Smokeless Tobacco’s Effects on Yeast’s Mutagenesis Rate
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2nd Year at PJAS
Smokeless Tobacco

• There are many different kinds of smokeless tobacco. (ex. chewing, dipping, etc.)
• Subject: Dipping tobacco
  ➢ Carcinogen
  ➢ Contains nicotine, which causes addiction
  ➢ Banned in several countries (not the United States)
Previous Studies

• Show that smokeless tobacco affects the cardiovascular system less than smoked tobacco
• Increased risk for mouth cancer
Skoal Ingredients

Some possibly harmful ingredients:

• Tobacco
  – N-Nitrosonornicotine (NNN)
    • Epidemiological studies from the USA, India, Pakistan, and Sweden provide sufficient evidence that smokeless tobacco causes oral cancer in humans
  – Benzopyrene
    • Found to play a role in the development of lung cancer

• Ethyl Alcohol
  – Ethanol is not a carcinogen. However, the first metabolic product of ethanol in the liver, acetaldehyde, is toxic, mutagenic, and carcinogenic
Question

• Do the ingredients in Skoal Original Fine Cut Wintergreen Smokeless Tobacco cause it to have significant mutagenic properties?
Yeast

- Commonly used model
- Tolerant and safe to culture
- Has similar reproduction, metabolism, and chemistry as other more advanced eukaryotic cells
  - Saccharomyces cerevisiae
- Special strain, unable to produce Lysine
Lysine

- Lysine’s codons are AAA and AAG

- There are defined **minus lysine** yeast mutants used in research.

- Lys 2 mutants are missing an enzyme function within the lysine biosynthesis pathway.

- Result – cells require lysine supplementation

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Saccharopina

Lysine

NADPH; NADP; a-Ketoglutarate

a-Ketoglutarate

AcCoA

CoA

HC Synthase

Homocirlate

Water

Homoaconitase LYS7

Homoisocitrate LYS4

a-Ketoadipate LYS12

a-Aminoadipate

a-Aminodipate

Semialdehyde LYS2

Glutamate

a-Ketoglutarate

ATP

PP

NADPH

NADP

Glutamate

NADPH

NADP

Water
Ames Test

- Developed to test the mutagenic and anti-mutagenic properties of various chemicals by Bruce Ames in 1970s.

- Ames used a minus histidine mutant Salmonella (single point substitution). Bacteria cannot synthesize histidine due to this mutation.

- Exposure to suspected mutagen correlated with increased reversion (mutation) rate.

- Visible colonies appearing on complete (-His) media evidence of mutation through reversion.

- Obviously, a lower limit on mutation rate, because only 1 DNA site in genome assayed.
Ames Test

- tryp-bacteria + chemical SER

petri dish with medium that allows only tryp+ mutants to grow

possible results:

- no colonies, chemical not a mutagen
- few colonies, chemical a mutagen
- many colonies, chemical a powerful mutagen
Modified Ames Test

• (-) Lys Yeast – Eukaryote

• The number of reverted colonies of yeast can be correlated with the rate of mutation.

• A reversion at that point can result in a reversion back to wild type yeast (lys +)

• Mutagen substitution – Smokeless Tobacco instead of heat
Ultraviolet Rays

- Light waves that have shorter wavelengths, thus greater energy, than visible light
- They range from 400nm to 10nm
- Given off from the sun but most are absorbed by the ozone layer
- Mutagen – Direct DNA Damage
Objective

- To assess the mutagenicity of smokeless tobacco (Skoal Original Fine Cut Wintergreen)
Hypotheses

- Null Hypothesis: Smokeless Tobacco will **not** have a significant effect on Yeast's Mutagenesis Rate

- Alternate Hypothesis: Smokeless Tobacco **will** significantly increase Yeast’s Mutagenesis Rate.
Materials

• (-) Lysine YEPD agar plates (1% yeast extract, 2% peptone, 2% dextrose, 1.5% agar)
• UV Light Hood (LD-50 on Yeast is 30 seconds)
• Sterile dilution fluid [SDF] (10mM KH2PO4, 10mM K2HPO4, 1mM MgSO4, .1mM CaCl2, 100mM NaCl)
• Klett spectrophotometer
• Sterile pipette tips and Micropipettes
• Vortex
• Sidearm flask
• Spreader bar
• Ethanol
• Micro burner
• (-) Lysine Saccharomyces cerevisiae (John Wolford lab, CMU)
• Rubber Gloves
• Test tubes
• Microtubes
• Test Tube Rack
• SDF Test Tubes
• Skoal Fine Cut Smokeless Tobacco
Procedure

1. A strain of yeast (-) Lys phenotype was grown for 2 days in YEPD media
2. A series of washes with SDF were performed on the sterile yeast pellet to remove any residual nutrients (lysine)
3. A 10% chewing tobacco extract was sterilized through a 0.22 micron syringe filters
5. The pellet in SDF was re-suspended
6. The following ingredients were pipetted into sterile microtubes. (Percents are by volume compared to stock solution)
## Test Tube Ingredients

<table>
<thead>
<tr>
<th></th>
<th>Water</th>
<th>Variable</th>
<th>Yeast</th>
<th>Volume</th>
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<tbody>
<tr>
<td>0%</td>
<td>0.8 mL</td>
<td>0 mL</td>
<td>0.2 mL</td>
<td>1 mL</td>
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<tr>
<td>0.1%</td>
<td>0.79 mL</td>
<td>0.01 mL</td>
<td>0.2 mL</td>
<td>1 mL</td>
</tr>
<tr>
<td>1%</td>
<td>0.7 mL</td>
<td>0.1 mL</td>
<td>0.2 mL</td>
<td>1 mL</td>
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</tbody>
</table>
Procedure (Continued)

7. The cells were allowed to sit for 15 min

8. 0.2 mL aliquots were spread onto 60 complete (-) Lys (10 each) agar plates (necessary to show cells that have reverted through mutation to wild type (+) lys)

9. The plates in tubes 7-12 were exposed to UV light for either 20, 40, or 60 seconds

10. All plates were allowed to incubate for 3 days at 32° C

11. The colonies were counted and recorded. Each colony assumed to have arisen from 1 cell
Results

Smokeless Tobacco’s Effects of Yeast’s Mutagenesis Rate

Number of colonies

Concentration of Smokeless Tobacco

0% | 0.1% | 1%
---|---|---
3 | 4 | 9

P-Value = 2.88E-09
UV’s Effects on Yeast’s Mutagenesis Rate

Number of Colonies

UV Exposure Time

0 sec  20 sec  40 sec  80 sec

P-value = 4.13E-12
Smokeless Tobacco’s Effects of Yeast’s Mutagenesis Rate

P-Value=3.08E-22

Number of Colonies

Concentration of Smokeless Tobacco

UV Exposure Time

0% 0.10% 1% 20 sec 40 sec 80 sec
Dunnett’s Test

- Alpha = .05  
  T-crit = 2.99

<table>
<thead>
<tr>
<th>Test</th>
<th>T-Value</th>
<th>Interpretation</th>
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<tbody>
<tr>
<td>0% vs. 0.1%</td>
<td>0.249118</td>
<td>Insignificant</td>
</tr>
<tr>
<td>0% vs. 1%</td>
<td>3.238665</td>
<td>Significant</td>
</tr>
<tr>
<td>0 sec vs. 20 sec</td>
<td>12.25417</td>
<td>Significant</td>
</tr>
<tr>
<td>0 sec vs. 40 sec</td>
<td>17.38521</td>
<td>Significant</td>
</tr>
<tr>
<td>0 sec vs. 80 sec</td>
<td>6.903762</td>
<td>Significant</td>
</tr>
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</table>
Conclusions

• The Null Hypothesis can be rejected for the 1% smokeless tobacco group, and also the 20 sec, 40 sec, and 80 sec UV light groups.

• The 1% results can be contributed to smokeless tobacco’s mutagenic properties.

• The 0.1% smokeless tobacco group did not have a significant effect.

• The lower colony counts in the 80 sec group may be due to UV light toxicity.

• UV light appears to be a stronger mutagen than smokeless tobacco.
Limitations and Extensions

- Limitations
  - Slightly out-of-synced plating which leads to slightly different exposure times to smokeless tobacco
  - Inability to control the exact amount of cells on each plate (minor difference overshadow by massive amount of cells)
  - Slight positioning differences in UV Oven
  - Inability to account for cell deaths due UV Light

- Extensions
  - Different model
  - Reduce lag time with lab assistants
  - Trypan Blue Assay to account for cell deaths
  - A future experiment testing smokeless tobacco’s effects on mammalian and cancerous cell lines to see if it promotes uncontrollable growth.
References

- http://dontdip.tamu.edu/ingredients.htm
- http://www.sciencemag.org/content/274/5286/430.abstract?ijkey=7dd94e096ea549bac90bce0ec51acb6422cbb1a4&keytype2=tf_ipsecsha