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# Immuno-toxicological Response In Monocytes To E-cigarette Flavor Chemicals And E-liquids

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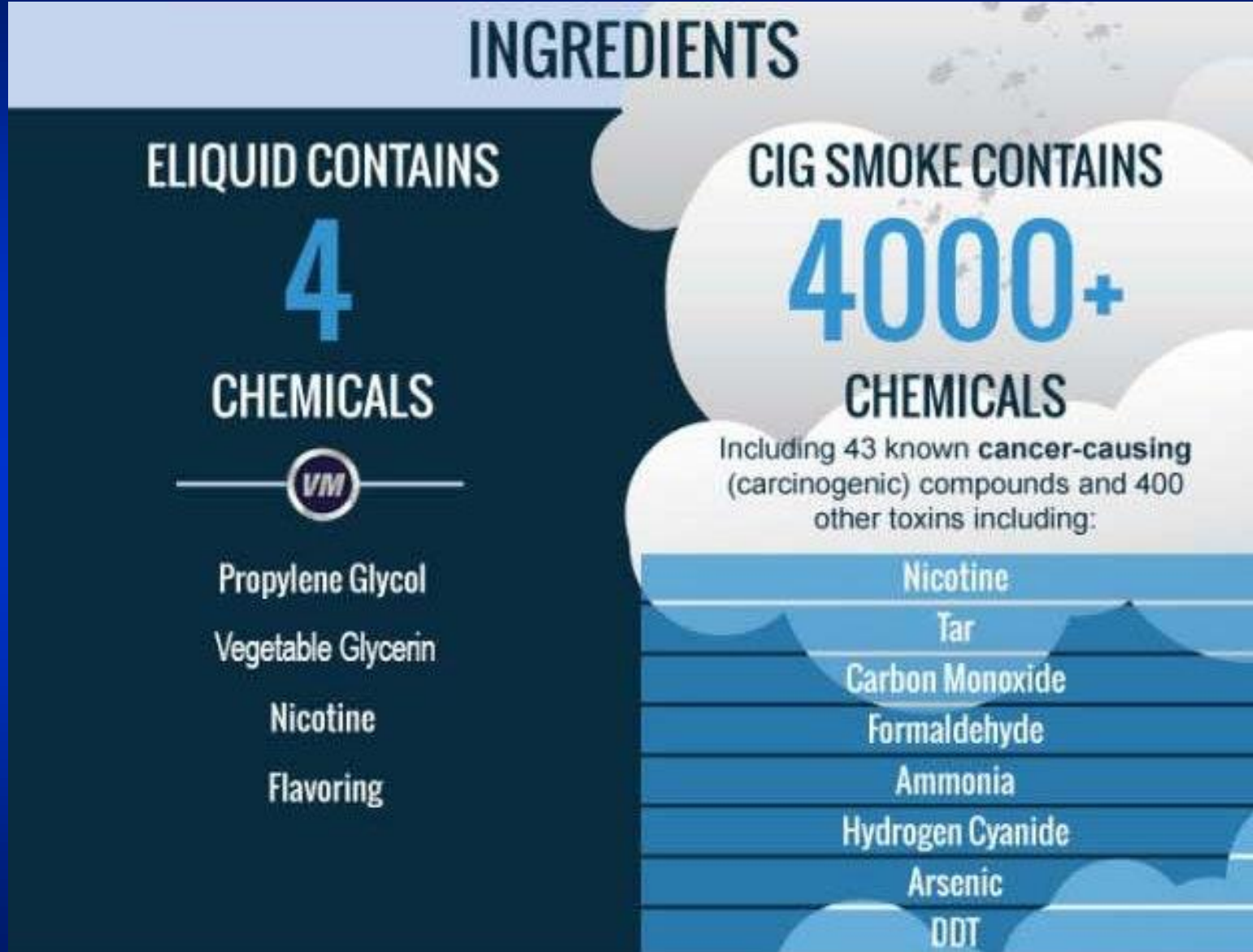
# Background

- E-cigarette sales in the U.S. were estimated at \$3.3 billion in 2015<sup>1</sup>
- Sales expected to grow nearly 50% per year through 2018<sup>1</sup>
- More than 460 different e-cigarette brands currently on the market<sup>2</sup>
- Over 7,700 unique e-cigarette flavors<sup>2</sup>
- American youth is the most exposed age group to e-cigarettes.
- E-cigarettes are claimed as a healthier alternative to traditional cigarettes, but the toxicological effects are not known.

• Sources: <sup>1</sup>K.L Marynak et al. Sales of Nicotine-Containing Electronic Cigarette Products: United States, 2015, AJPH.

• <sup>2</sup>Electronic cigarettes in the indoor environment, Chery Marcham, Embry Riddle Aeronautical University

# Misleading labeling by e-liquid producers



# Examples of available flavors on the market



## e-Liquid Flavors

MADE IN THE UNITED STATES  
LAB PRODUCED



### Exotic

- |                   |                            |
|-------------------|----------------------------|
| 1. Aphrodite      | 15. Iced Apple             |
| 2. Blue Palms     | 16. Gummy Punch            |
| 3. ButterHotz     | 17. Mythical Reserve       |
| 4. Dew Blast      | 18. Orange Sherbert        |
| 5. Fire Bomb      | 19. Papa Bear              |
| 6. Fruity Pebbles | 20. Papa Smurf             |
| 7. Cool Aid       | 21. Peguseuss              |
| 8. Pecan Pie      | 22. RazzBull               |
| 9. Infernomint    | 23. RazzLemade             |
| 10. Hive Nectar   | 24. Red Palms              |
| 11. Jamoconut     | 25. Red Hot Gummies        |
| 12. Kiwi Dream    | 26. ROKStar                |
| 13. Lime Sherbert | 27. Strawberry Fig         |
| 14. Lion's Blood  | 28. Sully                  |
|                   | 29. Watermelon-Bubblicious |



### Fruity

- |                      |                 |
|----------------------|-----------------|
| 30. Apple Snapz      | 39. Mogley      |
| 31. Blueberry        | 40. PMS         |
| 32. Grape            | 41. Pomegranate |
| 33. Greek Candi      | 42. Smoothie    |
| 34. Green Apple      | 43. Sour Berry  |
| 35. Juicy Lemon      | 44. Strawberry  |
| 36. Kiwi Strawberry  | 45. Tart Kiwi   |
| 37. Larry's Lemonade | 46. Watermelon  |
| 38. Lemon Meringue   |                 |



### Savory

- |                           |                            |
|---------------------------|----------------------------|
| 47. Apple Krisp           | 65. Coffee Delight         |
| 48. Apple Pie             | 66. Cotton Candy           |
| 49. Bahama Mama           | 67. Double Fudge Brownie   |
| 50. Banana Moon Pie       | 68. French Toast Krunch    |
| 51. Blue Belgian          | 69. Nanna Waffle           |
| 52. Blueberry Crumble     | 70. New England Cheesecake |
| 53. Boston Cream Pie      | 71. Ocean Blue Breeze      |
| 54. Bubblicious           | 72. Peaches N Cream        |
| 55. Buttery Nipple        | 73. Peanut Butter Crunch   |
| 56. Carmel Waffle         | 74. Reeses Cup             |
| 57. Cherry Banana Custard | 75. Sour Gummi             |
| 58. ChocoEclair           | 76. StarBux                |
| 59. Chocolate Almond      | 77. Strawberries N Cream   |
| 60. Chocolate Donut       | 78. Strawberry Graham      |
| 61. Cinaswirl Danish      | 79. Strawberry Ice Cream   |
| 62. Cinnamon Coffee Cake  | 80. Sugar Cookie           |
| 63. Cinnamon Roll         | 81. Vanilla Smoothie       |
| 64. Cinnamon Toast Crunch |                            |



### Tobacco

- |                    |                       |
|--------------------|-----------------------|
| 82. Blue-Bacco     | 86. Hannibal's Choice |
| 83. Camel Sands    | 87. Honeywood         |
| 84. Cherry Pipe    | 88. Latakia           |
| 85. Granny's Sweet | 89. Reds              |
|                    | 90. RY-More           |



### Menthol

- |                       |                     |
|-----------------------|---------------------|
| 91. Ande'z            | 97. Latakia Mintol  |
| 92. Berry Blast       | 98. Menthol Kings   |
| 93. Candy Cane        | 99. Paradise Cooler |
| 94. Cool Lights       | 100. RY-Mintol      |
| 95. Frescada          | 101. Turkish Mintol |
| 96. Kiwi Mint Breezer |                     |

## Nicotine Strengths

0mg 8mg 16mg 24mg

30mL Bottle - \$16.99

## Flavors of the Week 30mL Bottle for \$14.99!

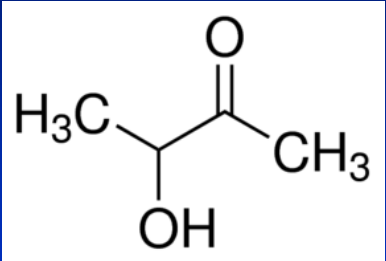
### 33. Greek Candi

Red and, Green Apple are back with a good friend to pump up the sweet!

### 81. Vanilla Smoothie

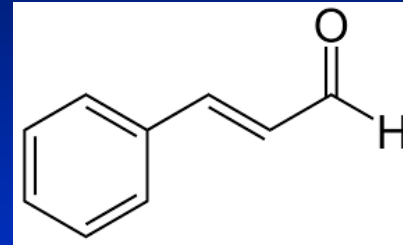
A creamy and, refreshing vape for those days of relaxation.

# Commonly used e-liquid flavoring chemicals



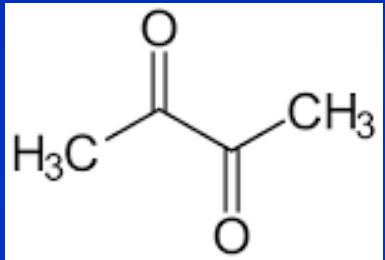
**Acetoin**

*Buttery flavor*



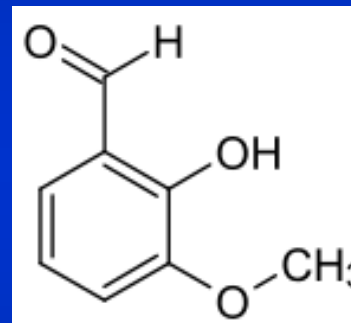
**Cinnamaldehyde**

*Cinnamon flavor*



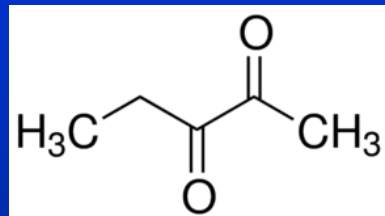
**Diacetyl**

*Buttery flavor*



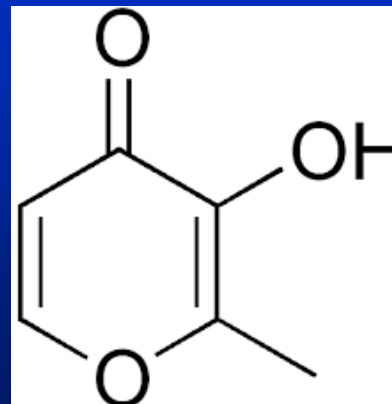
**O-Vanillin**

*Vanilla flavor*



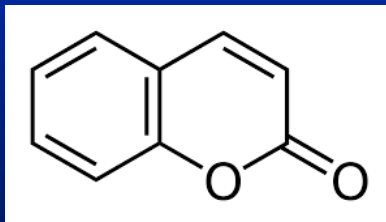
**2,3 Pentanedione**

*Buttery/cheesy flavor*



**Maltol**

*Malt flavor*



**Coumarin**

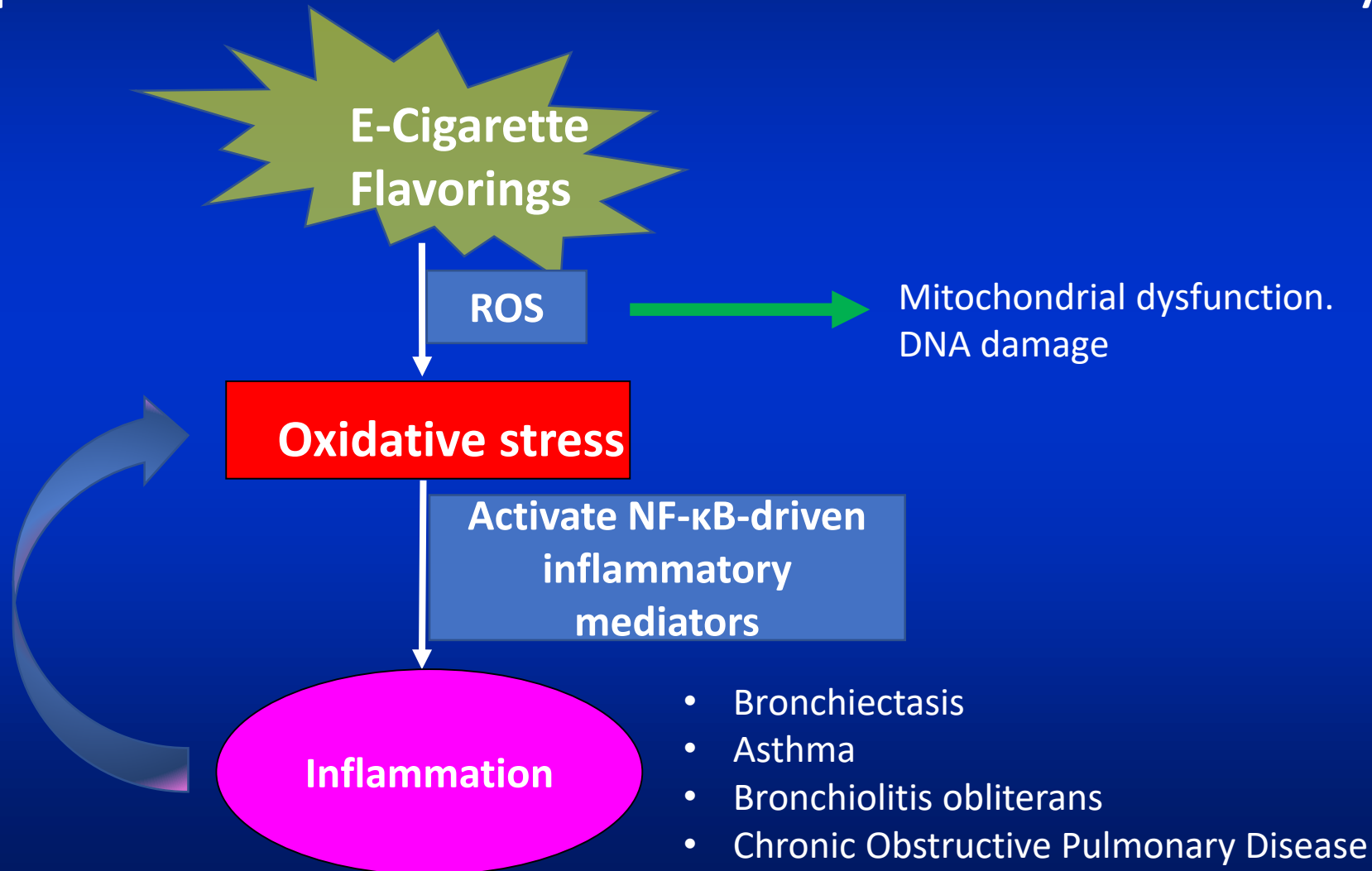
*Sweet flavor*



Flavors  
imparted by  
flavoring  
chemicals!

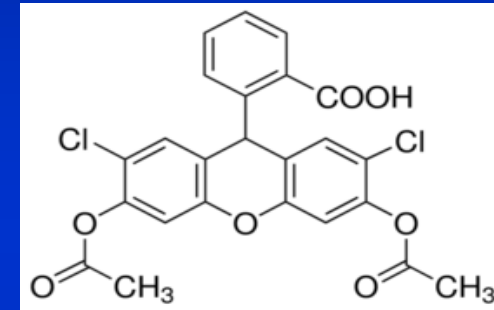
# Hypothesis

Flavors and flavoring chemicals produce reactive oxygen species (ROS) and the exposure to these flavors result in an inflammatory response.



# Cell-free ROS assessment in flavoring chemicals and flavors

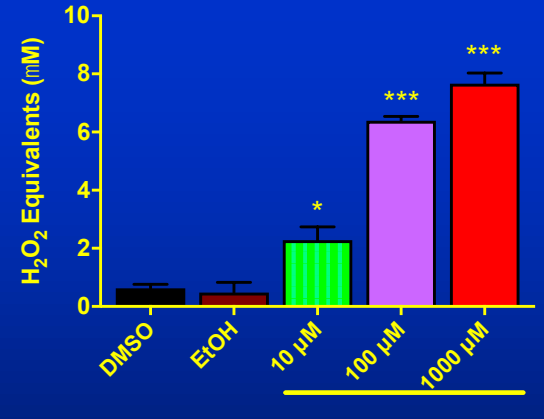
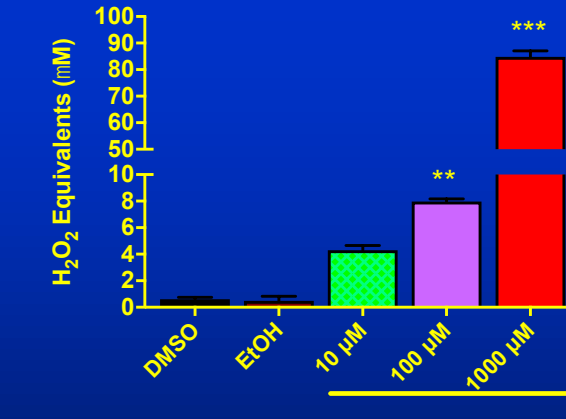
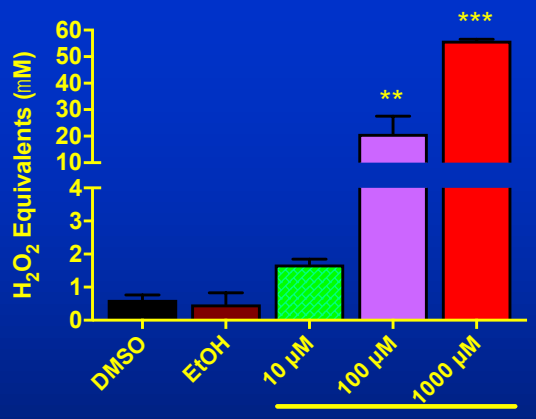
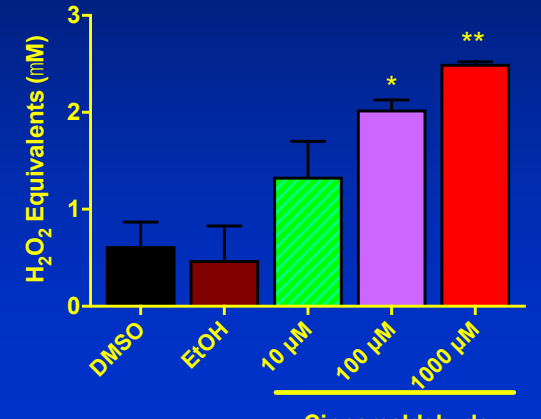
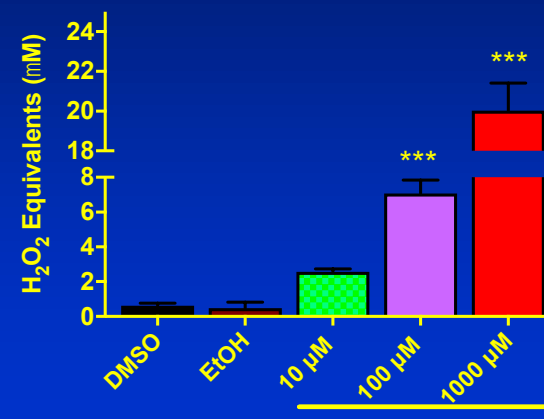
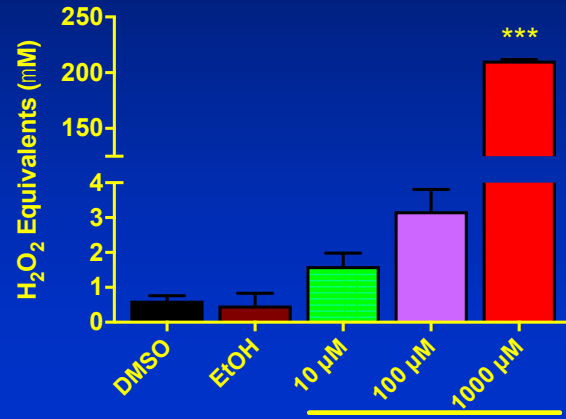
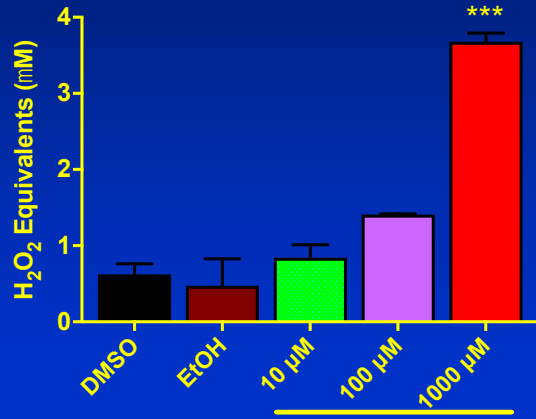
Principle: 2',7'-dichlorofluorescein diacetate (H<sub>2</sub> DCF-DA) fluorogenic probe.



E-liquid puffing was an adaptation of Behar profile:  
One puff/min for 10 minutes, puff duration 2.65 sec.

1. Prepare H<sub>2</sub>O<sub>2</sub> standards (0 though 50  $\mu$ M)
2. Addition of flavoring chemical or bubbling e-liquid aerosol (using SciReq InExpose through the DCFH solution).
1. Read fluorescence (ex 485 nm/em 535 nm)
2. Report OX/ROS as H<sub>2</sub>O<sub>2</sub> equivalents.

# Flavoring chemicals generate ROS



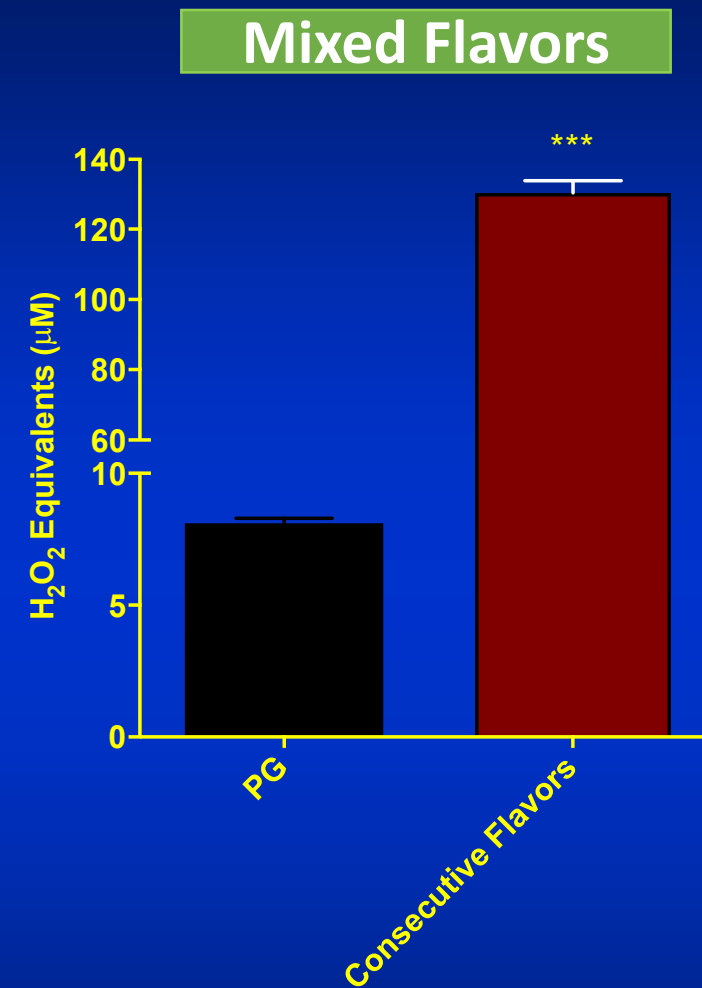
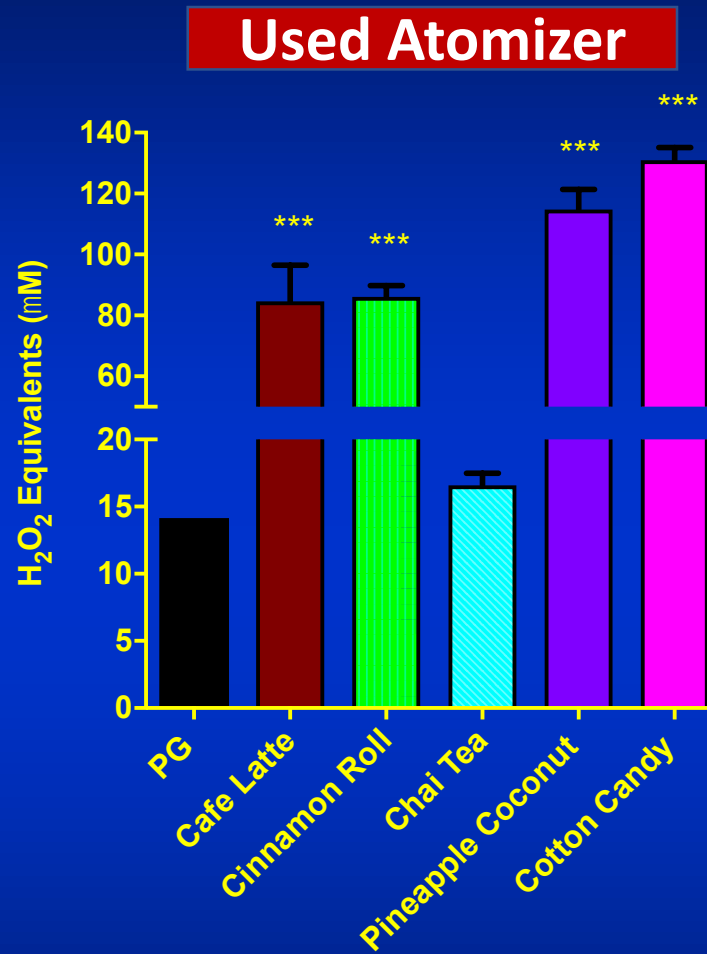
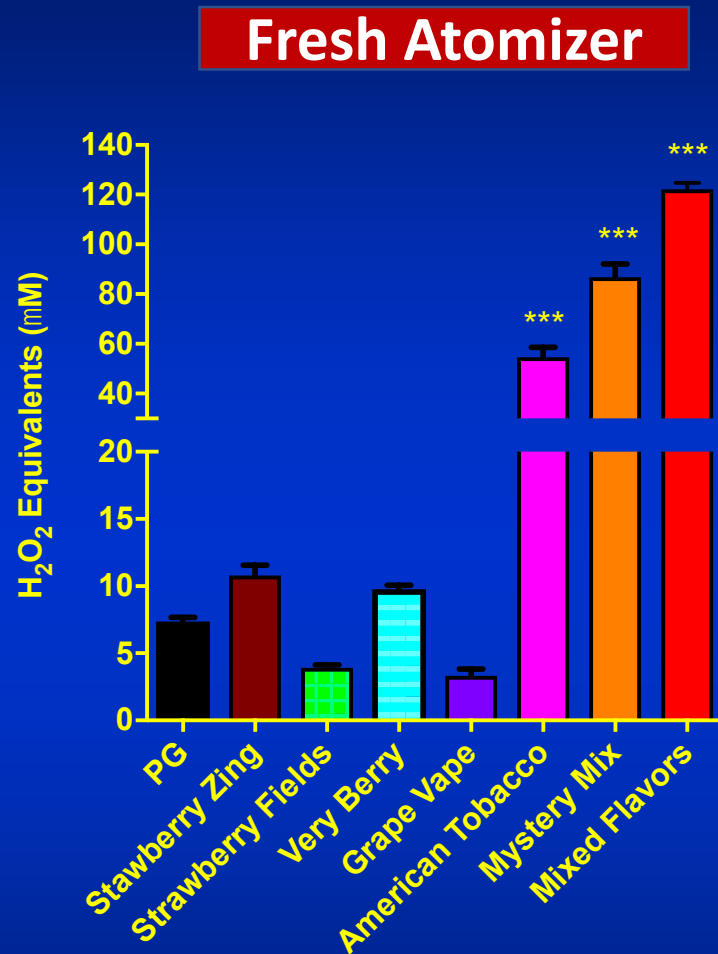
- Dose-dependent increase in ROS levels



# E-Liquids assessed in this study by flavor category

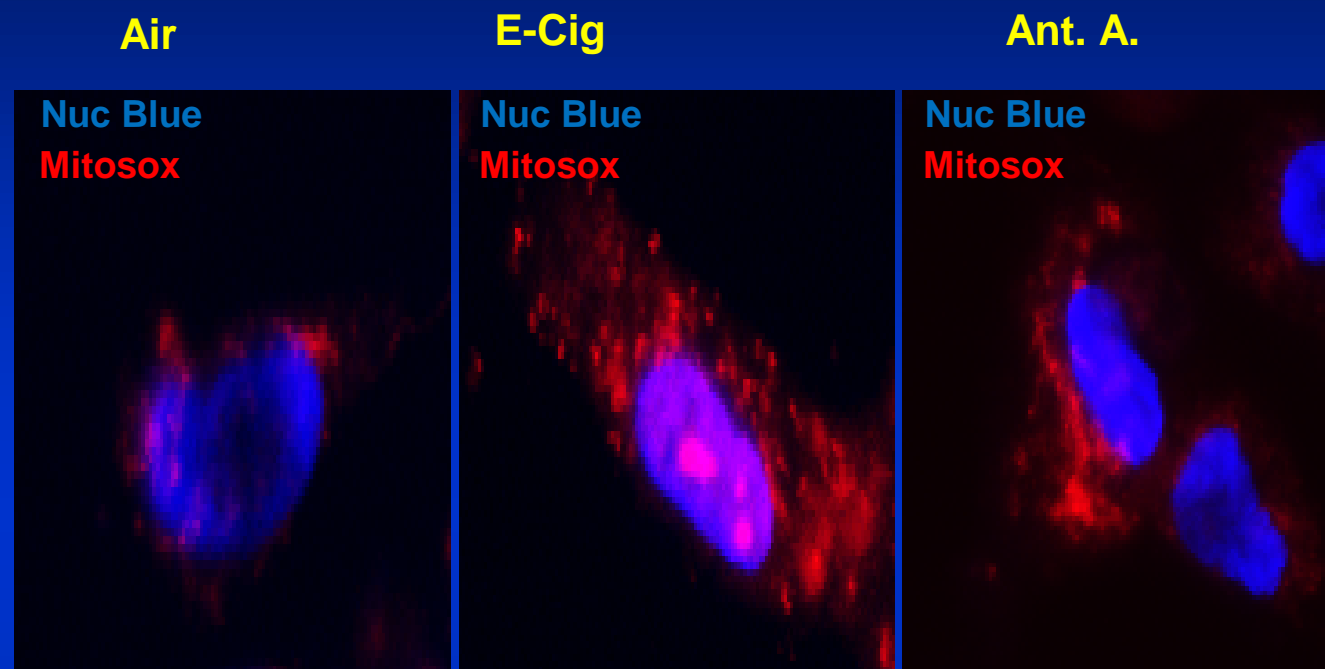
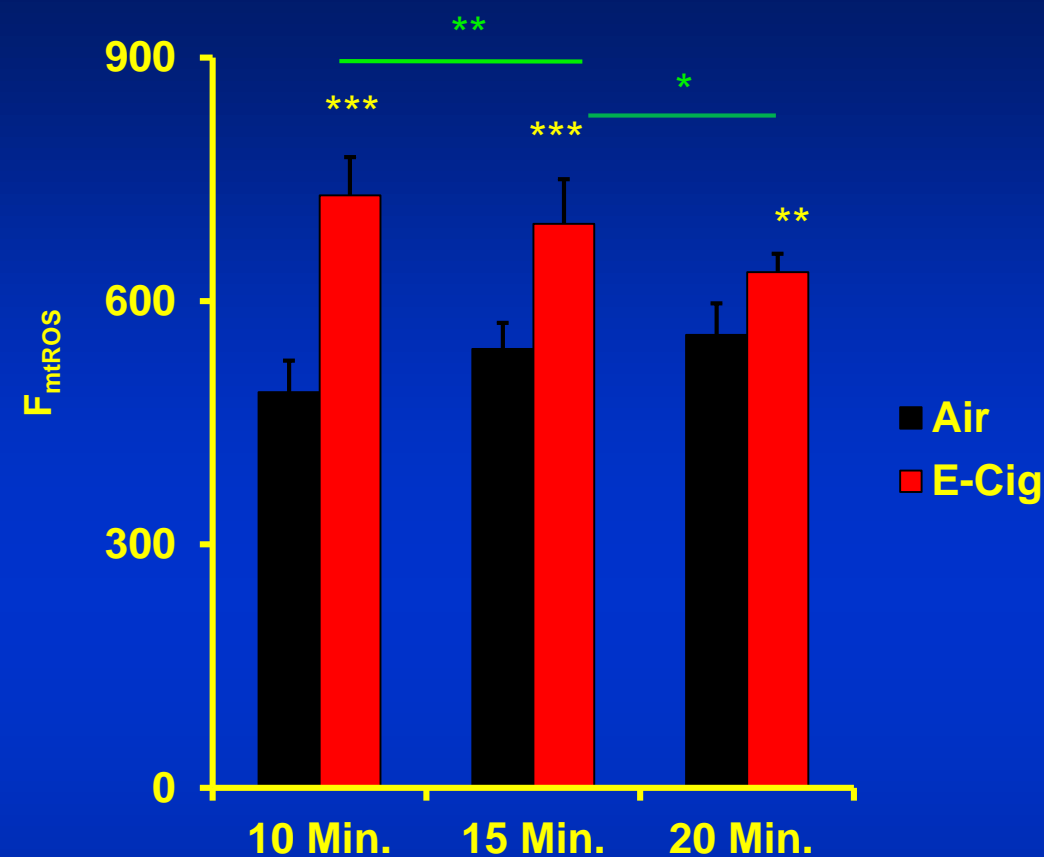
Alcohol	Berry	Cake	Candy	Coffee/Tea	Fruit	Menthol	Tobacco
Pineapple Coconut (Ecto)	Cherry (Smoker's Choice Rochester)	Apple Pie (Ecto)	Sweet Fishies (Ecto)	Cafe Royale (Cyber Liquids)	Mega Melons (Cuttwod)	Mystery Mix (Ecto)	American Tobacco (Ecto)
	Strawberry (Smoker's Choice Rochester)	Banana Nut Bread (Ecto)	Fruit Swirl (Ecto)	Cafe Latte (Ecto)	Tangerine (Smoker's Choice Rochester)		Classic Tobacco (Vape Dudes)
	Cherry (Ecto)	Cinnamon Roll (Vape Dudes)	Cotton Candy (Vape Dudes)	Chai Tea (Ecto)	Grape Vape (Vape Dudes)		Marbo (Upstate Vape)
	Very Berry (Vapor Drops)		Orange Creamsicle (Ecto)		Peaches N Cream (Drip)		9X Tobacco (Upstate Vape)
	Strawberry Fields (Vape Dudes)		Grape Jam (Vape Jam)		Pineapple Express (Drip)		Tobacco (Vapor Drops)
	Strawberry Zing (Vape Dudes)		Bird Brains (Cuttwood)		Melon Mania (Drip)		
	Berry Intense (Drip)		Euphoria (Cosmic Fog)		Peach (Ecto)		
					Plasma (Ecto)		

# Flavors generate ROS



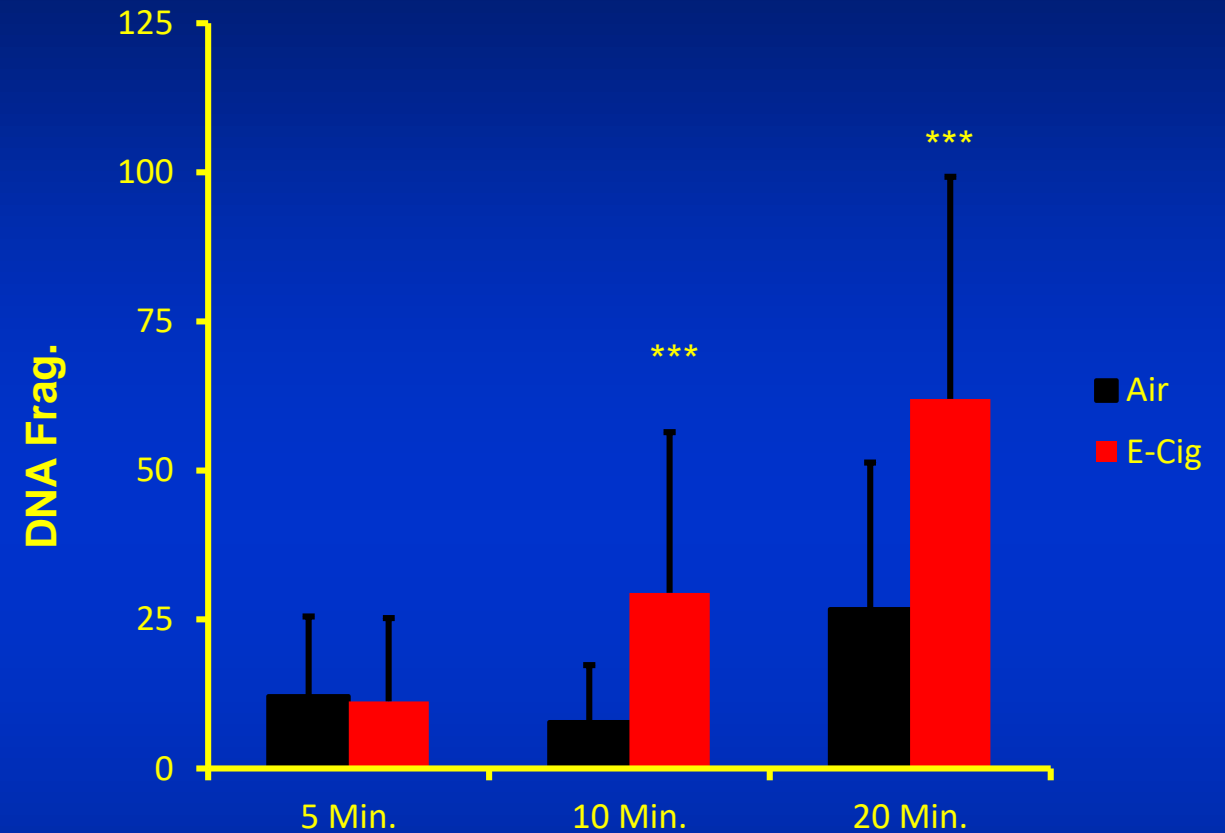
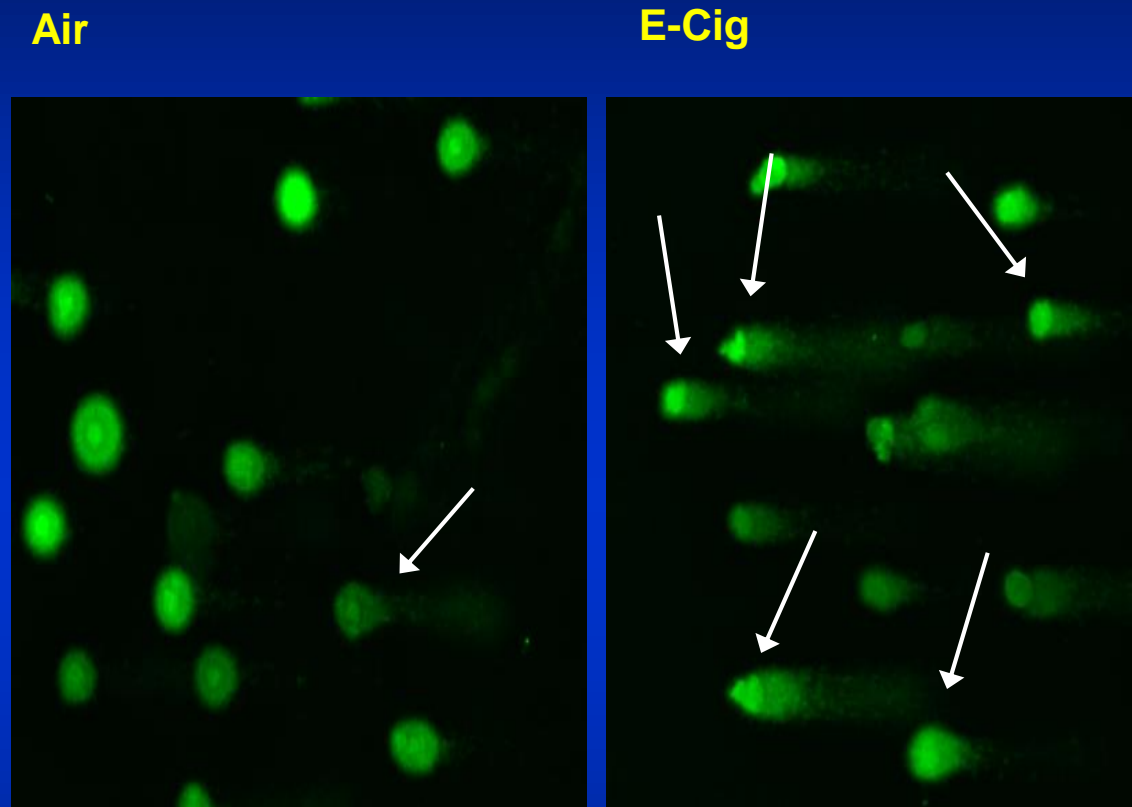
- Mixing flavors resulted in the highest ROS concentration.
- Consecutive flavors are comparable to mixing of flavors.

# E-cigarette Aerosol Causes Mitochondrial Dysfunction



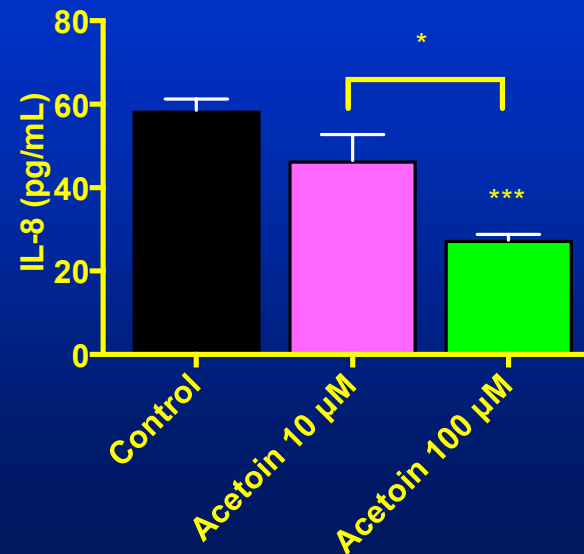
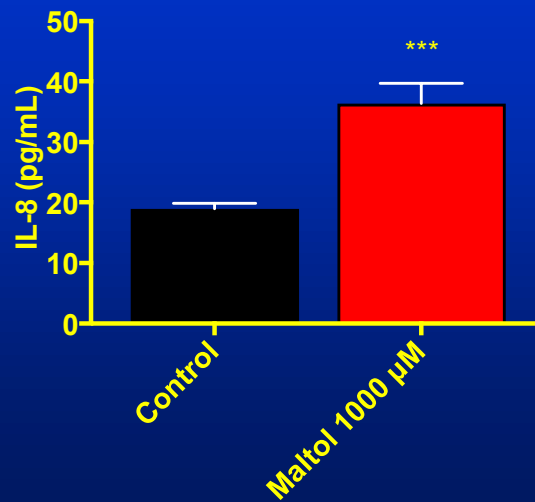
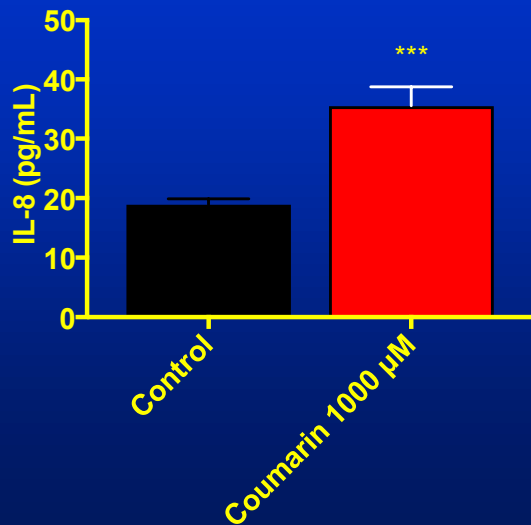
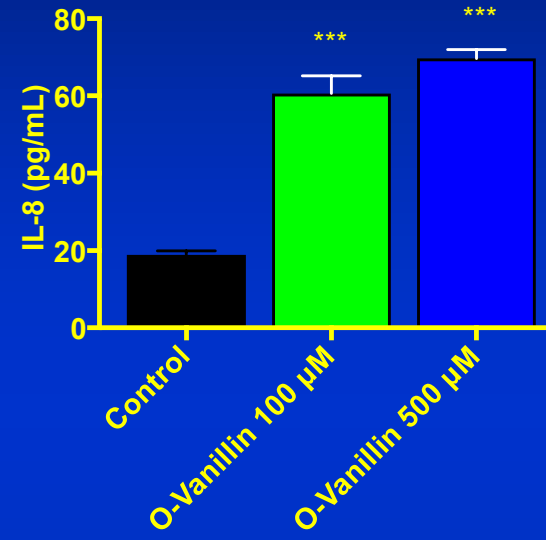
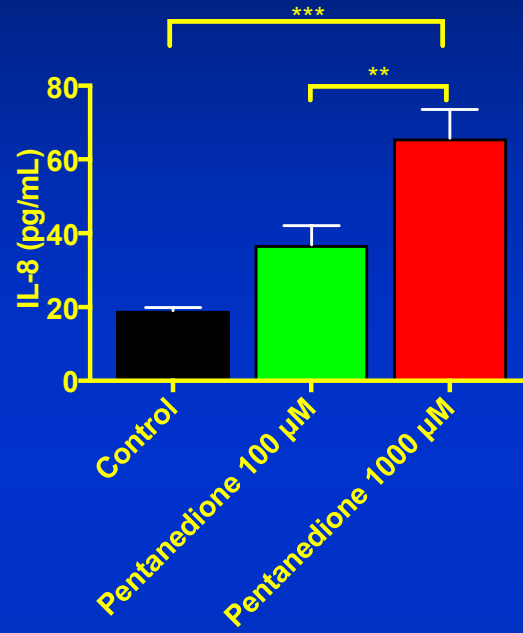
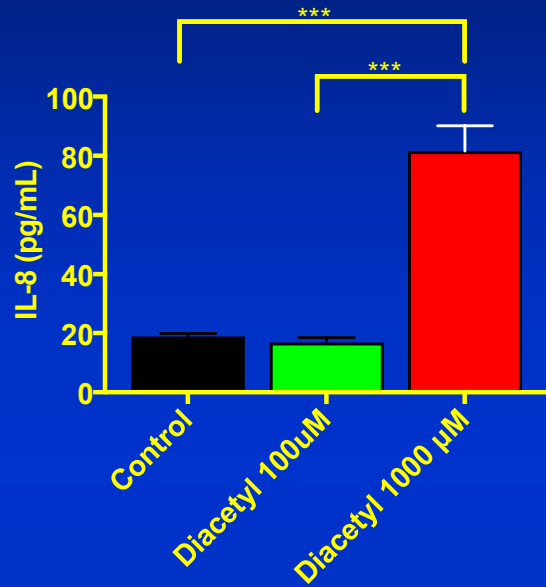
- Exposure to e-cig aerosol (classic tobacco) significantly increased mtROS level in HFL-1 cells. However, mtROS levels decreased with increased duration of exposure.
- Elevated mitochondrial ROS (in red) in e-cig group compared to the Air group (negative control). Antimycin A treatment (inhibition of oxidative phosphorylation) as positive control.

# E-cigarette Aerosol Causes DNA Damage



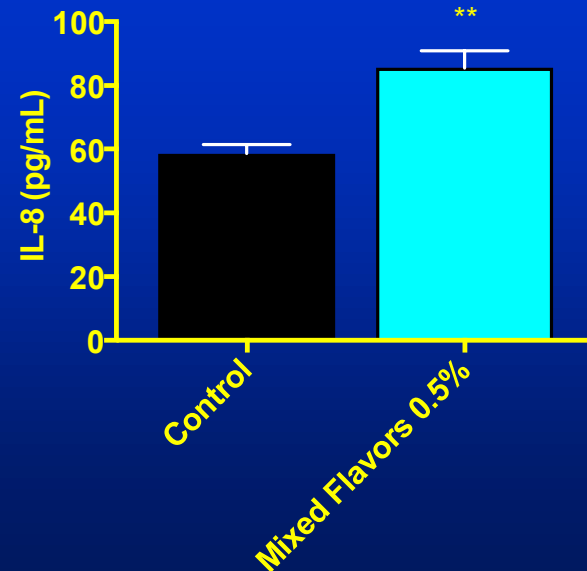
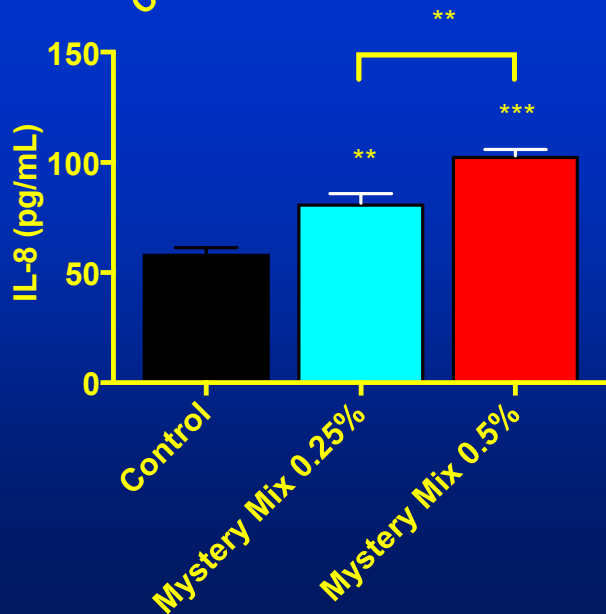
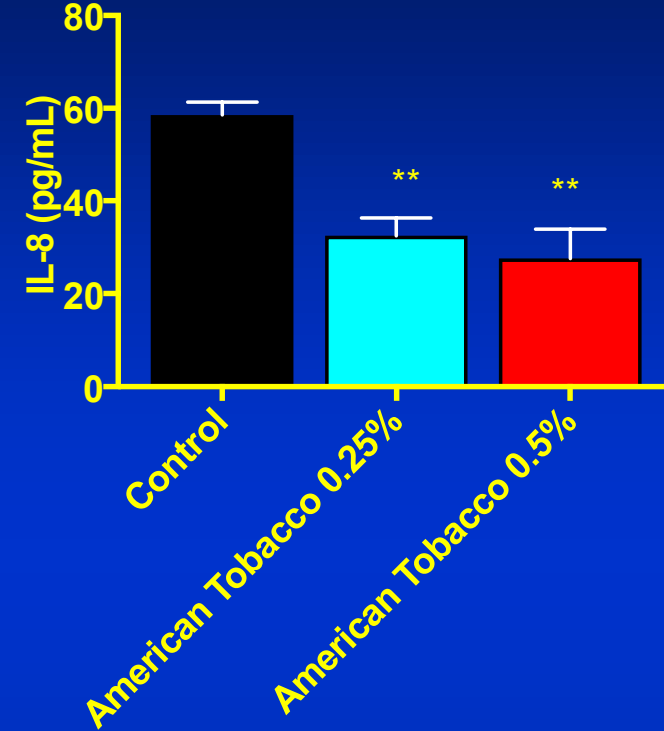
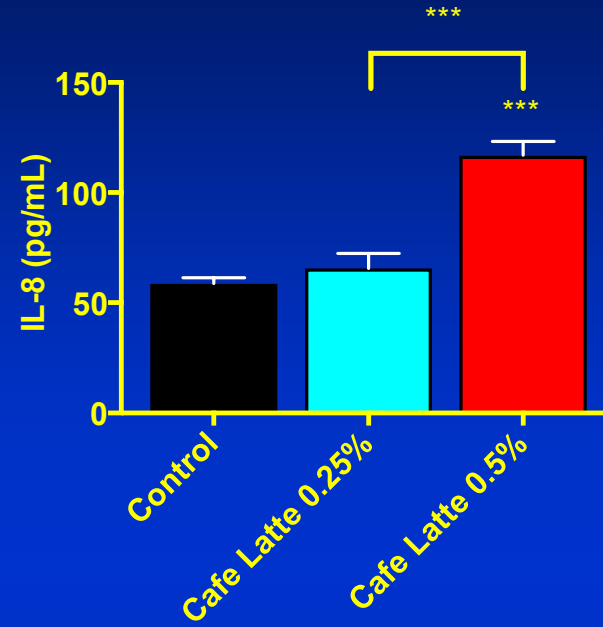
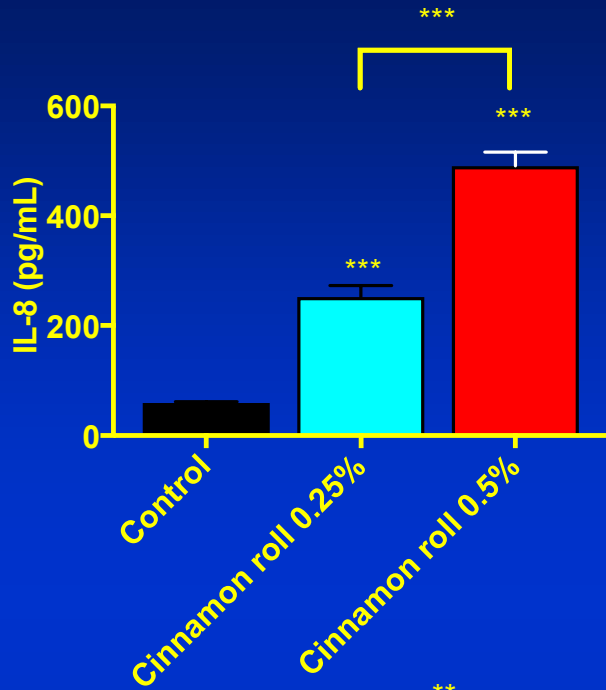
- Increased DNA fragmentation (Olive Tail Moment) in HFL-1 cells with increased duration of exposure.
- Exposure to classic tobacco e-cig aerosol significantly caused DNA damage.

# Flavor chemicals induce IL-8 chemokine in monocytes



- Diacetyl, Pentanedione, Vanillin, Coumarin, and Maltol induced IL-8 response.
- Acetoin suppressed IL-8 response.
- Acetoin, Pentanedione, Vanillin showed a dose-dependent response.

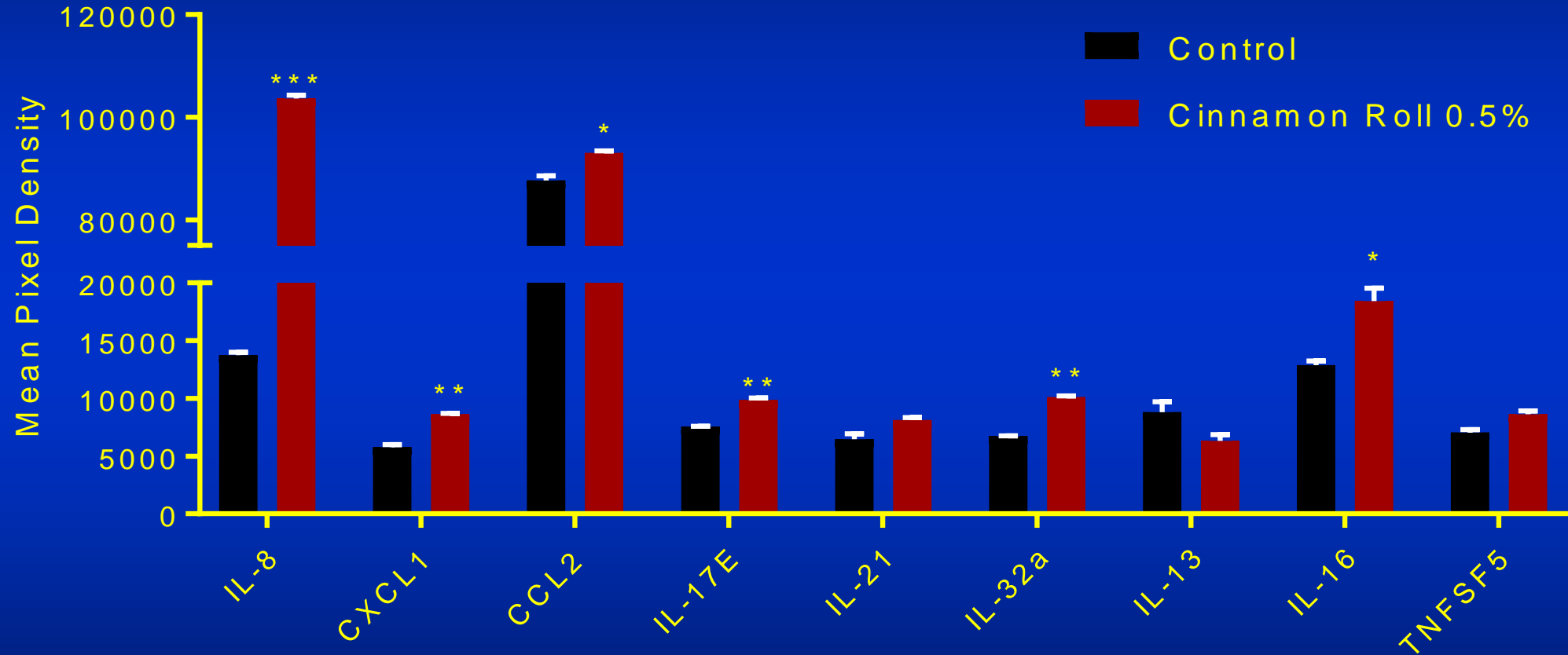
# Flavors induced IL-8 chemokine response in monocytes



- Cinnamon Roll, Café Latte, and Mystery Mix induced IL-8 response.
- American tobacco suppressed IL-8 response.
- Mixing flavors induced IL-8 significantly.

# Flavors induced pro-inflammatory cytokine response

Proteome profiler array on U937 conditioned media



- Cinnamon Roll significantly induced pro-inflammatory cytokine response.
- Cinnamon Roll lowered IL-13 (anti-inflammatory) response.

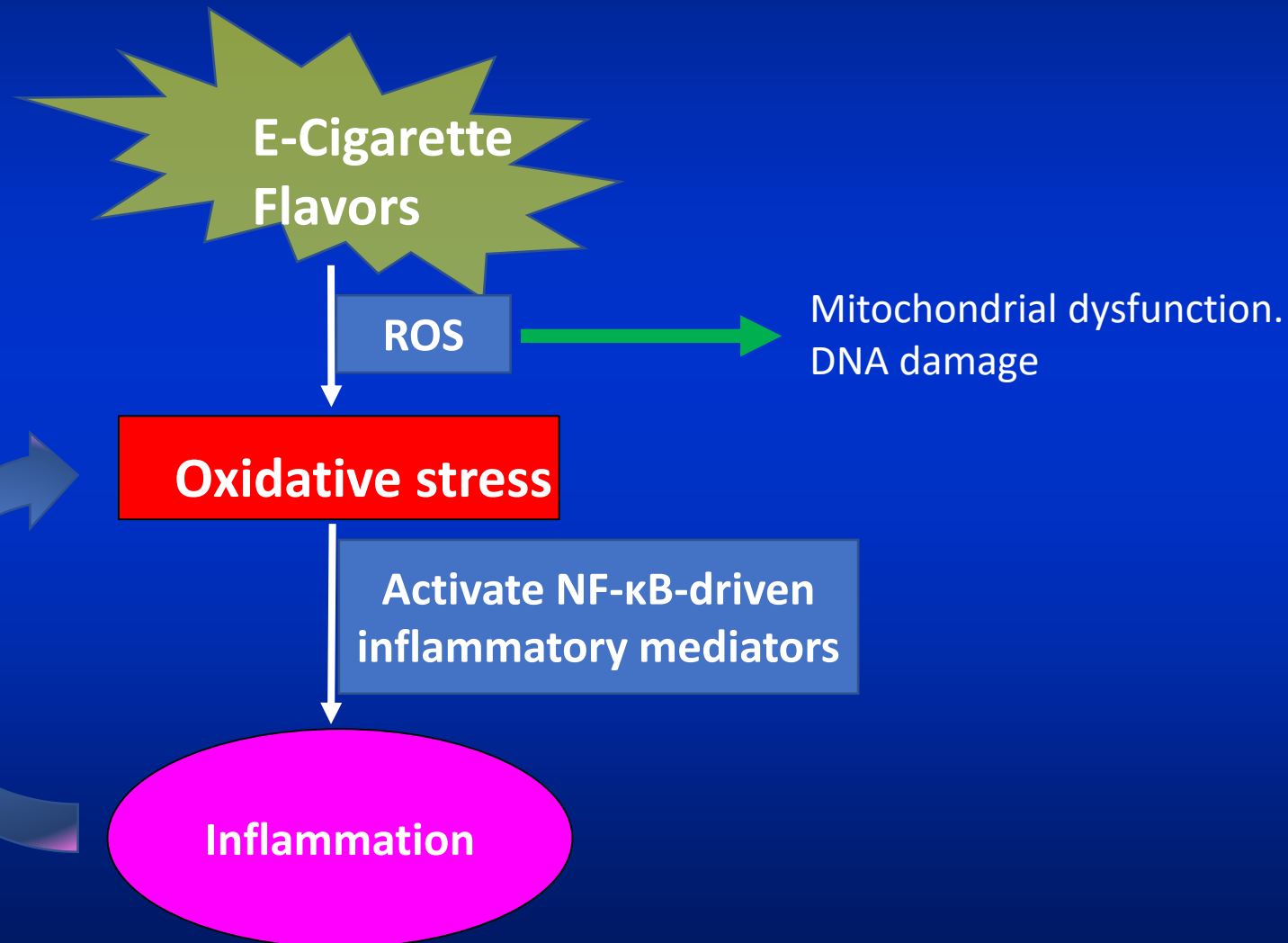
## Summary

- Flavors and flavoring chemicals generate significant ROS levels  
→ potential to cause oxidative stress.
- Exposure to e-cig aerosols induce mitochondrial dysfunction and DNA damage.
- Flavoring chemicals and flavors induce/augment an inflammatory response.
- Mixing e-liquids exacerbate/enhance these responses.



# Conclusions

- Flavors and flavoring chemicals induce an inflammatory response mediated by reactive oxygen species.



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ORIGINAL RESEARCH  
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## Inflammatory and Oxidative Responses Induced by Exposure to Commonly Used e-Cigarette Flavoring Chemicals and Flavored e-Liquids without Nicotine

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**Background:** The respiratory health effects of inhalation exposure to e-cigarette flavoring chemicals are not well understood. We focused our study on the immuno-toxicological and the oxidative stress effects by these e-cigarette flavoring chemicals on two types of human monocytic cell lines, Mono Mac 6 (MM6) and U937. The potential to cause oxidative stress by these flavoring chemicals was assessed by measuring the production of reactive oxygen species (ROS). We hypothesized that the flavoring chemicals used in e-juices/e-liquids induce an inflammatory response, cellular toxicity, and ROS production.

**Methods:** Two monocytic cell types, MM6 and U937 were exposed to commonly used e-cigarette flavoring chemicals; diacetyl, cinnamaldehyde, acetoin, pentanedione, o-vanillin, maltol and coumarin at different doses between 10 and 1,000  $\mu\text{M}$ . Cell viability and the concentrations of the secreted inflammatory cytokine interleukin 8 (IL-8) were measured in the conditioned media. Cell-free ROS produced by these commonly used flavoring chemicals were also measured using a 2',7'-dichlorofluorescein diacetate probe. These DCF fluorescence data were expressed as hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) equivalents. Cytotoxicity due to the exposure to selected e-liquids was assessed by cell viability and the IL-8 inflammatory cytokine response in the conditioned media.

**Results:** Treatment of the cells with flavoring chemicals and flavored e-liquid without nicotine caused cytotoxicity dose-dependently. The exposed monocytic cells secreted interleukin 8 (IL-8) chemokine in a dose-dependent manner compared to the unexposed cell groups depicting a biologically significant inflammatory response. The measurement of cell-free ROS by the flavoring chemicals and e-liquids showed significantly increased levels of  $\text{H}_2\text{O}_2$  equivalents in a dose-dependent manner compared to the control reagents. Mixing a variety of flavors resulted in greater cytotoxicity and cell-free ROS levels compared to the treatments with individual flavors, suggesting that mixing of multiple flavors of e-liquids are more harmful to the users.

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