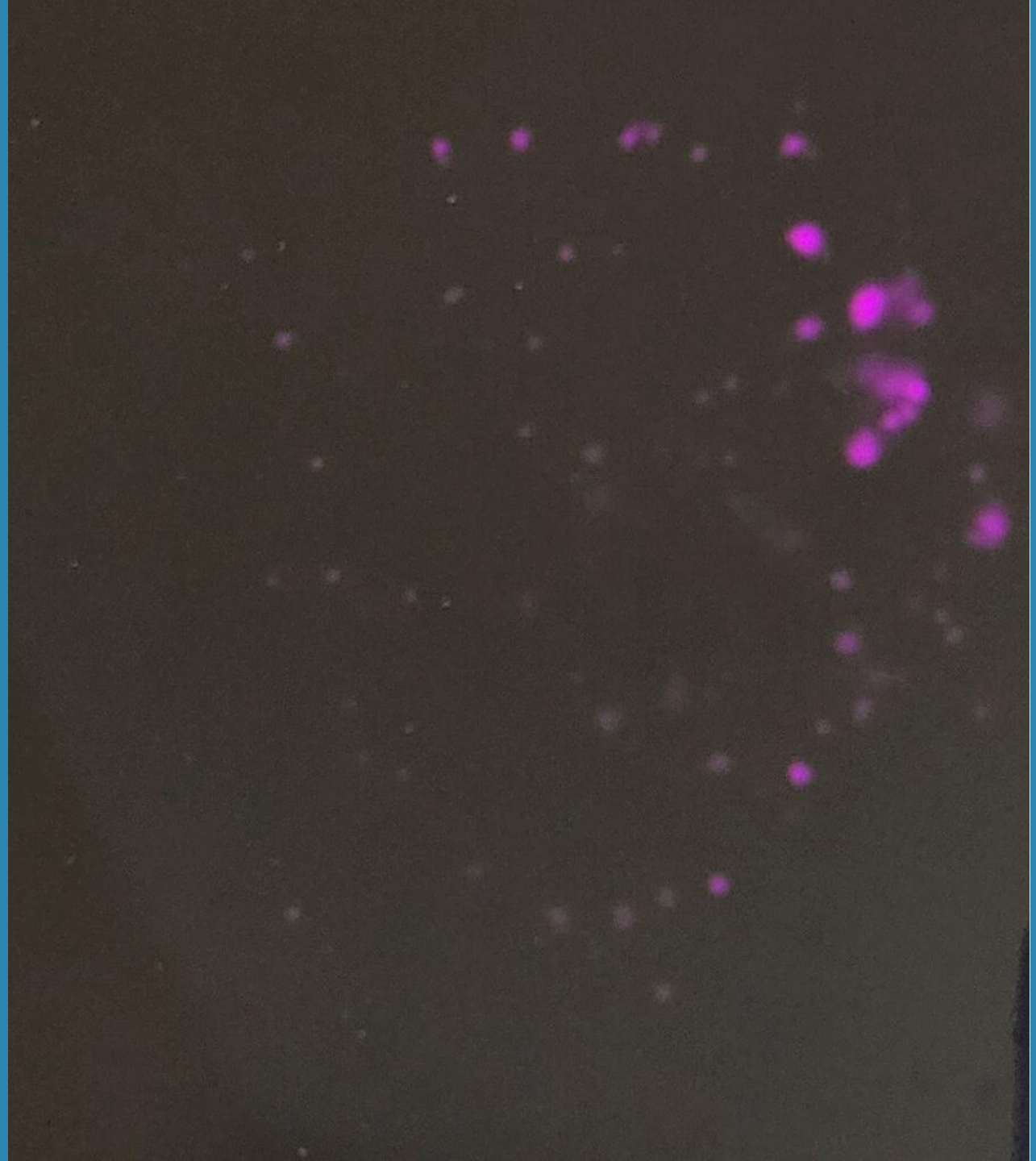

VOCs in the IVF Laboratory: Hiding in Plain Sight

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Jason Russack, MS

How many people are worried about VOCs in their lab?

How many people are doing something about the VOC concerns?

- Turns out, none of these things are fully protective from VOCs
- It’s a much more complicated story

HEPA filtration?

Using oil overlays?

Off gassing plastic ware?

Background

Air Pollution, Volatile Organic Compounds, and Common Sources

*Air pollution “greatest risk to overall environmental health”[1]

Volatile Organic Compounds (VOCs)

- Volatilize into air space, cause odor [2]
- Low boiling point, high vapor pressure [3,4]
- Indoor concentrations 2-10x outdoor (Up to 1000x)

VOCs in IVF

- Trends between fertilization rates in IVF and air quality [11,12]
  - Insect extermination → 3x decline in successful implantation [11]

- Laboratory Best practices: oil overlay, carbon air filtration, Class 100 Cleanroom
- Cairo Consensus [5]: formalized best practices guide for IAQ in ART
  - Recommendations for building materials, workstation, gas systems, layout
  - **< 500 µg/m3 Total VOC (TVOC), < 5 µg/m3 aldehydes**
  - **No mention of individual thresholds for specific species…**

**** What do these numbers mean? Why these thresholds?**

Laboratory Sources of VOCs

- Paints, cleaning supplies, building materials
- Smoking, personal care products
- Plasticware, incubator gases
- Road work, office fumigation
- Naturally occurring, forest fires

Cellular and Subcellular Impacts of VOC Exposure: what do we know?

- **Animal models**
- Acrolein, acetaldehyde/ethanol, toluene, xylene, formaldehyde: embryo/cytotoxicity studied with mouse and zebrafish [32-37]
- **Human Jurkat-T cells and fibroblasts**
  - Improper gene expression, cancer, inflammation [38,39]
  - Synergistic/antagonistic effects of VOC mixture [38,40,41]
- Better toxicity models may be developed…

Introduction

Combatting VOCs in the Laboratory

- No perfume, no smoking policies
- Selected low-VOC building materials
- Off-gas plastic consumables for longer periods
- VOCs were being reduced indiscriminately

Evolution of IVF

- *In-vitro* fertilization (IVF) has been steadily improving since its introduction
- However, there is still room for improvement
- Air quality contaminants have been suspect
- Specifically, VOCs could potentially hinder IVF outcomes

Gap in Our Understanding

- Relationship between VOCs and embryogenesis
  - IVF laboratory embryos may have direct contact with VOCs without maternal protection
  - The effect of VOCs can be direct and immediate

VOCs Selected for this Study

- Acetaldehyde
  - Metabolite of ethanol (Zimmerman *et al.,* 1995, *Reimers et al.,* 2004, Lau *et al.,* 1991)
  - **Most common VOC in the IVF laboratory**
- Styrene
  - Present due to polystyrene dishware
  - Aromatic hydrocarbon

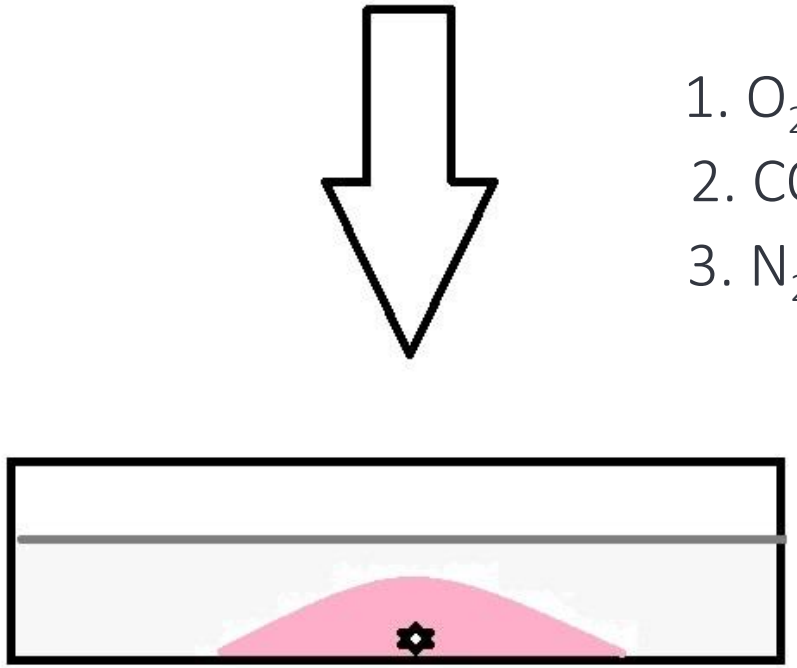
Acetaldehyde

- Naturally occurring – ripe fruits
- Toxicity of ethanol is largely due to this primary metabolite (Zimmerman *et al.,* 1995)
- Been shown to inhibit cell growth by delaying cell cycle progression and increasing the rate of death
- Largely hydrophilic

Styrene

- Synthetic chemical used in plastics manufacturing
- Aromatic hydrocarbon
- Largely hydrophobic
- Polystyrene, primary metabolite
- Also biologically damaging (Zimmerman *et al*., 1995)

Study Design



Incubator Gasses

1. O_2 at 5%
2. CO_2 at 6%
3. N_2 with 500ppb of acetaldehyde or styrene

500ppb x 89%N_2 = 445ppb

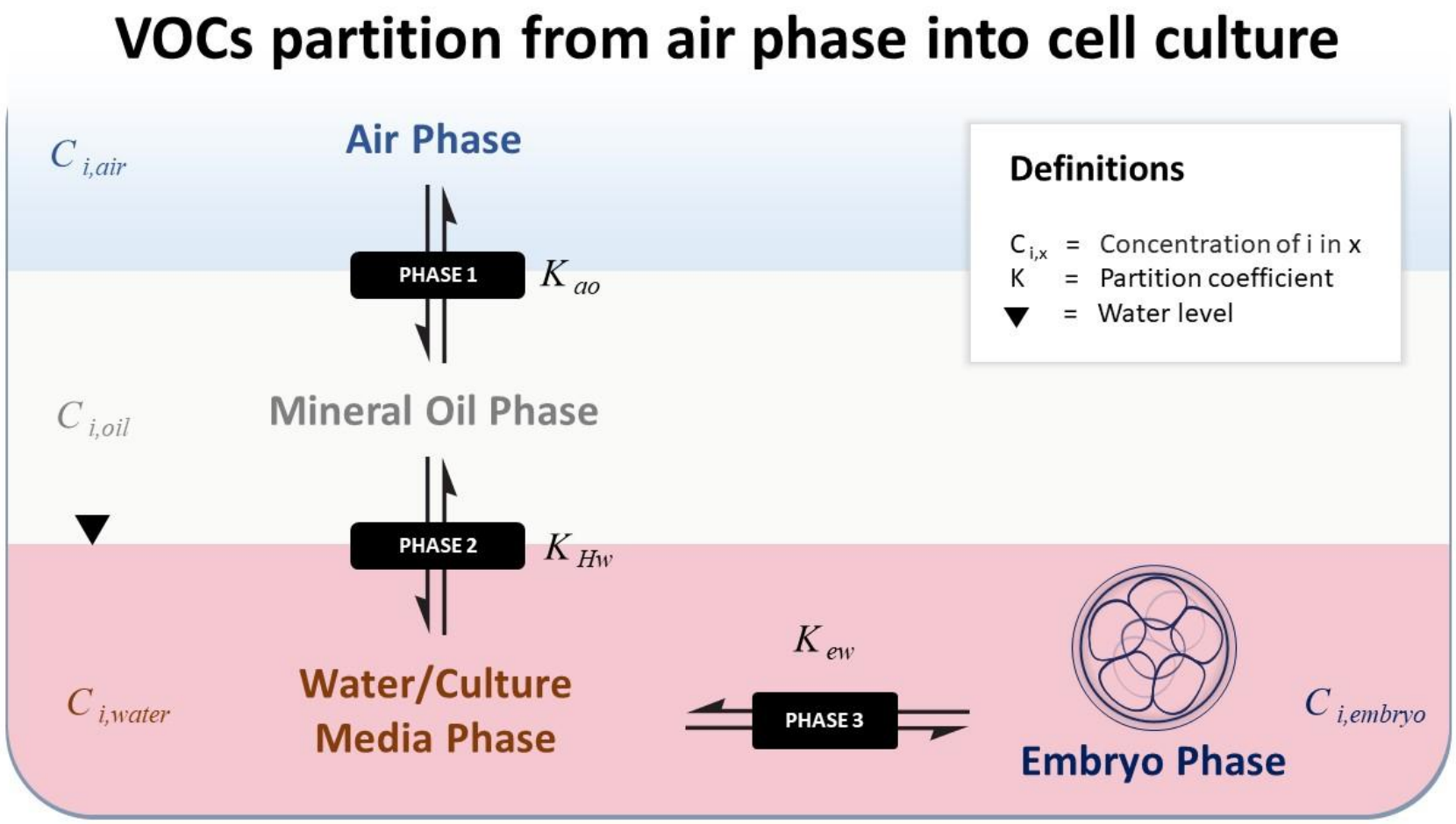
VOC Modes of Action

- VOCs transitioning through our culture systems
- Each VOC will interact differently
  - Hydrophilic vs hydrophobic
- Each VOC may have a different mechanism of toxicity

Markers Selected for This Study

- Cell Counts
  - Visual indicator of embryo health
- Reactive Oxygen Species (ROS)
  - Indicator of oxidative stress
- Apoptosis
  - Programmed cell death
- Genetic Changes

Equilibrium Partitioning Modeling



Equilibrium Partitioning Modeling

- Air-Oil and Oil-Culture Media Partition Coefficients [44-48]

$$K_{ial} = \frac{C_{ia}}{C_{il}}$$

$$K_{ilw} = \frac{C_{il}}{C_{iw}}$$

- Culture Media-Embryo partitioning [44]

$$K_{iwe} = \frac{C_{iw}}{C_{ie}} \approx \text{function of } K_{ow}$$

Modeling VOC Kinetics: Diffusion

(Solved) Mass Balance:

$$C_{il}(t) = C_{il0} \times e^{\frac{-k^{al}A_{al}}{V_l}(t-t_0)} + \frac{p_{i,max}}{RTK_{ial}}\left(1 - e^{\frac{-k^{al}A_{al}}{V_l}(t-t_0)}\right)$$

Oil Diffusivity

Oil Surface Area

Airborne concentration of VOC

Concentration of VOC "i" in Oil

Initial concentration

Oil Volume

Time

Air-Oil Partition Coefficient at Equilibrium

WOAH… That's a lot of MATH!

Tldr… (too long didn't read)…

- We can use mathematical/thermodynamic models to predict how different VOCs move
- We want to apply this knowledge to IVF
- Modeling the time to equilibrium harder than predicting end concentration
  - Tons of variables
  - Important because we think it happens **FAST**…

Specific Aims

1. Mouse embryos were grown in the presence of 445ppb of acetaldehyde or styrene for three days, and their development was compared to a negative control group with no added VOC in culture
    1. Hypothesis: The added VOC will affect mouse embryo development
    2. Null hypothesis: VOC will not affect mouse embryo development

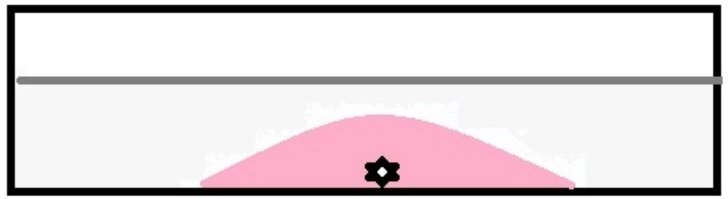
2. Mouse embryos that were grown in the presence of acetaldehyde or styrene were evaluated for levels of ROS and apoptosis as compared to the negative control group
    1. Hypothesis: The levels of ROS and apoptosis will be increased in the VOC test groups
    2. Null hypothesis: There will be no difference in ROS or apoptosis levels between the two groups and the negative control group

Specific Aims, continued

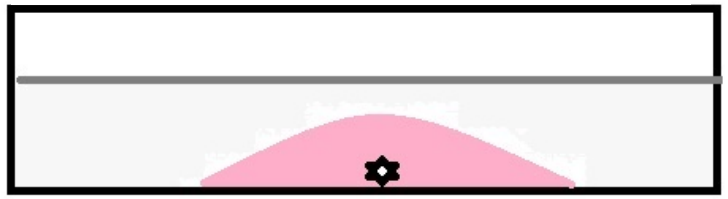
3. Determine genetic changes in the acetaldehyde-and styrene-exposed test groups as compared to the negative control group
    1. Hypothesis: There will be genetic changes in the test groups as compared to the negative control group
    2. Null hypothesis: There will not be genetic differences between the two groups and the negative control group

Specific Aims Flow Diagram

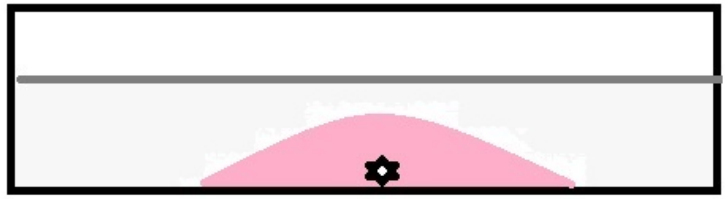
Negative Control Embryos



Acetaldehyde-exposed Embryos



Styrene-exposed Embryos



Aim 1:
Observation then cryopreservation

Aim 2:
Fluorescent Microscopy

Aim 3:
RT-qPCR

Methods

Mouse Embryos and Culture

- Mouse embryos used, $B_6C_3F_1$ x $B_6D_2F_1$
- Standard culture under oil
- Each VOC added through the nitrogen gas tank
- Culture for 3 days then cryopreserved for future testing

Gas Chromatography Testing

- To confirm each VOC transferred into the oil and the media
- Testing was outsourced to Colorado State University

Fluorescent Microscopy

- ThermoFisher Scientific EVOS™ M5000 Cell Imaging System
- Z-stack images after ROS and apoptosis staining
- Proprietary software analyzes fluorescent intensity
- Intensities are compared

RT-qPCR - Real-Time Quantitative Reverse-Transcriptase Polymerase Chain Reaction

- ThermoFisher QuantStudio™ RT-qPCR testing system
- GAPDH (glyceraldehyde 3-phosphate dehydrogenase) - reference gene

Genes Chosen for Testing

- **ErbB4** – an implantation receptor shown to be downregulated in oxidative stress conditions
  - Oxidative stress conditions during preimplantation culture can affect preimplantation receptors on blastocysts that may lessen the resulting IR (Paria *et al.,* 1999 and Egashira *et al.,* 2013)
- **Sirt3** – a mitochondrial deacetylase, involved with the regulation of electron transport in the mitochondria.
  - It is protective of *in-vitro* mouse preimplantation embryos and therefore would typically be upregulated in stressful conditions (Shafei *et al.,* 2020)
- **p53** – induces apoptosis and cell growth arrest when an embryo is under oxidative stress or DNA damage has occurred
  - Commonly called the "Guardian of the Genome" and has several well-known anti-cancer functions (Zhao *et al.,* 2021)

Statistical Analysis

- An unpaired t-test was used, comparing data from the acetaldehyde and styrene group to the negative control group using GraphPad Prism
  - Blastulation rate
  - Cell counts
  - Fluorescent intensity testing for ROS and apoptosis
  - $p<0.05$ considered statistically significant

Results and Discussion

Aim 1: Does the addition of 445ppb acetaldehyde or styrene to the gas phase of a triphasic IVF culture system affect mouse blastocyst development?

Time in culture = 72 hours +/- 1 hour				
	n	Degenerated/arrested/ cellular	Morula/early blast/blast/expanded blast	Hatching blast/hatched
Negative control	26	0	7 (27%)	19 (73%)
Acetaldehyde	51	3 (6%)	12 (24%)	36 (70%)
Styrene	37	**7* (19%)**	12 (32%)	18 (49%)

Embryo Development after 72 hours exposure to 445ppb acetaldehyde or styrene in a triphasic IVF culture system

*p<0.05, considered to be statistically significant, comparing degenerated/arrested embryos in the negative control group to the styrene-exposed group.

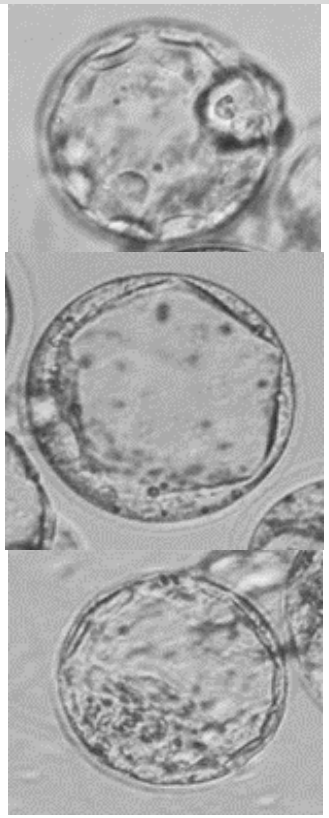
Visual Observation

Inverted microscope

Negative Control

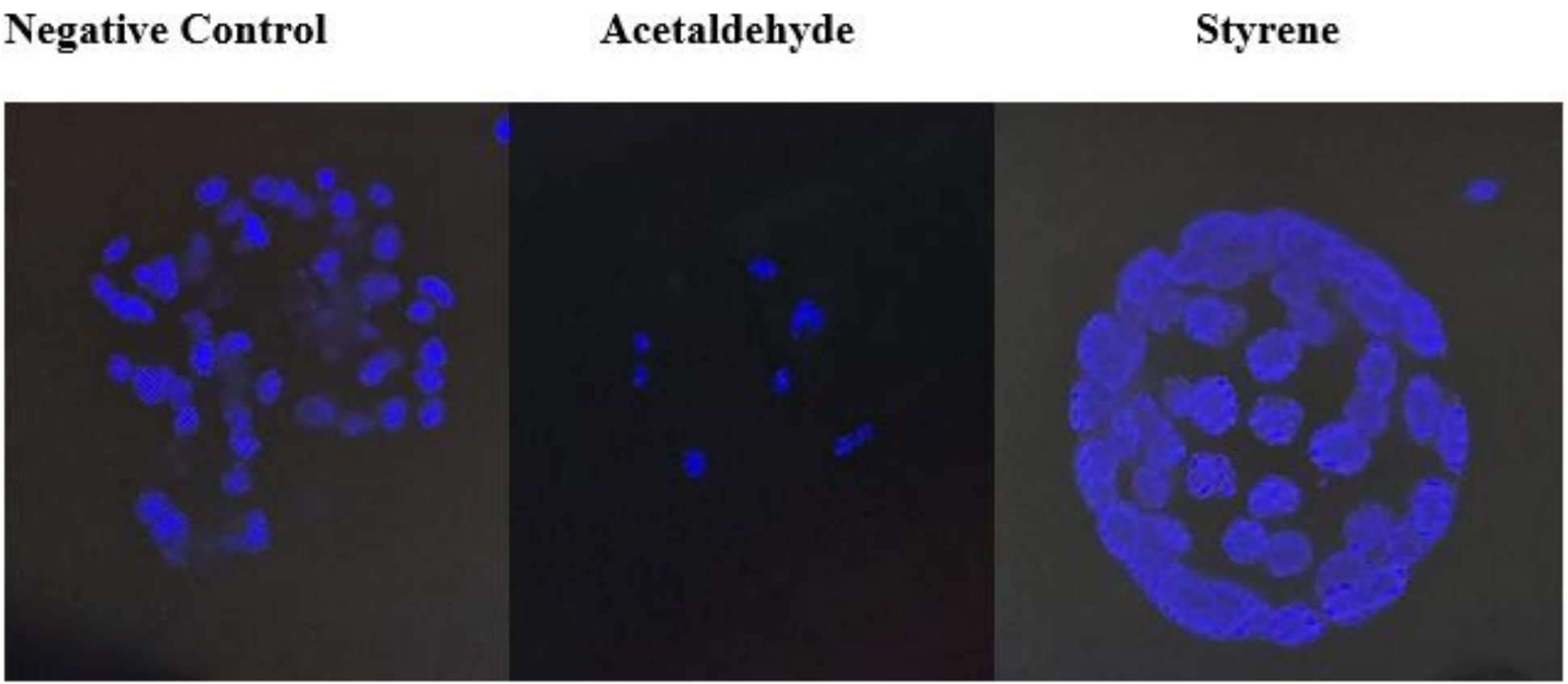
Acetaldehyde

Styrene



Mouse blastocyst development following 72 hours of exposure to 445ppb of acetaldehyde or styrene. Representative embryos at 400x.

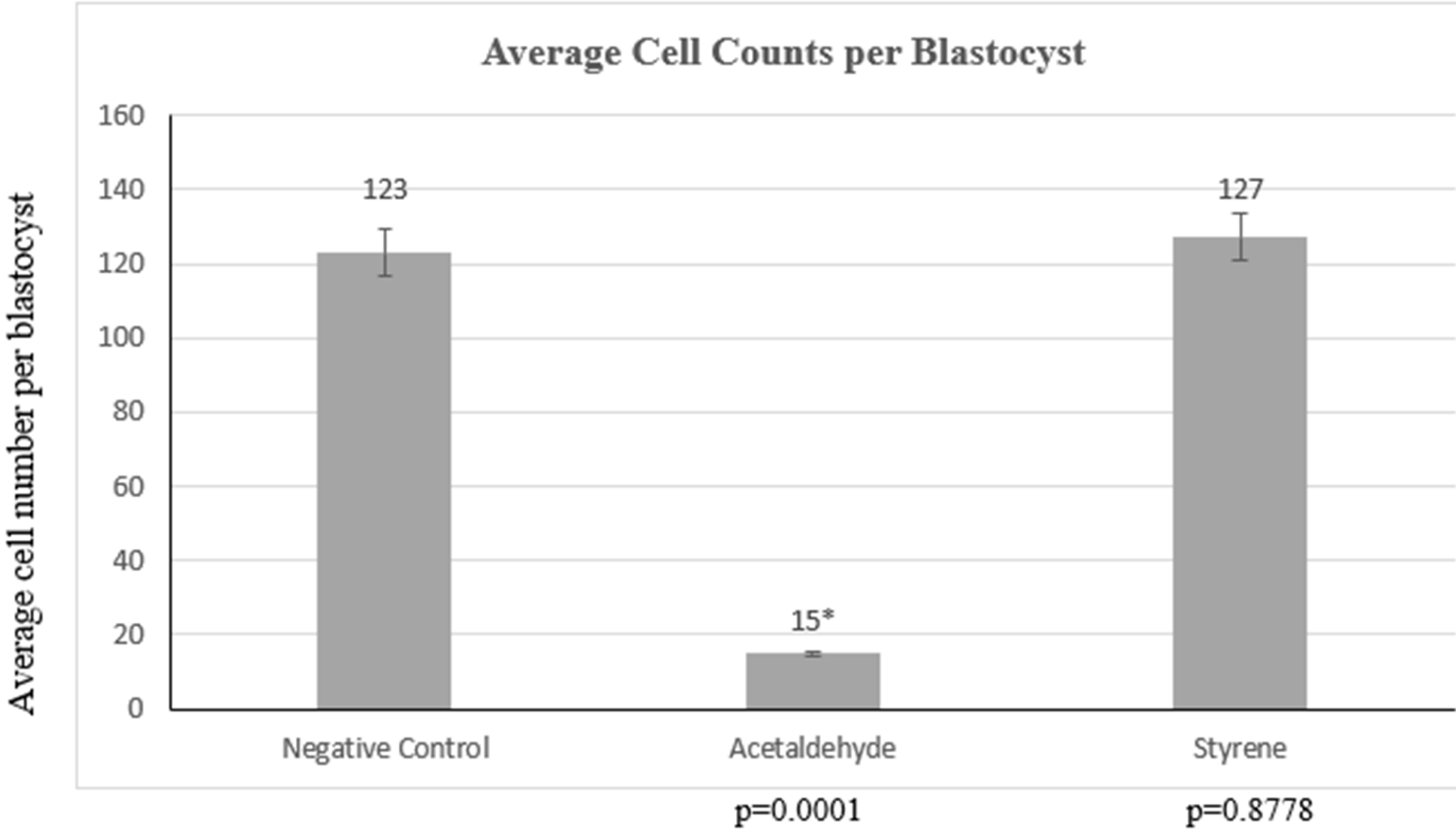
Cell counts



NucBlue™ nuclear counterstain z-stacks showing cell counts after 445pbb exposure of acetaldehyde or styrene. Representative embryos at 1116x.

Cell counts

Mouse embryo blastocyst cell counts following 72 hours of exposure to 445ppb of acetaldehyde or styrene. Negative control, n=5; Acetaldehyde group n=5; Styrene group n=5



Equilibrium Partitioning

Analyte	Media	Oil
Acetaldehyde	278 ppb	13 ppb
Styrene	84 ppb	670 ppb

Gas chromatograph results of concentrations of acetaldehyde and styrene in oil and media after 445ppb air exposure in a triphasic IVF culture system

Equilibrium Model Results for Acetaldehyde and Styrene

Equilibrium Concentrations in Culture Media and Oil Overlay, 500 ppb Air Phase [44]

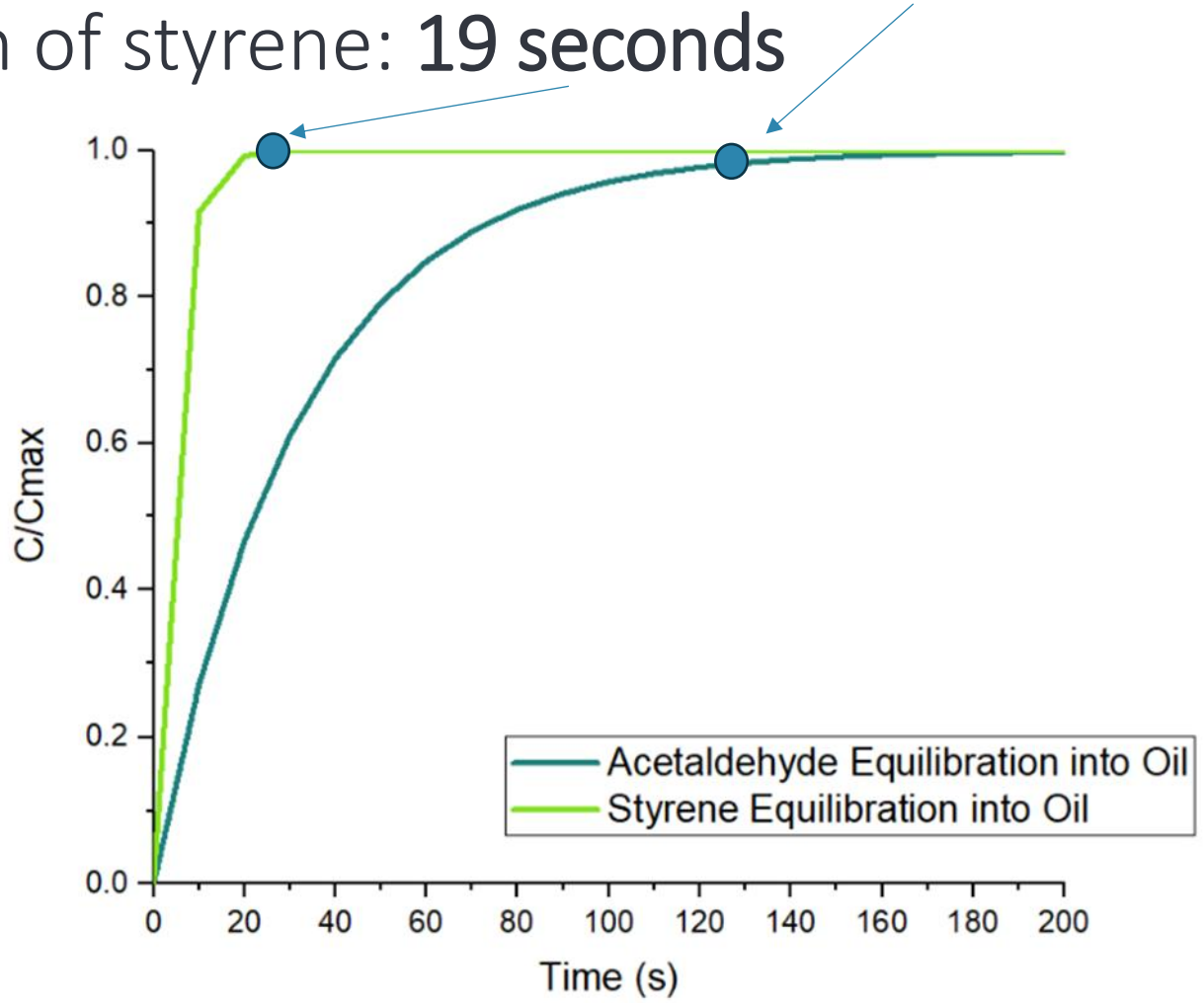
Analyte	GC-MS Measured Concentration	Equilibrium Model
Acetaldehyde in Mineral Oil*	13	19.5
Acetaldehyde in Culture Media*	278	428.5
Acetaldehyde in Embryo**	-	200.5
Styrene in Mineral Oil*	670	10220
Styrene in Culture Media*	84	11.5
Styrene in Embryo**	-	2223

Goal: Model within order of magnitude agreement of measured result

* Liquid concentrations reported in ppb (µg/L)
** Embryo concentrations reported in µg/kg embryo

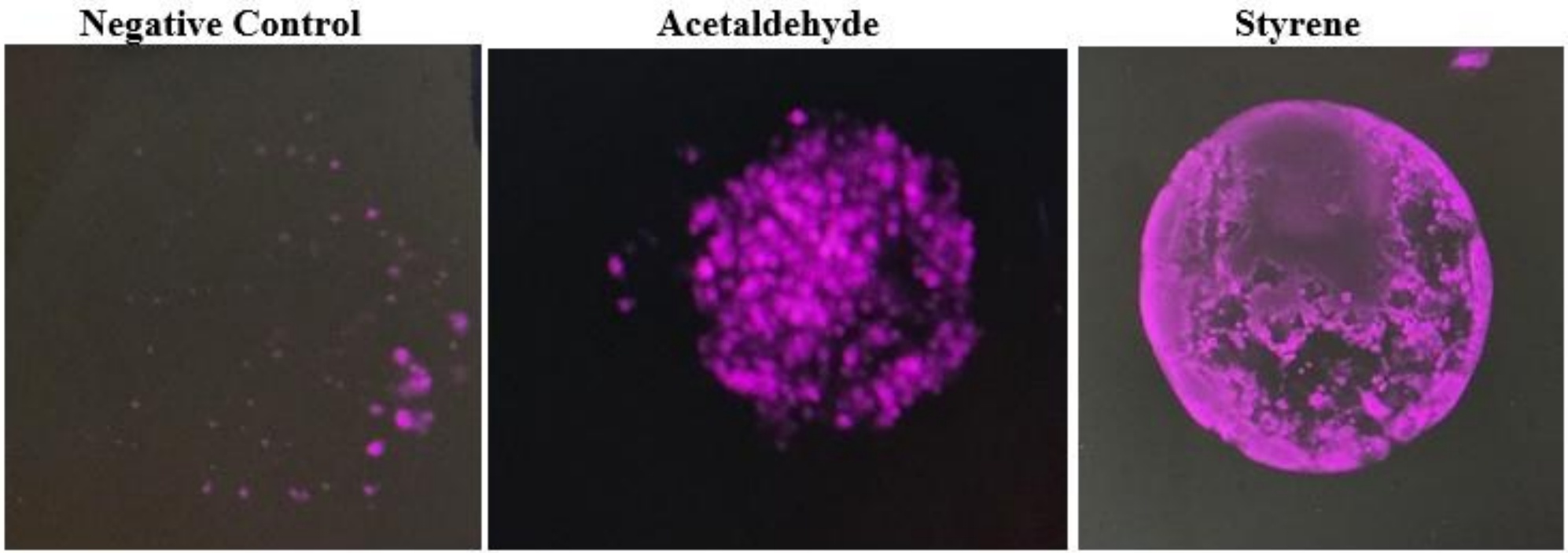
Kinetic Modeling Results

- Equilibration of acetaldehyde: **147 seconds**
- Equilibration of styrene: **19 seconds**



Equilibration into Dish Overlaid With Oil

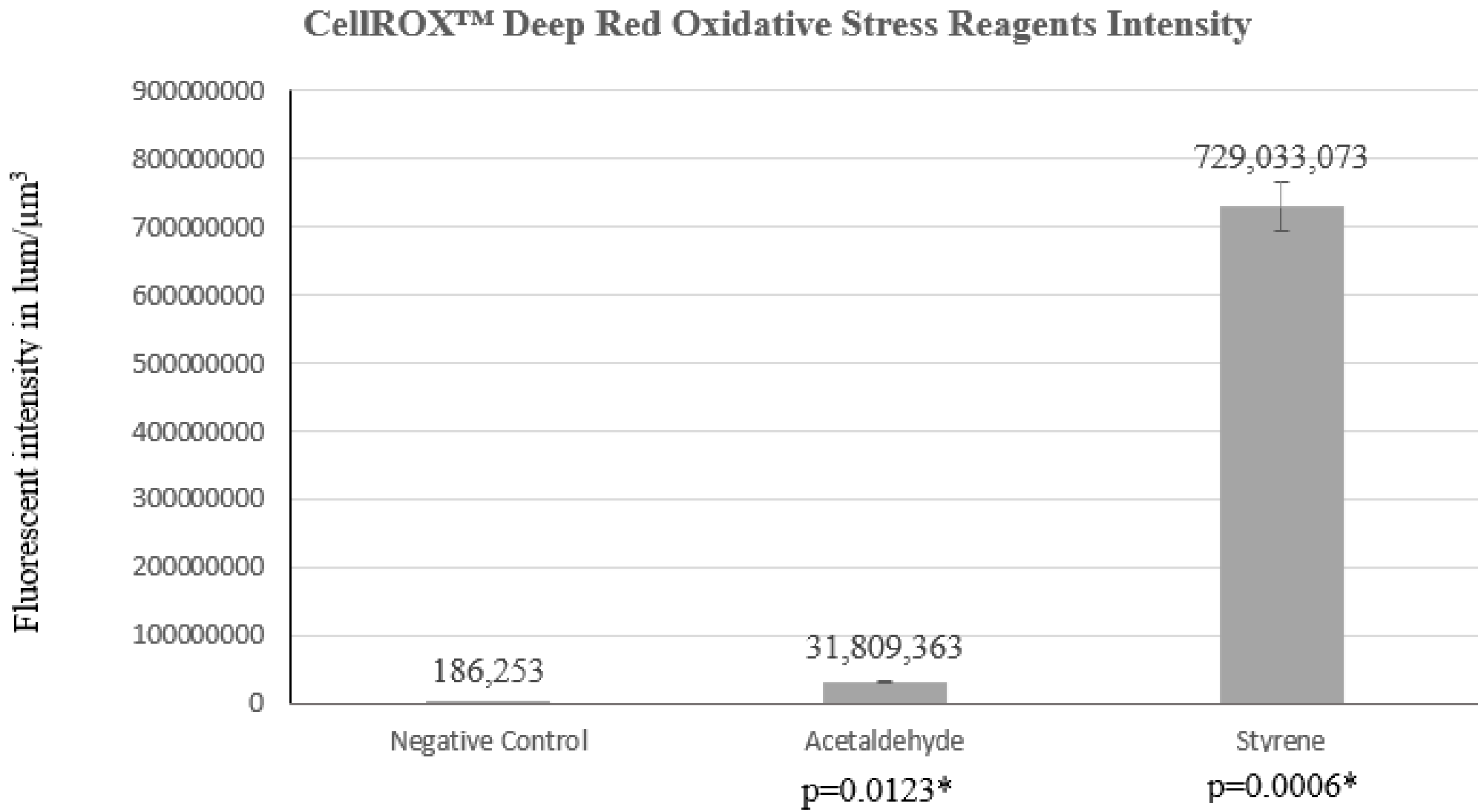
Aim 2: What are the effects of 445ppb acetaldehyde or styrene during preimplantation mouse growth on ROS and apoptosis levels in mouse blastocysts?



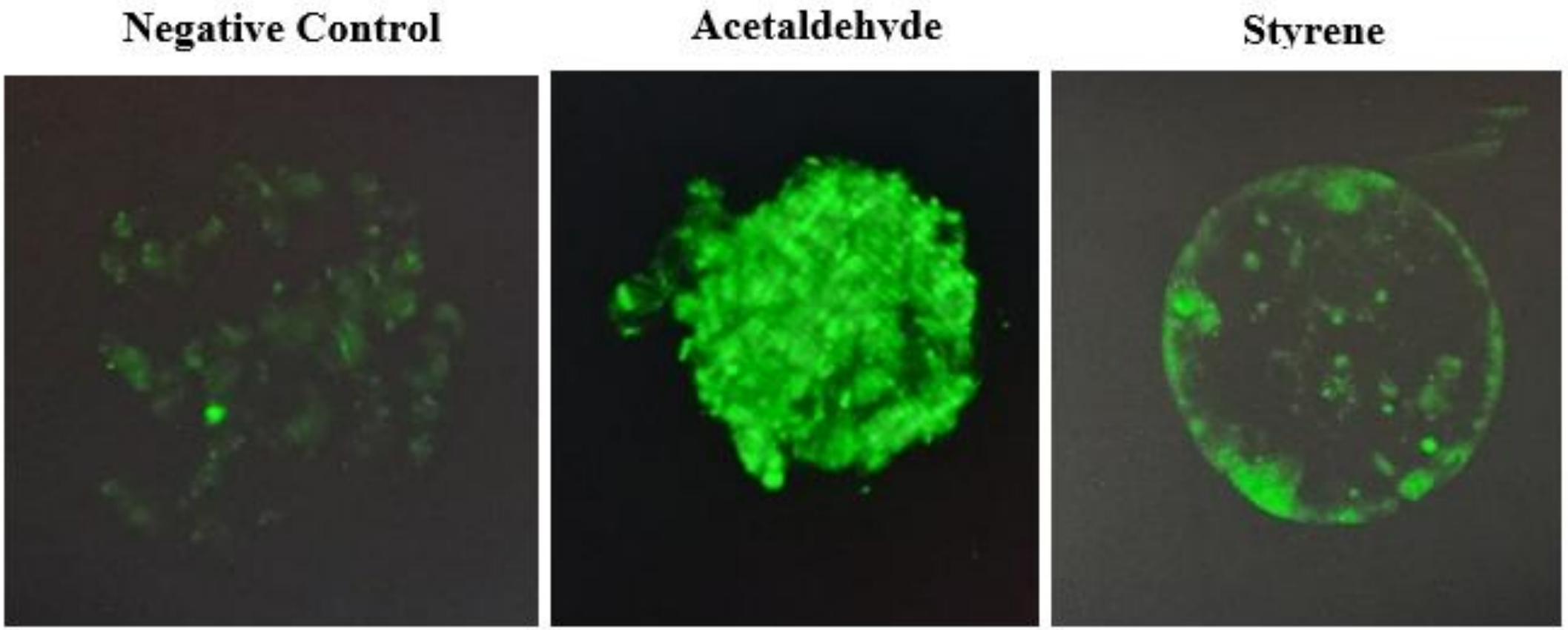
Reactive oxygen species fluorescent intensity levels with CellROX™ Deep Red in mouse preimplantation embryos after exposure to 445ppb of acetaldehyde or styrene. Representative embryos. Negative control n=5, Acetaldehyde n=5, styrene n=5

Fluorescent Intensities

Mouse embryo blastocyst reactive oxygen species levels following 72 hours of exposure to 445ppb of acetaldehyde or styrene. *p<0.05 considered statistically significant.



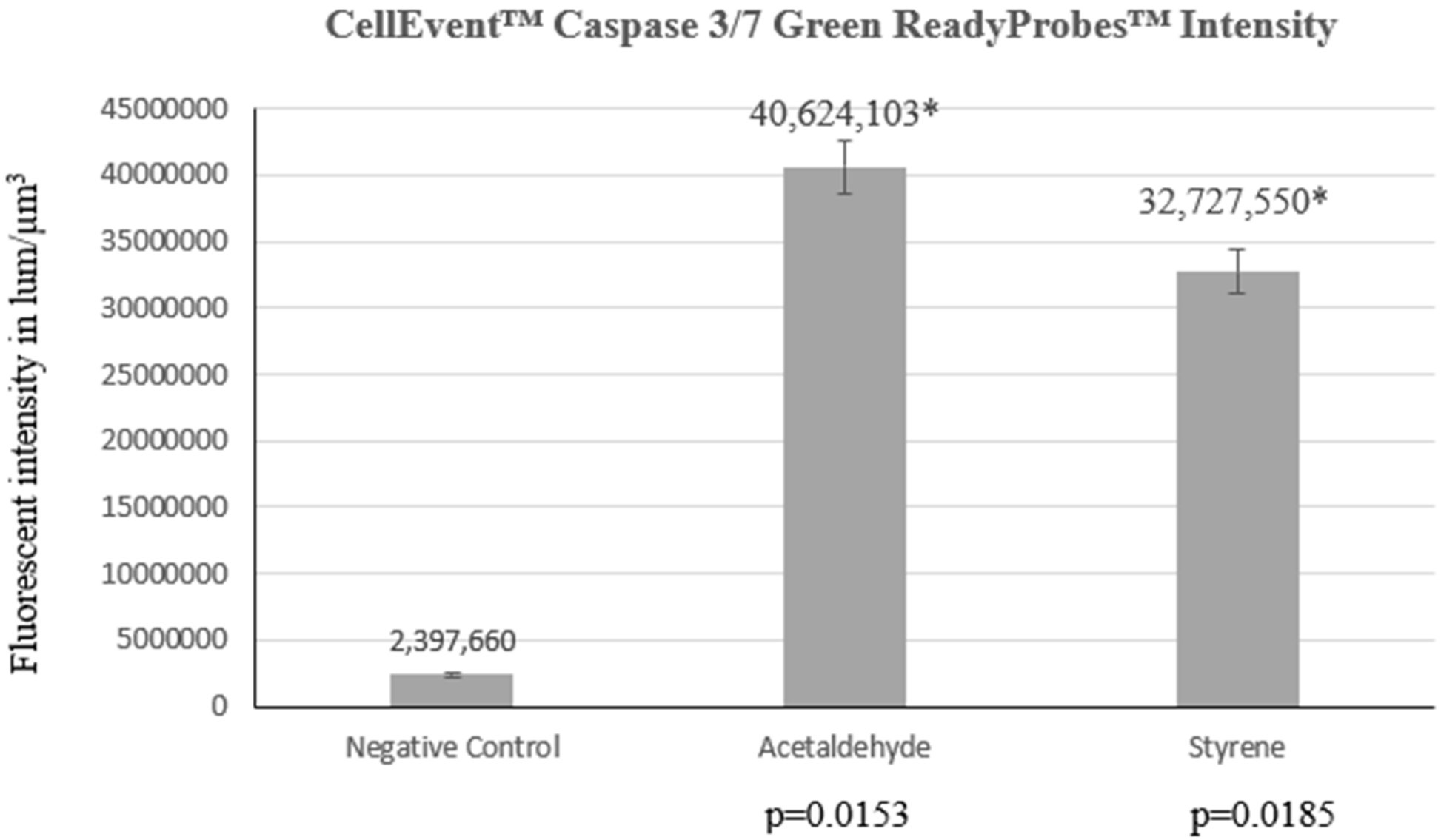
Aim 2: Apoptosis Levels



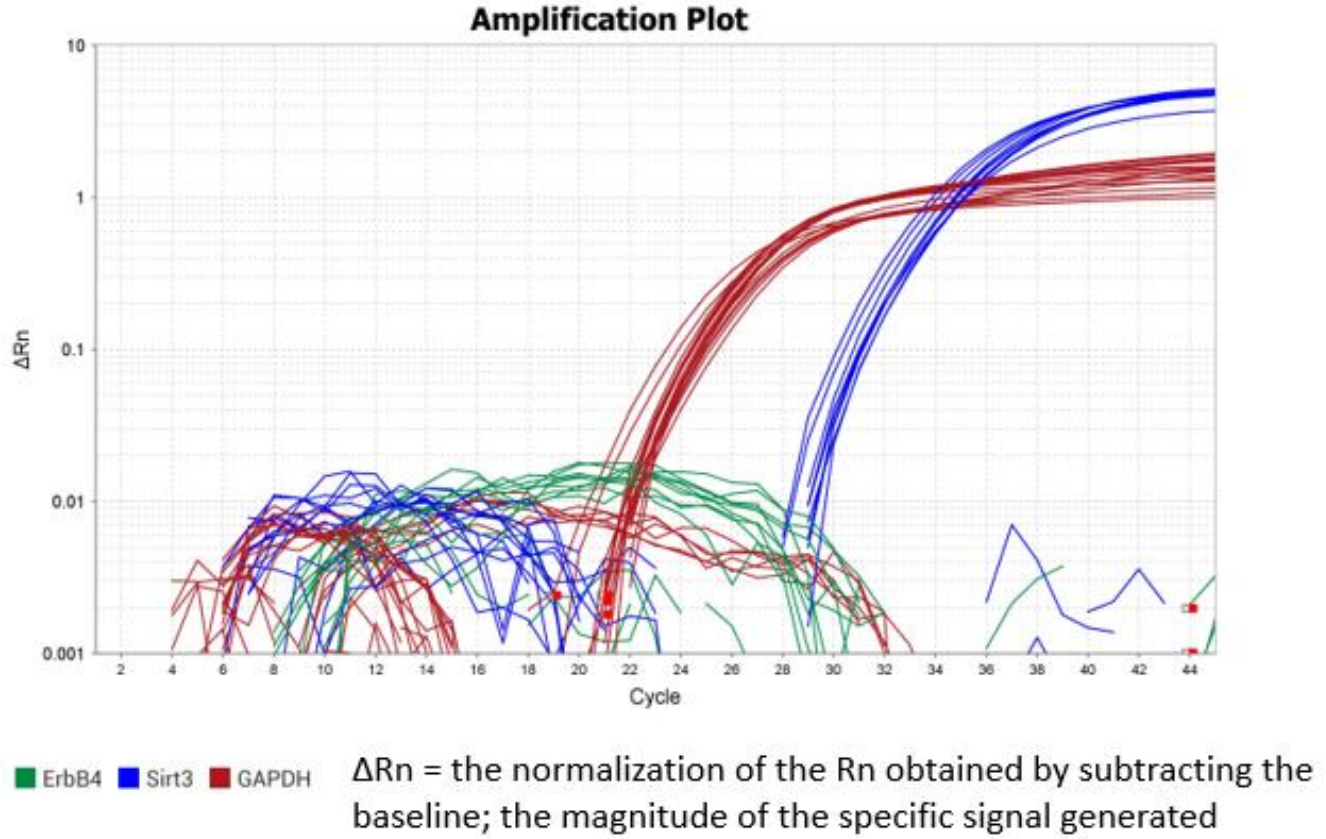
Apoptosis levels in preimplantation mouse embryos after 445ppb exposure to acetaldehyde or styrene as seen with CellEvent™ Green ReadyProbes™ Caspase 3/7 fluorescent stain. Representative embryos.

Apoptosis Intensity Levels

CellEvent™ Caspase 3/7 Green ReadyProbes™ intensity in mouse blastocysts after 72 hours of acetaldehyde or styrene exposure during preimplantation growth. *p<0.05 considered statistically significant.

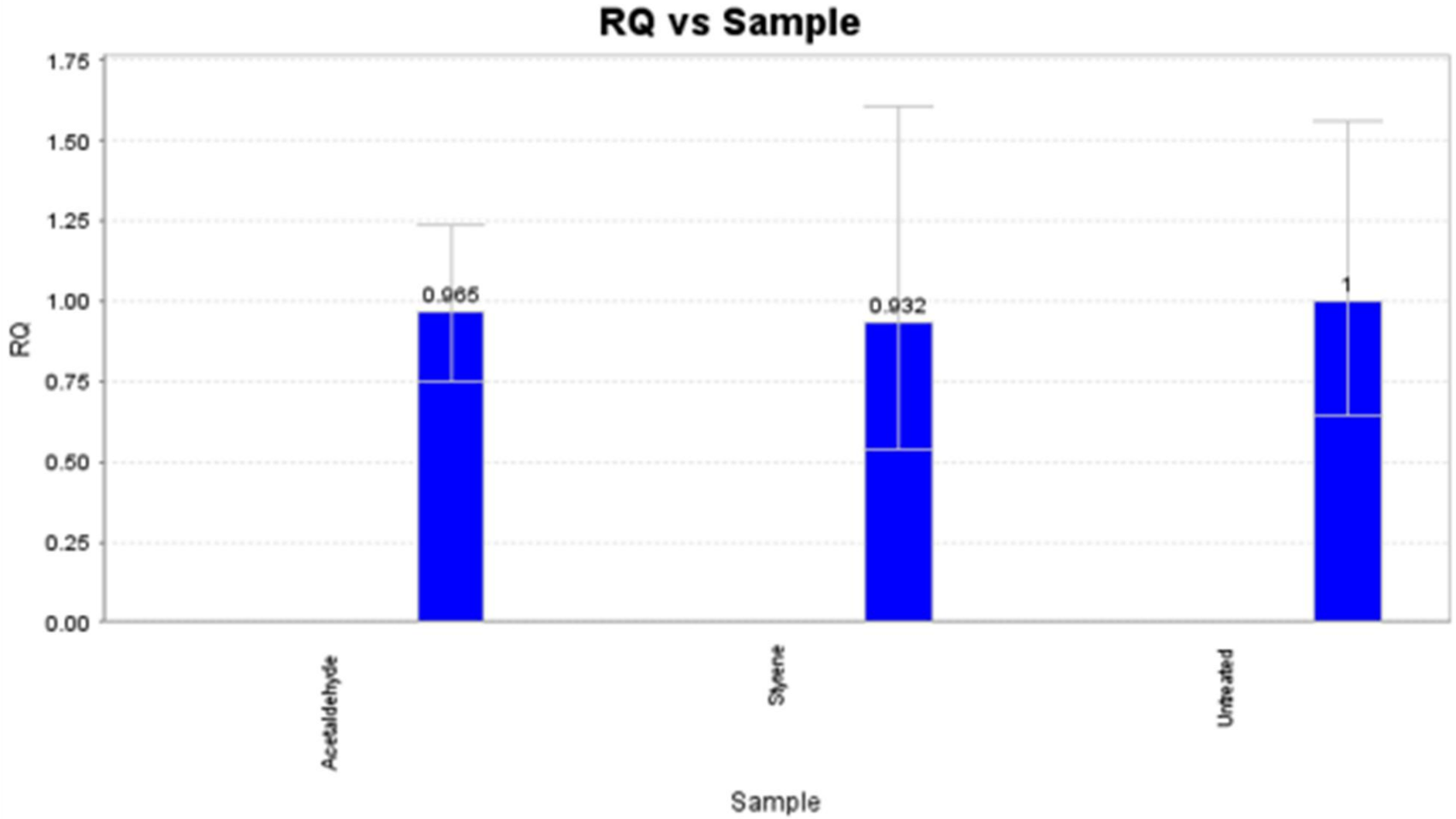


Aim 3: What genetic effects are seen in mouse embryos exposed to 445ppb of acetaldehyde or styrene during preimplantation *in-vitro* culture?



Amplification plot of RT-qPCR Run 1, including ErbB4, Sirt3 and GAPDH

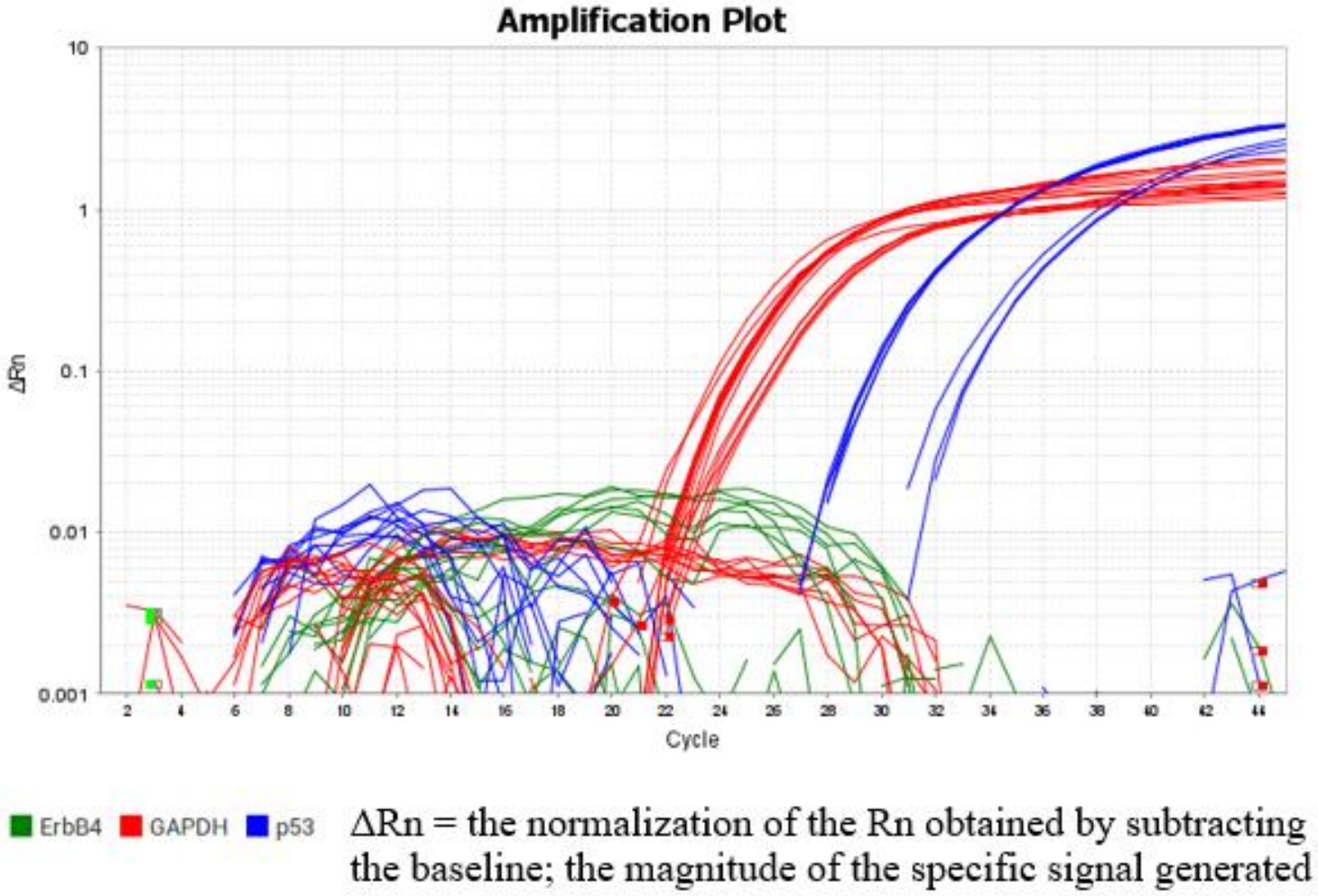
Relative Quantification



RQ = Relative quantification; the percentage of gene expression as compared to the untreated negative control

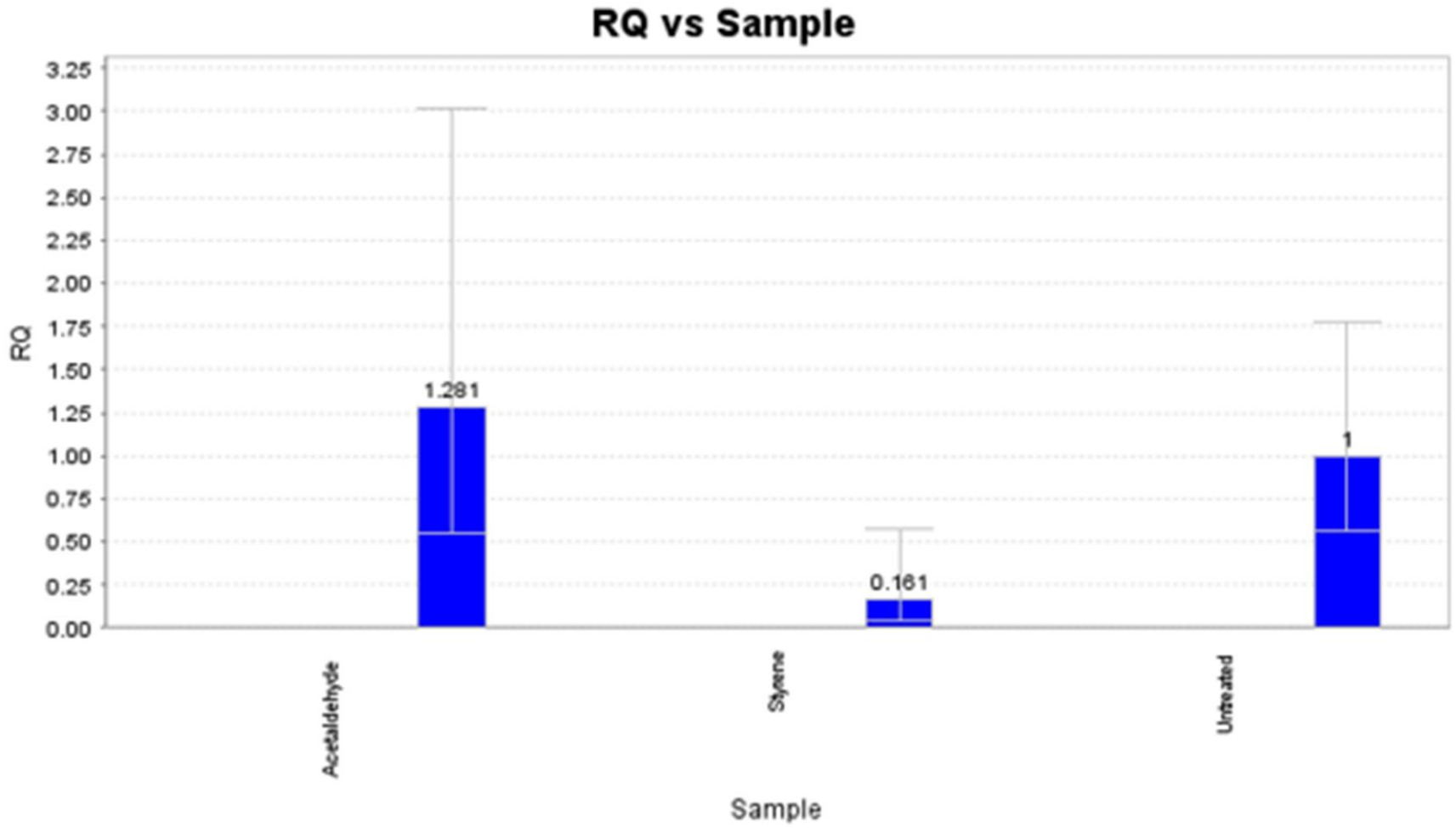
Relative quantification of Sirt3 in the acetaldehyde- or styrene-exposed group mouse blastocysts as compared to the negative intreated group. RT-qPCR Run 1.

Run 2: ErbB4 and p53



Amplification plot for RT-qPCR Run 2, including p53, ErbB4, and GAPDH

Relative Quantification



RQ = Relative quantification; the percentage of gene expression as compared to the untreated negative control group

Relative quantification of p53 in the acetaldehyde- or styrene-exposed group mouse blastocysts as compared to the negative intreated group. RT-qPCR Run 2.

Outcome of Genetic Tests

- Sirt3 showed no significant differences in either acetaldehyde or styrene group
- p53 showed a 6x downregulation in the styrene group

- GC testing showed both VOCs did partition into the media

- Suggests each VOC may have a different mechanism of action

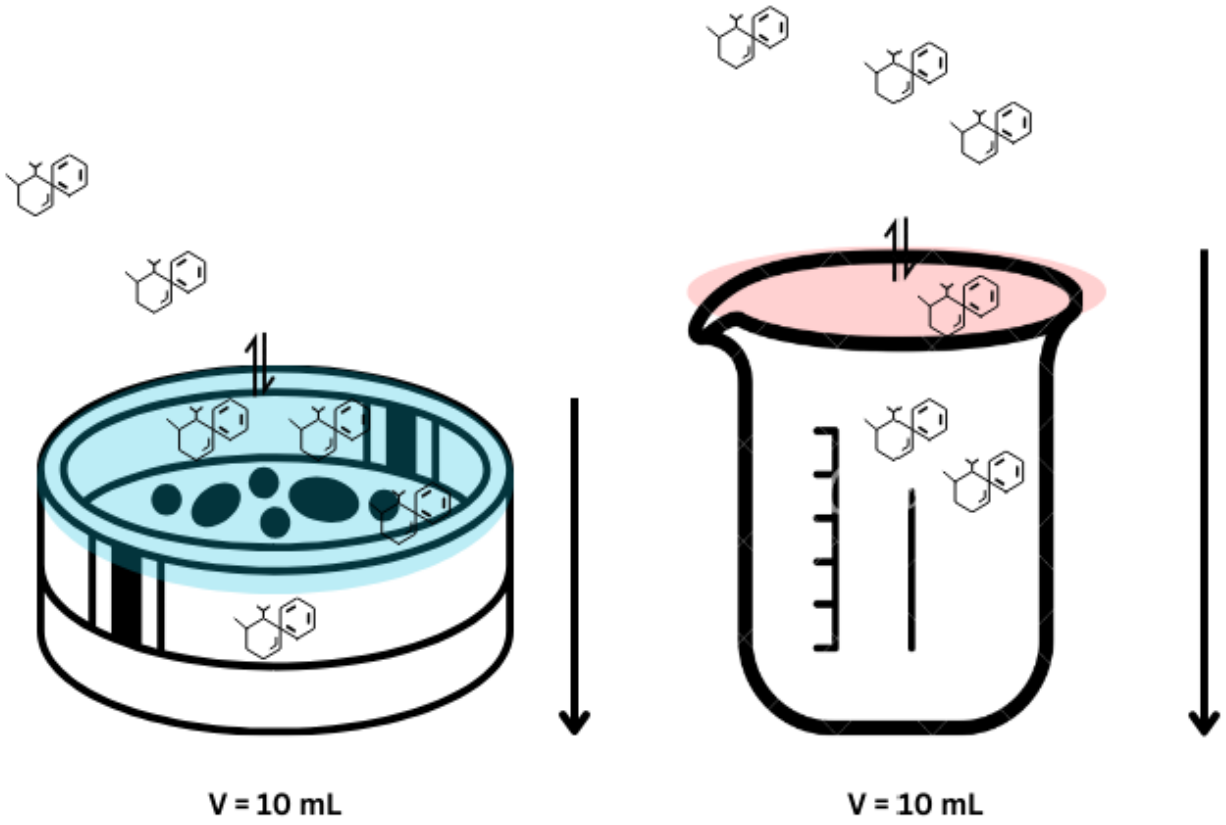
Summary

Discussion: Equilibrium Modeling

- Acetaldehyde partitioning uniformly well described by modeling
    - All results within an order of magnitude
- Styrene partitioning modeled well for media, not as reliable for oil
    - Styrene hydrophobicity is overestimated by K_{ial} constant
    - Possibility that plasticware used to transport samples attracted some styrene
- Shows promise

Discussion: Kinetic Modeling

Equilibration and the Impact of Minimizing Surface Area



Liquid resistance **greater** in the beaker due to greater depth.

Discussion: Kinetic Modeling

Trends in Model Parameters

- + = slower to equilibrate with an increase in a parameter
- - = faster to equilibrate with an increase in a parameter

By Increasing …	Impact
Airborne concentration of VOC	No change
Temperature	-
Air-Oil Equilibrium Partition Coefficient	+
Mass transfer coeffcieint	-
Surface Area of Oil	-
Volume of Oil	+

Summary

- Initial Project Unknowns
  - Acetaldehyde and styrene were chosen
    - Levels in IVF labs
    - Different chemical structures
  - Starting levels of 500ppb
    - Cairo Consensus benchmark of ~400-800ppb

- Has now been shown that VOCs cannot be considered one blanket category for IVF laboratories

Reactive Oxygen Species Levels

- Both acetaldehyde and styrene groups had increased levels of ROS
  - Acetaldehyde group – fewer cells
  - Styrene group – greater degeneration

- Different pathways affected?

Apoptosis Levels

- Levels of apoptosis in both acetaldehyde- and styrene-exposed embryo groups were increased
  - Different growth patterns
- Different VOCs tested here were likely processed by the embryos differently
- VOCs are not one collective group

Genetic Changes

- ErbB4
  - ErbB4 did not amplify in our hands. Future studies are necessary to assess the role of ErbB4 in increasing LBR
- Sirt3
  - Sirt3 was not expressed differently in either VOC group as compared to the negative control group
  - Therefore, this is not likely the path that is affected in acetaldehyde or styrene exposure

Genetic Changes, continued

- p53
  - Acetaldehyde-exposed embryos were not significantly different in p53 expression compared to negative control group
  - Styrene-exposed embryos had a 6x downregulation of p53
  - Damaged embryos with downregulated p53 may continue to develop
    - Threshold of damage?
    - Secondary reaction?

Genetic Changes, continued

- VOCs affect different pathways than oxidative stress
- Different VOCs act in different ways on the embryos under VOC stress
- Signaling pathways in preimplantation embryos are expansive and still being deciphered

Summary of Conclusions

- Different VOCs likely affect *in-vitro* embryos in different ways
  - Acetaldeyhde: similar blast rate but less cellular
  - Styrene: Higher degeneration, but blasts are of similar cell numbers
- VOCs cannot be considered as one compound
  - Acetaldehyde: Increased ROS and apoptosis, no change in p53
  - Styrene: Increased ROS and apoptosis, 6x downregulation in p53
- Oil does not protect *in-vitro* embryos from VOC exposure
- Each VOC compound will likely transition through the IVF culture system differently and have different effects on the embryos

Future Directions

- Other VOCs – partitioning and mechanisms
- Lower concentrations than 445ppb
- Higher and lower protein levels
- Differing viscosities of oil
- Humidified vs dry incubators
- Time-lapse/undisturbed culture

Future Directions

- Antioxidants – would they alleviate VOC stress?
- Air quality – VOC specific filtration, for lab and for incubators
  - HEPA filters are not specialized enough to remove VOCs
  - HEPA filter pore size = 0.3µm

Study Limitations

- Genetic markers run with RT-qPCR
  - Gene Ontology may have given a larger variety of genes
- Only two VOCs studied
  - Only 1 dose of each VOC examined
- Only one time-point examined
- Larger *n*

Key New Knowledge

01

VOC stress affects embryos differently than other stressors

02

Each VOC likely affects embryos in different ways

03

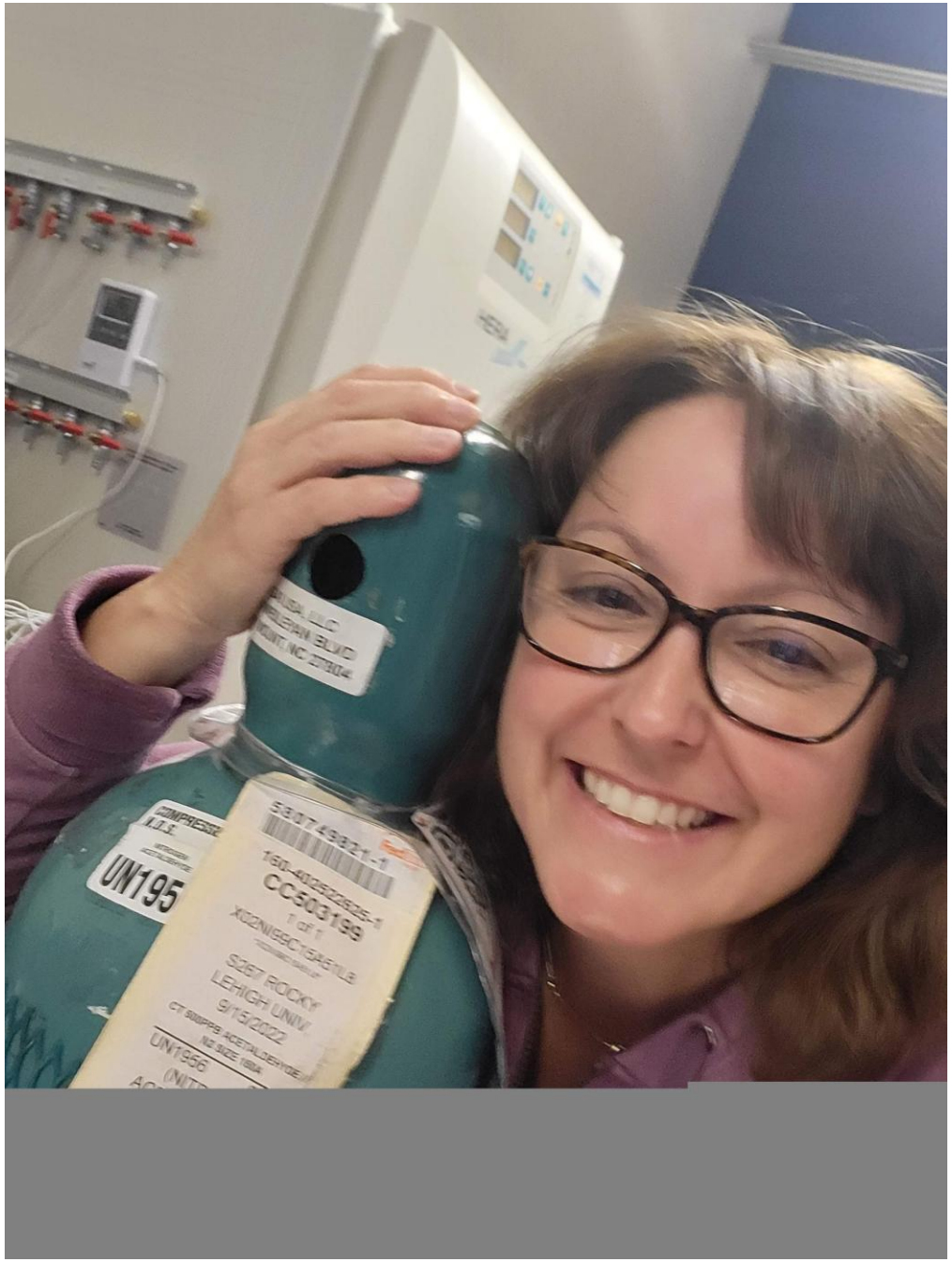
Oil does not protect *in-vitro* embryos from VOC exposure

Now we know:

- HEPA filters alone are not good enough to mitigate VOCs
- Oil overlays do not protect culture media and embryos from VOC exposure
- Offgassing consumables will not remove all the VOCs present
- You cannot be fast enough in the lab to keep VOCs from getting into your culture system

Thank you!

- My committee: Dr. Liang Yu, Dr. John Fox, Dr. Marlane Angle, Dr. Kimball Pomeroy, and Dr. Eva Schenkman
- Jason Russack, MS, Lehigh University
- ARTLAB in North Carolina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