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

Meetings

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

in vitro MNT

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		

in vitro MNT

❖ 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%.

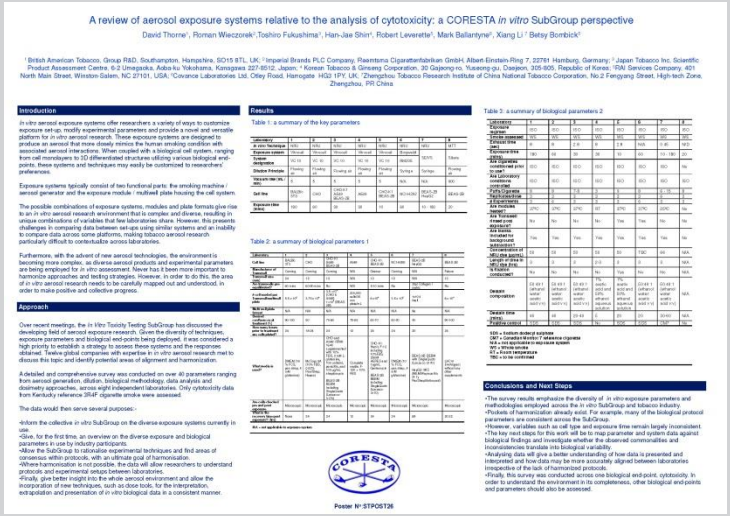
Upcoming Proficiency Studies

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

- **Poster: "Review of aerosol exposure systems relative to the analysis of cytotoxicity: a CORESTA *in vitro* Sub Group perspective": CORESTA Congress 2016**
  - **Authors: D. Thorne (BAT), R. Wieczorek (ITL), T. Fukushima (JT), H. Shin (KT&G), R.Leverette (RAI), Mark Ballantyne (Covance), Xiang Li (CNTC), Betsy Bombick (RAI)**


A review of aerosol exposure systems relative to the analysis of cytotoxicity: a CORESTA *in vitro* SubGroup perspective

David Thorne[1], Roman Wieczorek[2], Toshiro Fukushima[3], Han-Jae Shin[4], Robert Leverette[5], Mark Ballantyne[6], Xiang Li[7] Betsy Bombick[5]

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Introduction

In vitro aerosol exposure systems offer researchers a variety of ways to customize exposure set-up, modify experimental parameters and provide a novel and versatile platform for *in vitro* aerosol research. These exposure systems are designed to produce an aerosol that more closely mimics the human smoking condition with associated aerosol interactions. When coupled with a biological cell system, ranging from cell monolayers to 3D differentiated structures utilizing various biological end-points, these systems and techniques may easily be customized to researchers' preferences.

Exposure systems typically consist of two functional parts: the smoking machine / aerosol generator and the exposure module / multiwell plate housing the cell system.

The possible combinations of exposure systems, modules and plate formats give rise to an *in vitro* aerosol research environment that is complex and diverse, resulting in unique combinations of variables that few laboratories share. However, this presents challenges in comparing data between set-ups using similar systems and an inability to compare data across some platforms, making tobacco aerosol research particularly difficult to contextualize across laboratories.

Furthermore, with the advent of new aerosol technologies, the environment is becoming more complex, as diverse aerosol products and experimental parameters are being employed for *in vitro* assessment. Never has it been more important to harmonize approaches and testing strategies. However, in order to do this, the area of *in vitro* aerosol research needs to be carefully mapped out and understood, in order to make positive and collective progress.

Approach

Over recent meetings, the *In Vitro* Toxicity Testing SubGroup has discussed the developing field of aerosol exposure research. Given the diversity of techniques, exposure parameters and biological end-points being deployed, it was considered a high priority to establish a strategy to assess these systems and the responses obtained. Twelve global companies with expertise in *in vitro* aerosol research met to discuss this topic and identify potential areas of alignment and harmonization.

A detailed and comprehensive survey was conducted on over 40 parameters ranging from aerosol generation, dilution, biological methodology, data analysis and dosimetry approaches, across eight independent laboratories. Only cytotoxicity data from Kentucky reference 3R4F cigarette smoke were assessed.

The data would then serve several purposes:-

•Inform the collective *in vitro* SubGroup on the diverse-exposure systems currently in use.
•Give, for the first time, an overview on the diverse exposure and biological parameters in use by industry participants.
•Allow the SubGroup to rationalise experimental techniques and find areas of convergence within protocols, with an ultimate goal of harmonization.
•Where harmonization is not possible, the data will allow researchers to understand protocols and experimental setups between laboratories.
•Finally, give better insight into the whole aerosol environment and allow the incorporation of new techniques, such as dose tools, for the interpretation, extrapolation and presentation of *in vitro* biological data in a consistent manner.

Results

Table 1: a summary of the key parameters

Table 2: a summary of biological parameters 1

Table 3: a summary of biological parameters 2

Conclusions and Next Steps

•The survey results emphasize the diversity of *in vitro* exposure parameters and methodologies employed across the *In vitro* SubGroup and tobacco industry.
•Pockets of harmonization already exist. For example, many of the biological protocol parameters are consistent across the SubGroup.
•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.

Poster N° STPOST26

Poster No: STPOST26

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

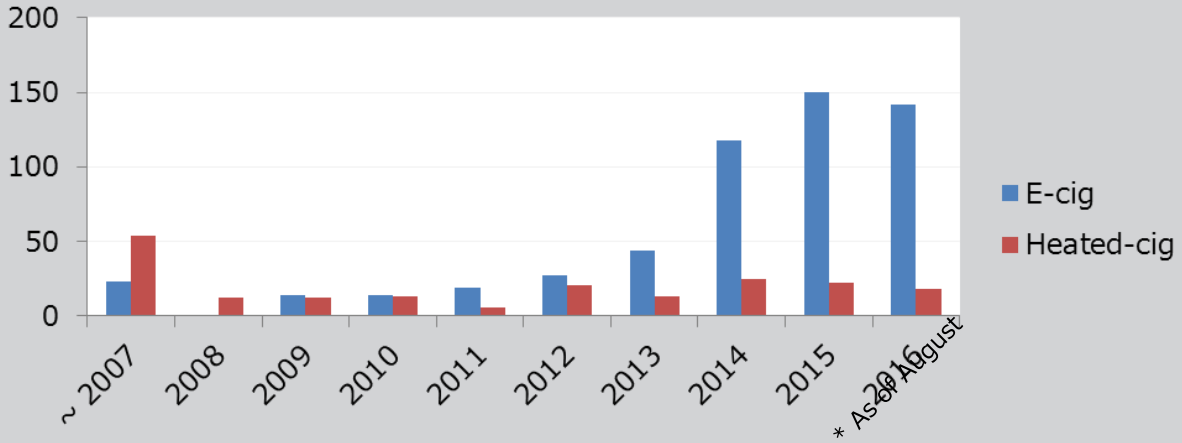
- Laboratory Exercises for pilot exercise complete
- Summary of results to be reviewed and after selection of best methods.
- Proof of principle phase – following confirmation of methods, testing of tobacco products will commence
- Participating/Contributing Labs: IIVS, BAT, ALCS, RAI, NCTR, JT, ITL,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

Literature Survey

- ***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

Literature Survey

- **Number of Literatures**
  - Academia : 32
  - Tobacco (e-cigarette) company: 17
- **Number of Literatures**



Literature Survey

- **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

Literature Survey

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

Literature Survey

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"

Literature Survey

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

Acknowledgement

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