

Toxicological evaluation of smokeless tobacco: 14- and 28-day feeding studies

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Introduction

- RJRT has introduced smokeless tobacco products in the US market and has conducted rodent toxicology studies in support of such products
- This poster presents 14- and 28-day dose-range finding studies. Goals were:
 - Define the toxicological effects of a smokeless tobacco blend and its aqueous extract administered to rodents in diet
 - Identify NOAEL [no body weight (bw) ↓]; LOAEL (minimal bw ↓); MTD (~10% bw or bw gain ↓) for subchronic studies
- 5 studies: 3x14-day (non-GLP); 2x28-day (GLP)
- Initially, rats/mice: tested at same doses
- However, sensitivity: mice < rats; thus, 2nd 14-day mouse study: ↑ doses
- Combined, dose ranges (mg nicotine/kg bw/day) tested were:
 - 14-day palatability studies: 0-240 (rats); 0-2400 (mice)
 - 28-day dose range-finding studies: 0-20 (rats); 2-200 (mice)

Materials and methods

Test articles, controls, and diets

- Test articles: smokeless tobacco blend (B); aqueous extract of tobacco blend (E)
- Aqueous extract (1:8): extracted at 100°F (37.8°C) for 1 hr => evaporated at 37.8°C => diluted to 38% solids
- Negative Control (C): diet; Positive Control: nicotine tartrate (NT)
- Abbreviations: males (M), females (F); doses, e.g., 0-2 mg nicotine/kg bw/day (0-2)
- Test articles characterization and stability:
 - Analytes of interest (e.g., nicotine, nitrosamines, phenols, PAHs, carbonyls, metals, agrochemicals)
 - Microbial endpoints (bacteria, yeast, mold, mycotoxins, e.g., aflatoxin)
- Test articles and positive controls: formulated into diets; matched for nicotine
- Diet base (all formulations): NTP-2000
- Diet formulation: weekly (14-day studies); monthly (28-day studies)
- Diet storage: room temperature
- Diets analyses-nicotine: formulation accuracy, stability, homogeneity

Experimental design and dose groups

14-day studies

- Determine palatability of diets with/without B, E, NT
- Define doses for 28-day studies
- N: 5 males/group (except sentinels: 10/group; mouse study 2 Control: 10/group)

28-day studies

- Determine toxicity of diets with/without B, E, NT
- Define doses for 90-day studies
- Assigned animals to: 1) core or 2) toxicokinetics (TK) groups
- Included Pair-fed Controls (to High Doses; rat study) to determine if B, E, NT induce additional effects when matched for the same amount of food consumed
- N: rat and mouse core: 10/sex/group; rat TK: 6/sex/group; mouse TK: 5 (Control) or 23/sex/group (treatments)

14-day studies

| # | Group | Target dose (mg nicotine/kg bw/day) | |
|----|-------------------|-------------------------------------|---------|
| | | Rat, Mouse 1 | Mouse 2 |
| 1 | Control | 0 | 0 |
| 2 | Blend | 0.2 | 40 |
| 3 | Blend | 2 | 80 |
| 4 | Blend | 4 | 160 |
| 5 | Blend | 8 | 240 |
| 6 | Blend | 20 | 400 |
| 7 | Blend | 40 | - |
| 8 | Extract | 0.2 | 40 |
| 9 | Extract | 2 | 80 |
| 10 | Extract | 4 | 160 |
| 11 | Extract | 8 | 240 |
| 12 | Extract | 20 | 400 |
| 13 | Extract | 40 | - |
| 14 | Nicotine tartrate | 2 | 40 |
| 15 | Nicotine tartrate | 8 | 80 |
| 16 | Nicotine tartrate | 20 | 160 |
| 17 | Nicotine tartrate | 40 | 240 |
| 18 | Nicotine tartrate | - | 400 |
| 19 | Sentinels | 0 | 0 |

28-day studies

| # | Group | Target dose (mg nicotine/kg bw/day) | |
|----|--|-------------------------------------|-------|
| | | Rat | Mouse |
| 1 | Control | 0 | 0 |
| 2 | Nicotine tartrate | 20 | 200 |
| 3 | Control diet pair-fed to nicotine tartrate | 0 | - |
| 4 | Blend | 0.2 | 2 |
| 5 | Blend | 2 | 20 |
| 6 | Blend | 8 | 80 |
| 7 | Blend | 20 | 200 |
| 8 | Control diet pair-fed to blend | 0 | - |
| 9 | Extract | 0.2 | 2 |
| 10 | Extract | 2 | 20 |
| 11 | Extract | 8 | 80 |
| 12 | Extract | 20 | 200 |
| 13 | Control diet pair-fed to extract | 0 | - |
| 14 | Sentinels | 0 | 0 |

Animals

- Rats: Wistar Han; mice: Swiss Webster/CD-1 (5-7 weeks old, Charles River Laboratories, Raleigh, NC)
- Before exposure: assigned healthy animals to treatment groups by sex and bw (PATH/TOX, 4 2 2, Xybio Medical Systems Corporation, Cedar Knolls, NJ)
- Animals: research conducted in AAALAC-accredited facility; maintained according to the Guide for the Care and Use of Laboratory Animals
- Food and fresh municipal water: supplied *ad libitum*

14-Day studies: endpoints

- Monobundity and mortality checks: 2x/day, 5 days/week, 1x/day weekends
- Detailed clinical observations: before exposure start, then 2x/week
- Individual body weights: before exposure start, then 1x/day, at termination
 - 2nd 14-day mouse study: if treated mean group bw ↓ >20% (2 consecutive days) => mice were fed Control diet
- Food consumption: 1x/day

Statistics

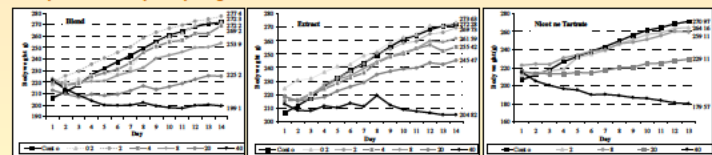
- Data (e.g., bw, food consumption) analyses: ANOVA, then Bartlett's test for variance homogeneity
- Homogeneous data: Dunnett's test
- Non-homogeneous data: Cochran and Cox's modified t-test
- Significance: 5%, 2-sided criteria
- Neurobehavioral data: SAS System (SAS Institute Inc., Cary, NC)
- Comparisons: Control vs Positive Control, Control vs Test Articles, Positive Control vs High Dose Test Articles, Blend vs Extract (corresponding doses), and Pair-fed Controls vs corresponding High Dose groups

28-Day studies: endpoints

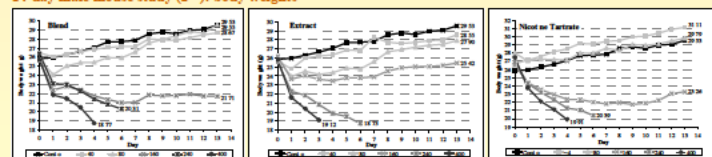
- Monobundity and mortality: 2x/day
- Detailed clinical examinations: before exposure start, then 1x/week (core), at necropsy (core)
- Body weights: before exposure, then 2x/week (core), at necropsy (core) TK: 1x/week
- Food consumption (core): 1x/week
- Food consumption rats, Pair-fed Controls (groups 3, 8, 13) and corresponding high dose groups (2, 7, 12): 1x/day
- Neurobehavioral FOB assessment: ~midpoint; 5 animals/sex/group (core, except pair-fed)
- Exposure control: plasma nicotine and cotinine concentrations. Separate TK studies:
 - Phase 1 (~midpoint): determined C_{max}/T_{max} (6 time points); Phase 2 (end): aimed for C_{max} at T_{max}
- End of study: clinical chemistry, hematology, coagulation parameters, tissues, organ weights (and related parameters)-assessed (core) in manner typical for classical 28-day toxicology studies

Results

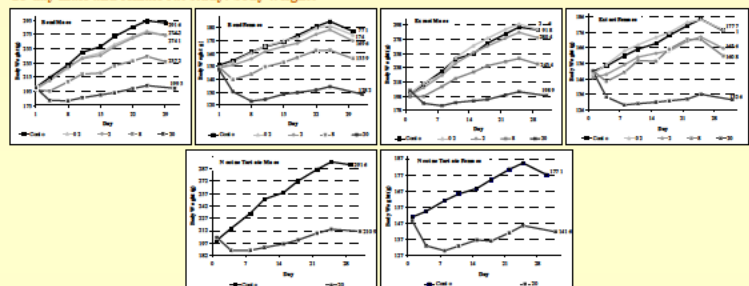
14-day male rat study: body weights



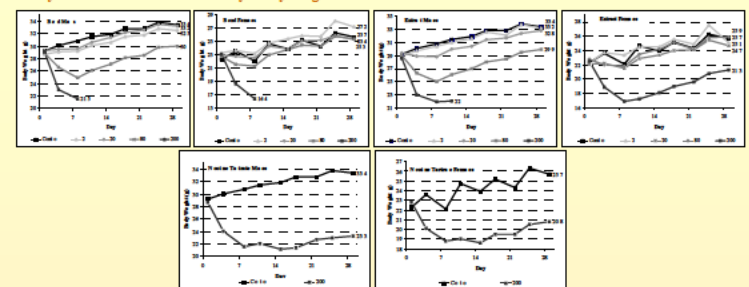
14-day male mouse study (2nd): body weights



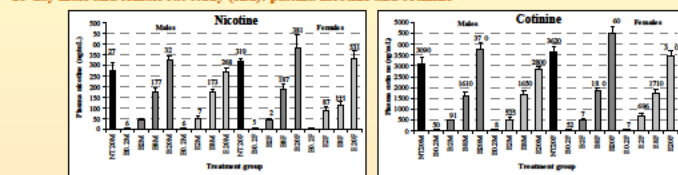
28-day male and female rat study: body weights



28-day male and female mouse study: body weights



28-day male and female rat study (end): plasma nicotine and cotinine



- B, E, NT exposures were appropriate: ↑ dose => ↑ plasma nicotine, cotinine; cotinine >> nicotine
- Blood collection times (Phase 2): 12 AM (rats); 10 AM (mice)
- Based on plasma nicotine, exposures to the lowest doses in these studies are relevant to smokeless tobacco users
- B, E, NT-induced effects: generally occurred at the highest doses (rats-8, 20; mice-80, 200):
 - ↓ Body weights and organ weight changes
 - Clinical signs (e.g., thinness, lethargy, hunched posture, paleness, rough hair coat, tremors)
 - Slight neurobehavioral changes (↓ rectal temperature; arousal and emotionality alterations)
- Necropsy revealed no gross lesions
- B, E-induced effects generally paralleled NT-induced effects (nicotine: limiting factor)
- Food consumption at highest doses: ↓
- Body weights: High Dose groups < matching Pair-fed Controls
- Sensitivity: rats > mice; male mice > female mice

Conclusions

- 28-day Wistar Han data-consistent with previous 90-day Sprague-Dawley data¹ (both-rat tobacco feeding studies):
 - Previous: 9 mg nicotine/kg/day => 14% bw ↓
 - Current: 8 mg nicotine/kg/day => 7-12% bw ↓; 20 mg nicotine/kg/day => 21-27% bw ↓
- Both rat studies indicate MTD range: 5-9 mg nicotine/kg/day
- Doses for 90-day studies were selected mainly based on ↓ body weights:
 - 0.3, 3, 6 mg nicotine/kg/day (rats)
 - 6, 60, 120 mg nicotine/kg/day (mice)

¹Krauter GR, Potts RJ, Mould AP (2008). Comparative toxicity of nicotine and a tobacco pellet to Sprague-Dawley rats in a 90-day feeding study. American College of Toxicology Annual Meeting, Tucson, Arizona.