences)
- Monitoring Mammalian Cells in Real-Time: Dr. Yama Abassi (ACEA)

❖ Three internal presentation shared

- US FDA deeming regulation update : Dr. Monica Lee (Altria Client Services)
- EU TPD-II testing: Dr. Jacqueline Miller Holt (JT International)
- Literature Survey: Dr. Kazuo Erami (JT)



Institute of In Vitro Sciences (IIVS)

❖ Workshop Series

➤ Technical Workshop for Goblet Cell Hyperplasia, Mucus Production, and Ciliary Beating Assays

- Laboratory Exercises for pilot exercise complete
- Summary of results to be reviewed and after selection of best methods.
- Proof of principle phase – following confirmation of methods, testing of tobacco products will commence
- Participating/Contributing Labs: IIVS, BAT, ALCS, RAI, NCTR, JT, ITL, PMI
- Tissue Vendors: MatTek and Epithelix



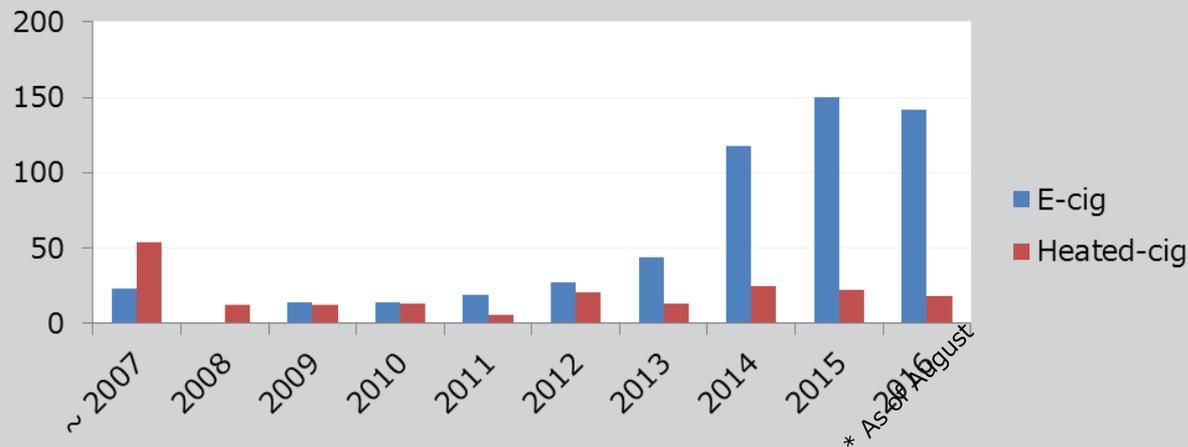
❖ Preliminary Survey : “Methods of in vitro toxicity testing with Emerging Products” : Kazuo Erami, Yoko Sawamoto, Toshiro Fukushima (JT)

- Duration : 2008 – 2016 (August)
- Survey targets
 - Aerosol generation method (e.g., machine vaping condition, puff count)
 - Sample preparation method (e.g., aerosol bubbling in medium)
 - Exposure method (e.g., submerged)
 - Dose level/Dose unit for data analysis
 - Endpoint targeted
 - Positive response
- 584 papers for e-cigarettes & 201 papers for heated cigarettes were found
 - 50 papers were found to be relevant to our interests

❖ Number of Literatures

- Academia : 32
- Tobacco (e-cigarette) company: 17

❖ Number of Literatures



- ❖ **Organization: 17 papers from Tobacco (E-cig) company, 32 papers from academia**
- ❖ **Aerosol Generation Method: No publication was found using CRM81**

Vaping condition	E-cigarette	Tobacco Vapor
Health Canada Intense	4	11
ISO	0	1
Original Regimen	15	1

- ❖ **Sample Preparation Method**

Aerosol collection	E-cigarette	Tobacco Vapor
According to CS collection	3	5
Bubbling aerosol into medium/PBS	14	7
Cold trap	1	0
Direct exposure	8	2

❖ Exposure Method

Exposure method	E-cigarette	Tobacco Vapor
Submerge	20	11
Air liquid interface	12	2

❖ Dose levels / Dose Unit

Dose unit	E-cigarette	Tobacco Vapor
Aerosol (TPM) weight	3	3
Puff	2	5
% of mother solution	8	2
Nicotine equivalent	4	1
Stick	0	1
OD	1	0
Dilution rate (for direct exposure)	4	2

❖ Endpoints

➤ IVT recommended battery

- Ames, MN, MLA, NRU

➤ Genotoxicity

- Gamma H2AX, SCG

➤ Carcinogenesis

- Cell Transformation Assay, *Anchorage-independent growth assay*

➤ Cytotoxicity

- MTT, LDH, Trypan Blue exclusion, Apoptosis (e.g., Cytochrome C release, Mitochondrial mass, Mitochondrial perturbation), Cell membrane permeability, Cell cycle, Cell proliferation (e.g., CCK-8), Cell morphology, Ceramide determination, Transcellular electrical resistance

➤ Oxidative Stress / Antioxidant response

- *Anchorage-independent growth assay*

➤ Inflammatory response / Cell migration (associated with Inflammation)

- *Cytokine release (e.g., IL-8, TNF- α), Metalloproteinase release (e.g., MMP-9), Expression of inflammatory markers (e.g., CD11b, CD66b), TEM assay, Chemotaxis assay, Wound healing, Cell invasion assay*

➤ Adhesion

➤ Developmental Toxicity

- *Cardiac development (e.g., Zebrafish, Human embryonic stem cells)*

➤ Comprehensive

- *Gene expression, Protein expression, Transcriptome analysis, Signaling pathway activation*

“Direct bubbling into culture medium” or “Direct aerosol exposure”

❖ Summary

- It is found that a variety of methods for aerosol generation, sample preparation, exposure to biological tissues and data analysis.
- The aerosol derived from “E-cigarettes” mostly do not show positive response on CORESTA IVTSG recommended battery (i.e., Ames, MN, MLA, NRU), however, positive response of oxidative stress and inflammation are observed in many researches with samples prepared by bubbling into culture medium, however, most literature do not mention and/or do not take care about potential technical artefacts (e.g. osmolality).

❖ Next step

- The group may propose to have a workshop (at the next IVTSG meeting; TBD) to discuss an additional “CORESTA recommended battery” (endpoints, assays, technical guidance).



Acknowledgement

- ❖ **Holger Behrsing (IIVS), Yama Abassi (ACEA)**
- ❖ **April Brys & Battelle : Hosting the spring meeting**
- ❖ **Betsy Bombick & RAI: Supporting the spring meeting**
- ❖ **Wendy Wagstaff & Alex Hauleithner: Statistical Analysis**
- ❖ **Elisabeth Weber (JTI/Oekolab): Study Coordinator of MN study**
- ❖ **T. Fukushima, Y. Sawamoto, K. Erami : Literature Review, Cigarette preparation & shipment, Nicotine & Water Analysis**
- ❖ **Monica Lee (ALCS), Jacqueline Miller Holt (JTI): Regulatory Monitoring**

❖ Sincere thanks to the members of the IVT SG for their contributions

