Experimental and Toxicologic Pathology 64 (2012) 15–24

Contents lists available at ScienceDirect

Experimental and Toxicologic Pathology

journal homepage: www.elsevier.de/etp

Toxicological evaluation of smokeless tobacco: 90-Day rodent feeding studies

Eugenia H. Theophilus*, Johnnie R. Hayes, Ryan J. Potts, Paul H. Ayres, Chandra D. Williams, Charles D. Garner

Research and Development, R. J. Reynolds Tobacco Company, Bowman Gray Technical Center, Winston-Salem, NC 27105, USA

ARTICLE INFO

Article history:
Received 29 March 2010
Accepted 25 May 2010

Keywords:
Smokeless tobacco
Toxicology
In vivo
Mice
Rats

ABSTRACT

This manuscript presents data from 90-day toxicology studies designed to characterize the subchronic effects of a smokeless tobacco blend and an aqueous extract of that blend when administered to rodents in NTP-2000 feed. Positive control (nicotine tartrate) and treatment groups were matched for a range of nicotine levels. The doses evaluated were 0.3, 3, and 6 mg nicotine/kg body weight/day in Wistar Hannover rats and 6, 60, and 120 mg nicotine/kg/day in CD-1 mice. Variables evaluated included plasma nicotine and cotinine, body weights, feed consumption, clinical observations, clinical and anatomic pathology (including organ weights), and histopathology. Plasma nicotine and cotinine levels were dose-responsive. Key effects such as body weight reductions and organ weight changes occurred in rats and mice predominantly at the highest doses of test articles and positive control in the absence of treatment-related gross or histopathological changes. Organ weight changes were attributed mainly to the lower body weights of treated vs. control groups. The blend- and extract-induced effects generally paralleled each other and the nicotine-induced effects. Based on these studies, the doses evaluated spanned the no observable adverse effect level, the lowest observable adverse effect level and the maximum tolerated dose.

1. Introduction

The purpose of this manuscript is to present data from two new, 90-day subchronic toxicology studies, conducted to further characterize the toxicological effects of smokeless tobacco and tobacco extract ingestion in rats and mice. These studies are important for three main reasons. The first reason is that various organizations, including the Life Sciences Research Office (LSRO, 2008), have summarized the effects of smokeless tobacco and pointed out a need to add to the weight of scientific evidence, as more smokeless tobacco products are introduced into the market. Such products include dissolvable products, designed to be entirely ingested. These new studies, in part, address that need. The second reason is that these studies clarify similarities between ingesting tobacco and tobacco extract. The extract was included, in part, as a bridge between these new studies and the many epidemiology studies available for snus. Snus users typically swallow the tobacco extract. The third reason is that Krautter et al. (2008) have reported the 90-day effects of ingesting tobacco in Sprague–Dawley rats. These studies confirm the reproducibility of the previously reported effects in two other rodent models (Wistar Hannover rats and CD-1 mice).

The studies were conducted at Battelle, Columbus, OH, USA and were compliant with Food and Drug Administration's Good Laboratory Practices (21 Code of Federal Regulations 58). Palatability and dose-range finding studies (14- and 28-day) were conducted prior to the 90-day studies (Theophilus et al., 2009). Based on the shorter-term studies, the range of doses selected for the 90-day studies (Table 1) was designed to span the no observable adverse effect level, the lowest observable adverse effect level, and the maximal tolerated dose.

2. Materials and methods

2.1. Test articles, controls, and diets

The test articles used in diets were: (1) a smokeless tobacco blend (B, 26 mg nicotine/g tobacco) and (2) a water extract of that tobacco blend (E, 23 mg nicotine/g tobacco). The extract (1 part tobacco blend: 8 parts potable water) was produced at 100 °F (1 h) and was filtered (final extract: 38% total solids). The 100 °F was selected to mimic the normal oral temperature in humans. Test articles were stored frozen (≤0 °C).

The positive control used in diets was nicotine hydrogen tartrate salt (NT, purity ≥98%; Sigma–Aldrich Co., St. Louis, MO). The negative control was NTP-2000 diet.

Abbreviations: ANOVA, analysis of variance; B0.2M, treatment groups include group, dose, gender (e.g., B0.2M, that is blend, 0.2 mg nicotine/kg body weight/day, male); B, tobacco blend; C, negative control; E, tobacco extract; F, females; LSRO, Life Sciences Research Office; M, males; NCI, National Cancer Institute; NTP, National Toxicology Program; NT, positive control-nicotine tartrate; PFC, pair-fed control; TK, toxicokinetics.

* Corresponding author. Tel.: +1 336 741 1536; fax: +1 336 741 6285.
E-mail address: theophe@rjrt.com (E.H. Theophilus).

0940-2993/$ – see front matter © 2010 Elsevier GmbH. All rights reserved.
doi:10.1016/j.etp.2010.05.013

Table 1
90-Day rat and mouse study designs.

No.	Group	Target dosage of nicotine (mg/kg/day)*	No. animals/group				Dose group abbreviations	
			Males		Females		Males	Females
			Core	TK[a]	Core	TK		
Rats								
1	Control	0	20	6	20	6	CM	CF
2	Nicotine tartrate	6	20	6	20	6	NT6M	NT6F
3	Tobacco blend	0.3	20	6	20	6	B0.3M	B0.3F
4	Tobacco blend	3	20	6	20	6	B3M	B3F
5	Tobacco blend	6	20	6	20	6	B6M	B6F
6	Tobacco extract	0.3	20	6	20	6	E0.3M	E0.3F
7	Tobacco extract	3	20	6	20	6	E3M	E3F
8	Tobacco extract	6	20	6	20	6	E6M	E6F
Mice								
1	Control	0	20	10	20	10	CM	CF
2	Nicotine tartrate	120	20	10	20	10	NT120M	NT120F
3	Tobacco blend	6	20	10	20	10	B6M	B6F
4	Tobacco blend	60	20	10	20	10	B60M	B60F
5	Tobacco blend	120	20	10	20	10	B120M	B120F
6	Tobacco extract	6	20	10	20	10	E6M	E6F
7	Tobacco extract	60	20	10	20	10	E60M	E60F
8	Tobacco extract	120	20	10	20	10	E120M	E120F

* Corresponding concentrations of test articles or positive control in diet spanned 0.02–0.4% (rat study) and 0.2–4% (mouse study).
[a] Nicotine/cotinine analysis; TK = toxicokinetics (plasma nicotine and cotinine).

Test articles were targeted to match nicotine contents because: (1) nicotine toxicity was expected to be limiting; (2) analytical methods exist for measuring nicotine; and (3) a principal tobacco constituent had to be used to standardize the tobacco (complex mixture). Thus, nicotine was used for dosing and for monitoring feed formulations and rodent exposures.

Test articles were analyzed for tobacco constituents and standard microbial endpoints and test article stability during use and storage was established. Diets were mixed with test articles or positive control, prepared monthly, and stored at room temperature. The diets were analyzed to verify the nicotine content (Krautter et al., 2008) and stability and homogeneity were confirmed. The NTP-2000 certified powdered diet used for formulations was purchased from Harlan Teklad Inc., Madison, WI.

2.2. Experimental design

The 90-day studies were designed to determine the subchronic toxicological effects of feeding diets with and without test articles or positive control to rodents. Treatment groups are shown in Table 1. There was also a sentinel group in each study to monitor animal health. Endpoints monitored were typical of modern, standard 90-day studies with an additional toxicokinetic (TK) component (plasma nicotine and cotinine).

2.3. Animals

The Wistar Hannover (Wistar Han) rat and the Swiss Webster/CD-1 mouse were selected because both animal models are generally accepted as appropriate for toxicology studies. Wistar Han rats (4–5 weeks old, 105–179 g at Day 1) and CD-1 mice (4–5 weeks old, 20–32 g at Day 1) were acquired from Charles River Laboratories, Raleigh, NC.

Study animals were cared for according to the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996). Institutional Animal Care and Use Committees approved the protocols. Animal care programs were fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care.

Study animals were housed in animal rooms with 12 h light/12 h dark cycles; 64–79 °F; 30–70% relative humidity; and airflow minimum of 10 air changes/h. Feed and fresh municipal water (provided via an automatic watering system) were supplied *ad libitum*.

Before exposures, animals were randomized by body weights using the PATH/TOX SYSTEM (4.2.2, Xybion Medical Systems Corporation, Cedar Knolls, NJ). After randomization, mean group body weights were not significantly different ($p \leq 0.05$).

2.4. Endpoints measured

Consistent with currently accepted toxicology guidelines, moribundity and mortality checks were performed twice/day (morning and afternoon). Clinical observations data were collected on core groups before exposure start, then weekly throughout the study, and on the day of scheduled necropsy.

Body weights were determined before group allocation, then weekly, and at study termination. Mean group body weights and percent body weight gains were calculated. Feed consumption (core groups) was measured weekly.

Ophthalmic examinations were conducted on core groups by a staff veterinarian before study start and near study end. A mydriatic drug was used for these exams.

For exposure evaluation, 6 rats/gender/group or 10 mice/gender/group were included in each dose group for determination of plasma nicotine and cotinine concentrations using a validated liquid chromatography–mass spectrometry method (Krautter et al., 2008). Blood sampling occurred on Weeks 2, 4, 8, and 13 for rats and on Weeks 3, 5, 9, and 14 for mice. Blood was collected retro-orbitally at time points targeted around 12 a.m. for rats and 10 a.m. for mice, based on results from corresponding 28-day TK studies (Theophilus et al., 2009). TK study animals were anesthetized with CO_2/O_2. Blood was collected into tubes containing ethylenediaminetetraacetic acid anticoagulant. Samples were placed on wet ice until centrifuged. Plasma was transferred into appropriately labeled tubes that were placed on dry ice until stored in a freezer (−60 °C to −80 °C). After blood collection, animals were returned to their home cages. These animals were euthanized at study termination with no further data collected.

Clinical chemistry, hematology, and coagulation were evaluated in all core animals, and urinalysis assessments were performed on 10 animals/core group at necropsy. Animals were fasted overnight. Blood samples were collected under CO_2/O_2 anesthesia. The tubes contained EDTA anticoagulant for blood samples collected for hematology. The tubes used for serum chemistry determinations contained a serum separator gel. Sodium citrate was used as the anticoagulant for the prothrombin time coagulation assay. Clinical chemistry included aspartate aminotransferase, bilirubin, gamma glutamyl transferase, albumin, albumin/globulin ratio, alkaline phosphatase, glucose, triglycerides, cholesterol, creatinine, globulin, total protein, urea nitrogen, calcium, chloride, phosphorus, potassium, and sodium. Hematologic parameters included erythrocyte count, hematocrit, hemoglobin, leukocyte count, leukocyte differential count, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, mean corpuscular volume, platelet count, and reticulocyte count. Coagulation parameters included prothrombin time (rats only). Urinalysis included urine appearance, color, volume, pH, glucose, protein, specific gravity, and microscopic examination of sediment.

Terminal body weights were determined and external features of the animals were evaluated prior to euthanasia, followed by necropsy (conducted under the supervision of a board-certified pathologist). Each necropsy included: examination of the external surface of the body; all orifices; the cranial, thoracic, abdominal and pelvic cavities and their contents; and collection of all tissues typical of 90-day studies, as well as gross findings. Tissues collected were: gross lesions, adrenal glands, bone with articular surface and marrow (femur), brain (cerebrum, cerebellum, medulla), clitoral gland, epididymides, esophagus, pharynx, trachea, eyes (with optic nerve), harderian glands, heart, kidneys, large intestine (cecum, colon, rectum), liver (median lobe and left lateral lobe), lungs with bronchi, mesenteric lymph node, mammary gland (females only), nasal cavities and turbinates, ovaries (without oviduct), oral cavity, pancreas, parathyroid/thyroid gland, pituitary gland, preputial glands, prostate, salivary gland (mandibular), sciatic nerve, seminal vesicles, skeletal muscle (biceps femoris), skin, small intestine (duodenum, jejunum, ileum), spinal cord (cervical, thoracic, lumbar), spleen, sternum, bone marrow, stomach (fore-stomach and glandular), testes, thymus, tongue, urinary bladder, uterus, vagina, Zymbal glands. Tissues were fixed in 10% neutral buffered formalin (NBF), with the exception of testes, which were preserved in Bouin's fixative and subsequently transferred to 70% ethanol, and eyes with optic nerve were fixed in Davidson's fixative then transferred to 10% NBF. In addition, all fixed tissues from C and high dose groups were processed to slides and stained with hematoxylin and eosin for histopathologic examination.

Core group absolute organ weights, organ/body weight, and organ/brain weight ratios were determined. Organs weighed were: adrenal glands (rats), brain, epididymides, heart, kidneys, liver (with gall bladder, mice), lungs, pituitary gland (rats), prostate, thyroid/parathyroid gland (rats), seminal vesicles (rats), spleen, ovaries (without oviduct, rats), testes (without epididymides), thymus, salivary glands, (mandibular), uterus (with cervix).

2.5. Statistical analyses

Data were statistically analyzed with the PATH/TOX software, using a one-way analysis of variance (ANOVA), followed by Bartlett's test for variance homogeneity. If data were homogeneous, Dunnett's test was performed. If data were non-homogeneous, Cochran and Cox's modified *t* test was performed. Statistical tests were carried out to 5% (two-sided). Comparisons included C vs. NT, B, E; NT vs. high dose B, E; and corresponding B vs. E dose groups.

3. Results

3.1. Body weights, body weight gains, and feed consumption

The key findings in the 90-day studies were related to body weight reductions. Tables 2 and 3 present the rat group mean body weights vs. time data in males and females, respectively. Tables 4 and 5 present mouse group mean body weights vs. time data in males and females, respectively. At the end of the studies, rat body weights of the high dose treatment groups vs. corresponding C were 9.2–13.7% lower while those of mice were 6.1–14.5% lower. The higher doses induced clear body weight differences with time vs. C and body weight changes were dose-responsive for B and E.

Feed consumption data were variable. In both studies, feed consumption of treated groups vs. C tended to be lower at the highest doses (data not shown).

3.2. Survival and clinical signs

There were no treatment-related mortalities in the rat and mouse studies (one B3F rat died accidentally). There were no treatment-related clinical signs of toxicity in the rat and mouse studies as treated animals were similar to C in overt behavior, general health, and appearance. In rats, clinical signs included abrasion, alopecia, and red eye discharge. In mice, clinical signs included hunched posture, rough coat, genitalia swelling, discoloration, or tissue mass, tail or foot abrasion or ulceration, thinness, eye opacity, and lethargy. These abnormalities occurred in only a few animals in different dosage groups and were considered to be sporadic in occurrence and minor in severity.

3.3. Toxicokinetics

Table 6 shows TK data for rats and mice from the end of the study. For both rats and mice, the plasma nicotine and cotinine values increased with a corresponding increase in B and E administered dose, indicating a clear dose-responsiveness. The high dose groups exhibited similar nicotine and cotinine concentrations, indicating similar systemic exposures at similar administered doses of nicotine. For mice, plasma values showed a gender effect as both nicotine and cotinine concentrations were consistently lower in females than in males.

3.4. Hematology, serum chemistry, urine analysis, and ophthalmic exams

In both rats and mice, group mean hematology data, group mean absolute white blood cell differential count data, group mean coagulation data (prothrombin time, rats), group mean serum chemistry data, and urinalysis data indicated that, although there were some statistically significant differences, there were no NT, B, or E treatment-related effects (data not shown). At the ophthalmic exams, corneal crystals were noted in several animals both at the beginning and end of the study (no treatment-related effects).

3.5. Organ weights

Tables 7 and 8 present group mean terminal body weights and group mean absolute organ weights for male and female rats, respectively. Predominantly in the NT and the higher B or E dose groups, statistically significant decreases were noted in absolute organ weights vs. C in males and/or females, e.g., heart, kidneys, liver, seminal vesicles, prostate, thymus, pituitary, thyroid glands, adrenal glands, and spleen. The reductions in body weights at the

Table 2
Rats: group mean absolute body weights (g)—males.

Group	Mean SD	Day 1	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49	Day 56	Day 63	Day 70	Day 77	Day 84	Day 91	%Change*
CM	Mean	156.1	192.0	235.5	265.6	291.0	308.3	326.1	339.3	351.4	362.5	372.2	383.5	392.7	398.6	0
	SD	9.2	10.0	11.0	12.0	14.6	15.6	17.1	18.6	18.5	19.6	20.5	22.3	22.5	22.5	
NT6M	Mean	155.2	184.3	222.2^{A}	247.3^{A}	267.9^{A}	280.1^{A}	292.9^{A}	303.8^{A}	315.3^{A}	324.6^{a}	332.2^{a}	339.1^{a}	346.2^{a}	348.1^{a}	−12.7
	SD	10.8	11.9	12.1	12.7	15.8	17.5	20.0	21.5	21.9	23.8	24.2	26.0	26.6	27.4	
B0.3M	Mean	155.8	191.6	236.2	267.5	293.3	310.5	328.7	342.2	354.7	366.1	376.2	384.4	393.7	399.1	+0.1
	SD	11.5	13.8	15.3	17.4	19.5	21.0	22.7	25.0	26.7	27.4	28.5	29.5	30.2	31.7	
B3M	Mean	156.7	189.2	229.2	255.7	278.5	293.7	308.3	319.9^{A}	327.8^{A}	341.2^{a}	350.5^{a}	357.1^{a}	366.8^{a}	372.5^{a}	−6.5
	SD	12.0	14.5	17.7	21.9	22.9	24.8	26.3	26.2	27.1	29.4	29.7	30.3	31.3	31.4	
B6M	Mean	155.1	182.5	218.6^{A}	242.8^{A}	261.1^{A}	271.7^{A}	287.3^{A}	298.9^{A}	306.6^{A}	318.3^{a}	325.1^{a}	330.1^{a}	339.9^{a}	343.9^{a}	−13.7
	SD	11.8	13.0	15.4	19.4	22.6	25.0	25.9	25.7	26.3	27.8	27.9	28.1	29.8	29.6	
	N	20	20	20	20	20	20	20	20	20	20	20	20	20	20	
E0.3M	Mean	156.6	189.9	232.6	260.7	284.2	298.4	317.5	330.0	339.5	353.6	364.8	372.1	383.3	388.1	−2.6
	SD	10.6	12.6	14.2	14.9	18.7	20.9	22.8	24.0	25.4	28.2	30.7	30.7	31.2	31.1	
E3M	Mean	156.4	188.1	227.9	258.8	281.7	295.7	313.6	325.0	333.3	349.1	359.9	364.9^{a}	376.8	383.0	−3.9
	SD	10.2	10.8	12.3	14.9	18.3	19.1	22.8	23.9	25.8	28.6	29.6	30.4	31.6	33.6	
E6M	Mean	156.0	183.2	222.0^{A}	250.6^{A}	271.0^{A}	285.9^{A,c}	302.0^{A,c}	314.6^{A,c}	322.6^{A,c}	333.8^{a,c}	344.1^{a,c}	348.9^{a,c}	358.4^{a,c}	361.8^{a,c}	−9.2
	SD	11.8	12.1	12.4	12.7	12.5	12.6	12.9	12.9	13.9	12.4	13.0	12.5	13.0	14.0	

Multiple comparisons were made according to the letters listed below. Capitals indicate significant difference ($p \leq 0.05$, Dunnett's test). Lower case letters indicate significant differences ($p \leq 0.05$, modified *t* test). A = CM vs. NT6M, B0.3M, B3M, B6M, E0.3M, E3M, E6M. B = NT6M vs. B6M, E6M. C = corresponding blend vs. extract dose groups (B0.3M vs. E0.3M, B3M vs. E3M, B6M vs. E6M). Number of animals/group = 20. SD = standard deviation.

* End of study: % change vs. CM.

Table 3
Rats: group mean absolute body weights (g)—females.

Group	Mean SD	Day 1	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49	Day 56	Day 63	Day 70	Day 77	Day 84	Day 91	%Change*
CF	Mean	123.9	141.4	159.7	172.9	179.5	188.1	199.6	206.5	210.9	216.3	220.3	222.7	227.7	227.1	0
	SD	10.2	10.8	11.8	12.5	12.4	12.6	14.8	15.8	13.9	14.6	16.8	16.0	14.0	15.6	
NT6F	Mean	123.8	132.5^{A}	146.5^{A}	163.4^{A}	166.3^{A}	173.3^{A}	181.3^{A}	186.7^{A}	188.5^{A}	193.9^{A}	198.0^{A}	199.5^{A}	202.4^{A}	200.0^{A}	−11.9
	SD	9.1	7.4	8.8	10.7	9.5	10.0	11.7	10.5	10.2	10.4	11.4	12.0	13.6	12.2	
B0.3F	Mean	125.7	141.0	159.4	174.6	180.9	192.1	200.8	206.6	208.4	216.0	220.6	222.0	223.6	225.9	−0.5
	SD	8.4	11.6	10.9	10.8	11.1	12.4	14.4	11.7	12.0	13.7	15.2	13.3	12.9	13.4	
B3F	Mean	124.5	137.5	151.2	164.8	173.7	181.8	187.6^{A}	194.5^{A}	198.2^{A}	201.2^{A}	205.3^{A}	207.8^{A}	208.8^{A}	210.8^{A}	−7.2
	SD	10.0	11.0	11.1	11.9	11.2	13.6	12.2	14.3	12.4	14.1	13.5	15.4	13.9	15.8	
B6F	Mean	123.7	135.8	152.0	161.6^{A}	169.5^{A}	178.2	182.2^{A}	186.2^{A}	191.0^{A}	194.2^{A}	196.6^{A}	195.7^{A}	198.5^{A}	202.1^{A}	−11.0
	SD	10.4	12.1	12.7	14.6	15.1	17.7	15.8	17.6	17.0	18.1	18.8	20.2	18.1	18.8	
E0.3F	Mean	125.7	143.0	158.9	173.4	184.3	191.1	198.8	206.6	209.9	213.3	217.6	222.8	224.5	226.6	−0.2
	SD	9.1	10.3	11.4	10.8	12.5	13.4	15.6	13.3	12.8	15.4	15.2	14.7	14.2	15.8	
E3F	Mean	127.5	141.3	158.5^{C}	173.0^{C}	182.1^{C}	189.8^{C}	195.9^{C}	203.7^{C}	204.9	211.1^{C}	213.3	217.3	218.1	218.3	−3.9
	SD	6.8	9.0	8.1	7.7	11.7	10.9	9.9	10.9	12.7	13.1	12.9	14.4	15.5	15.1	
E6F	Mean	124.1	133.0	148.1^{A}	162.4^{A}	170.8	177.7	182.9^{A}	188.7^{A}	191.3^{A}	195.1^{A}	197.1^{A}	201.2^{A}	199.6^{A}	201.5^{A}	−11.3
	SD	8.3	8.6	7.9	7.6	8.2	9.2	8.9	9.2	9.6	10.5	9.6	9.4	9.8	11.0	

Multiple comparisons were made according to the letters listed below. Capital letters indicate significant difference ($p \leq 0.05$, Dunnett's test). Lower case letters indicate significant difference ($p \leq 0.05$, modified *t* test). A = CF vs. NT6F, B0.3F, B3F, B6F, E0.3F, E3F, E6F. B = NT6F vs. B6F, E6F. C = corresponding blend vs. extract dose groups (B0.3F vs. E0.3F, B3F vs. E3F, B6F vs. E6F). *N* (number of animals/group) = 20 except B3F Days 77, 84, and 91 (*N* = 19). SD = standard deviation.

* End of study: % change vs. CF.

Table 4
Mice: group mean absolute body weights (g)—males.

Group	Mean SD	Day 1	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49	Day 56	Day 63	Day 70	Day 77	Day 84	Day 91	%Change*
CM	Mean	28.2	30.6	32.1	33.7	34.6	35.3	36.5	36.9	37.5	38.2	38.6	38.7	39.1	39.2	0
	SD	1.6	1.7	2.0	2.1	2.1	2.6	2.1	2.4	2.4	2.1	2.9	3.1	3.0	2.9	
NT120M	Mean	28.5	25.8[a]	27.1[A]	28.3[A]	30.1[A]	30.2[A]	31.4[A]	32.0[A]	32.8[A]	33.4[A]	33.7[A]	33.6[A]	34.0[A]	34.0[A]	−13.3
	SD	1.5	1.8	2.0	2.5	2.8	2.1	2.5	2.3	2.3	1.8	2.2	2.2	2.0	2.0	
B6M	Mean	28.1	30.1	32.1	33.3	34.6	35.6	36.6	37.2	38.0	37.4	38.0	38.4	38.9	39.7	+1.3
	SD	2.2	1.9	1.9	2.2	1.7	1.9	2.2	2.0	2.5	2.8	2.5	2.8	2.9	2.7	
B60M	Mean	28.0	28.1[a]	29.8[A]	31.4[A]	32.9	33.1[A]	34.3[A]	34.5[A]	35.4	35.5[A]	36.0[A]	35.9[A]	36.6[A]	36.4[A]	−7.1
	SD	1.9	3.0	2.8	2.9	2.6	2.5	2.6	3.1	2.8	3.8	3.1	3.6	2.9	3.4	
B120M	Mean	28.5	24.9[a]	26.0[A]	28.1[A]	29.7[A]	30.5[A]	31.2[A]	32.1[A]	33.1[A]	33.3[A]	33.5[A]	33.7[A]	34.3[A]	33.8[A]	−13.8
	SD	2.0	2.6	2.4	2.4	2.7	2.8	2.9	3.1	3.2	3.1	2.8	3.3	2.9	2.8	
E6M	Mean	28.1	30.2	31.8	33.0	34.1	35.0	35.9	36.4	37.3	37.7	37.7	38.4	39.6	39.3	+0.3
	SD	1.8	1.4	2.9	1.8	2.2	2.0	2.1	2.4	3.0	2.8	2.6	2.4	2.9	2.9	
E60M	Mean	28.4	28.7[a]	30.4	30.9[A]	32.5[A]	33.5	34.5	35.1	36.0	35.7[A]	36.3	36.5	36.6[A]	36.4[A]	−7.1
	SD	1.5	2.2	2.5	2.7	2.6	3.0	3.2	3.0	2.5	3.2	3.1	2.7	3.0	2.8	
E120M	Mean	28.5	25.8[a]	27.2[A]	28.7[A]	30.1[A]	31.5[A]	31.7[A]	32.4[A]	33.0[A]	33.7[A]	33.5[A]	34.1[A]	34.2[A]	33.5[A]	−14.5
	SD	2.5	2.3	3.0	2.6	2.5	2.7	2.6	2.9	2.6	2.6	2.7	2.7	2.7	2.4	

Multiple comparisons were made according to the letters listed below. Capital letters indicate significant difference ($p \leq 0.05$, Dunnett's test). Lower case letters indicate significant difference ($p \leq 0.05$, modified t test). A = CM vs. NT120M, B6M, B60M, B120M, E6M, E60M, E120M. B = NT120M vs. B120M, E120M. C = corresponding blend vs. extract dose groups (B6M vs. E6M, B60M vs. E60M, B120M vs. E120M). Number of animals/group = 20. SD = standard deviation.

* End of study: % change vs. CM.

Table 5
Mice: group mean absolute body weights (g)—females.

Group	Mean SD	Day 1	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49	Day 56	Day 63	Day 70	Day 77	Day 84	Day 91	%Change*
CF	Mean	22.1	23.7	24.5	24.6	25.4	26.1	27.3	28.1	28.7	28.8	29.4	29.4	30.2	29.7	0
	SD	1.1	1.4	1.8	1.4	2.1	1.8	1.8	2.7	2.5	2.4	2.5	2.8	2.9	2.8	
NT120F	Mean	22.3	22.7	23.8	23.8	24.8	24.9	25.6[A]	25.4[A]	26.1[A]	26.2[A]	26.9[A]	27.5[A]	27.6[a]	26.7[A]	−10.1
	SD	1.1	1.2	1.1	1.4	1.3	1.4	1.6	1.6	1.7	1.6	2.0	2.2	2.2	2.1	
B6F	Mean	22.5	23.7	24.6	25.1	25.3	25.6	24.7[A]	26.6	26.9[A]	27.6	27.6[A]	28.4	29.3	28.3	−4.7
	SD	1.2	1.3	1.6	1.5	1.4	1.8	1.2	1.8	2.1	2.6	2.2	2.3	2.5	2.4	
B60F	Mean	22.7	23.5	23.9	24.7	25.4	25.1	26.2	26.8	26.8[A]	26.9[A]	27.4[A]	28.1	28.1[a]	27.5[A]	−7.4
	SD	1.1	1.1	1.5	1.3	1.4	1.5	1.8	2.0	2.3	1.6	2.0	2.3	2.0	2.2	
B120F	Mean	22.5	22.8	23.9	25.1[B]	25.4	25.8	26.4	26.6 B	27.2	27.6 B	27.5[A]	28.2	28.5[a]	27.9[A]	−6.1
	SD	0.9	1.3	1.3	1.5	1.3	1.2	1.6	1.5	2.0	1.5	1.8	2.0	1.7	1.8	
E6F	Mean	22.5	23.3	24.1	24.4	25.6	26.4	26.6[c]	27.2	27.6	28.3	28.3	29.1	29.7	28.8	−3.0
	SD	1.0	1.1	1.2	1.0	1.3	1.4	2.0	1.3	1.6	2.0	1.9	2.0	1.9	1.9	
E60F	Mean	22.7	23.2	24.4	24.7	25.7	26.3[C]	26.3	25.6[A]	27.2	27.3	27.7	28.7	29.0	28.0	−5.7
	SD	1.1	1.0	1.3	1.4	1.7	1.7	1.6	1.7	1.8	1.9	2.2	2.4	2.8	1.8	
E120F	Mean	22.6	22.5[A]	23.7	24.9	25.2	25.3	25.9	26.4[A]	26.2[A]	26.9[A]	27.5[A]	27.7	27.6[a]	26.7[A,C]	−10.1
	SD	1.4	1.5	2.0	1.9	2.0	1.7	1.7	1.6	1.5	1.6	2.2	1.8	1.2	1.4	

Multiple comparisons were made according to the letters listed below. Capital letters indicate significant difference ($p \leq 0.05$, Dunnett's test). Lower case letters indicate significant difference ($p \leq 0.05$, modified t test). A = CF vs. NT120F, B6F, B60F, B120F, E6F, E60F, E120F. B = NT120F vs. B120F, E120F. C = corresponding blend vs. extract dose groups (B6F vs. E6F, B60F vs. E60F, B120F vs. E120F). Number of animals/group = 20. SD = standard deviation.

* End of study: % change vs. CF.

Table 6
Plasma nicotine and cotinine (end of study): rats and mice.

Rats[a]			Mice[a]		
Group	Nicotine (ng/ml)	Cotinine (ng/ml)	Group	Nicotine (ng/ml)	Cotinine (ng/ml)
		Males			
NT6M	93.1 ± 12.0	741 ± 40	NT120M	441 ± 28	6640 ± 590
B0.3M	6.35 ± 1.6	55 ± 4	B6M	11.0 ± 4.8	204 ± 45
B3M	63.2 ± 11.3	574 ± 32	B60M	470 ± 75	4410 ± 260
B6M	134 ± 15	1080 ± 40	B120M	382 ± 112	6500 ± 1400
E0.3M	4.6 ± 0.4	52 ± 2	E6M	16.4 ± 10.1	134 ± 35
E3M	49.4 ± 12.4	485 ± 26	E60M	360 ± 56	4630 ± 400
E6M	96.3 ± 8.7	881 ± 38	E120M	565 ± 167	6150 ± 880
		Females			
NT6F	116 ± 26.0	953 ± 110	NT120F	243 ± 62	2830 ± 430
B0.3F	3.5 ± 0.9	52 ± 4	B6F	4.8 ± 2.1	96 ± 22
B3F	56.6 ± 13.5	441 ± 62	B60F	120 ± 25	2270 ± 330
B6F	125 ± 18	1150 ± 60	B120F	291 ± 74	4350 ± 690
E0.3F	3.1 ± 0.7	43 ± 6	E6F	2.6 ± 0	82 ± 11
E3F	88.8 ± 14.8	637 ± 67	E60F	152 ± 47	2530 ± 570
E6F	94.1 ± 19.1	1190 ± 190	E120F	270 ± 83	3980 ± 650

[a] Group means ± standard errors.

higher doses were attributed to reduced palatability of the dosed feed (and/or possibly nicotine appetite suppressant effects). These reductions in body weights also led to an increase in the organ/body weight values for the brain, testes, salivary glands, adrenal glands, epididymides, pituitary, and liver. Decreases in the organ/body weights were seen for thyroid and uterus.

Tables 9 and 10 present group mean terminal body weights and group mean absolute organ weights for male and female mice, respectively. The NT and high B or E exposure groups showed decreases in absolute organ weights vs. C in males and/or females for heart, kidneys, liver, epididymides, salivary glands, brain, and lungs. There were corresponding body weight reductions attributed to reduced palatability of the dosed feed (and/or nicotine appetite suppressant effects). These reductions in body weights likely led to decreases in organ weights and increases in organ/body weights (vs. C) for brain and uterus.

3.6. Gross pathology and histopathology

For both rats and mice, a few macroscopic findings were observed at necropsy, none of which were NT, B, or E treatment-related. Tables 11 and 12 present the microscopic observations summary (incidence, severity) scores for rats and mice, respectively (only data from organs that displayed changes are shown). Microscopic histological changes were graded semi-quantitatively with the following severity scale: 1 (minimal)-barely detectable change, unlikely to be of biological significance, 2 (mild)-change likely to have minor functional significance, 3 (moderate)-change likely to have clinical significance and 4 (marked)-change approaching maximal extent. A few microscopic changes were observed. However, these changes were typical of background changes in untreated animals, were generally of minimal severity (<1), typically occurred in a small number of animals (with some exceptions), and were interpreted to be neither toxicologically nor biologically significant (not NT, B, E-related).

4. Discussion

The Life Sciences Research Office (LSRO, 2008) has undertaken an effort to determine how smokeless tobacco products rank on the risk continuum spanning from cigarettes to smokeless tobacco use. LSRO has reviewed the scientific evidence available for smokeless tobacco products and, for some effects (e.g., lung cancer, chronic obstructive pulmonary disease), has determined that smokeless tobacco products present a lower risk than cigarettes. LSRO has also encouraged the conduct of additional studies to further define the effects of smokeless tobacco consumption. These studies are consistent with LSRO recommendations.

The results of these studies are consistent with results from a previous study (Krautter et al., 2008). In that study, Sprague–Dawley rats were fed diets containing powdered tobacco pellet or NT at 0, 1.8, 5.3, and 9 mg nicotine/kg/day for 90 days and were assessed for clinical, hematological, macroscopic, and histopathologic changes (C and high dose groups). Plasma nicotine and cotinine were measured at 4, 9, and 13 weeks and indicated a good dose–response relationship. All animals survived and there were only minor changes in hematology and clinical chemistry at the end of the study. The significant effects measured were dose-dependent decreases in body weights, body weight gains, and feed consumption vs. C (no histopathology changes). At the end of that study, the mean absolute body weight reductions for the 9 mg nicotine/kg/day were 13–15% (NT and tobacco pellet groups) vs. C. At the end of the rat study reported here, the body weight reductions for the 6 mg nicotine/kg/day NT, B, and E groups were 9–14% vs. C. The effects measured in the current studies are consistent with the effects measured in the previous study (e.g., body and organ weight reductions, lack of treatment-related histopathological changes). However, similar body weight reductions vs. C occur at a lower dose in Wistar Han vs. Sprague–Dawley rats (6 vs. 9 mg nicotine/kg body weight/day, respectively).

The effects of B and E generally paralleled the effects of NT (e.g., body weights reductions). Based on this effect parallelism, nicotine was likely a key component that altered diet palatability (and may have acted as an appetite suppressant) and was a limiting factor in terms of how much tobacco could be incorporated into the diets without eliciting toxicity (e.g., excessive body weight loss). Nicotine was useful in evaluating the stability of the test articles and diets and the homogeneity of the diets containing the test articles. In addition, plasma nicotine and cotinine were reliable exposure indicators.

Human exposure to smokeless tobacco results in typical concentrations of plasma nicotine of ~30 ng/ml at steady state (range ~10–50 ng/ml) (LSRO, 2008; NCI, 1992). Exposures to 0.3–3 mg nicotine/kg/day (rats) and 6 mg nicotine/kg/day (mice) elicit plasma nicotine levels relevant to typical consumers of smokeless tobacco products.

Table 7
Rats: group mean terminal body weights (g) and group mean absolute organ weights (g)—males.

Group	Mean SD	Terminal body weight	Adrenal glands	Brain	Epididymides	Heart	Kidneys	Liver	Lungs	Pituitary gland	Prostate	Salivary gland	Seminal vesicles	Spleen	Testes	Thymus	Thyroid glands
CM	Mean	379.7	0.055	2.018	1.2641	1.125	2.169	8.683	3.128	0.013	1.139	0.634	1.275	0.588	3.605	0.394	0.031
	SD	23.1	0.008	0.103	0.0964	0.141	0.202	0.682	0.653	0.002	0.122	0.055	0.178	0.062	0.389	0.061	0.005
NT6M	Mean	329.2[a]	0.058	2.007	1.2186	0.952[A]	1.946[A]	7.394[A]	2.870	0.012	0.981[A]	0.646	1.092[A]	0.554	3.661	0.330[A]	0.031
	SD	27.5	0.006	0.072	0.0848	0.079	0.169	0.982	0.558	0.002	0.156	0.050	0.167	0.095	0.296	0.057	0.005
B0.3M	Mean	382.0	0.054	2.052	1.2885	1.075	2.080	8.518	3.108	0.011	1.224	0.644	1.135	0.639	3.695	0.406	0.030
	SD	31.3	0.010	0.077	0.1222	0.109	0.230	0.949	0.653	0.002	0.153	0.088	0.205	0.108	0.249	0.058	0.005
B3M	Mean	353.6[a]	0.057	2.040	1.2741	1.031[A]	2.099	8.288	2.969	0.013	1.135	0.673	1.182	0.608	3.771	0.344	0.033
	SD	30.2	0.010	0.116	0.1000	0.093	0.222	1.049	0.692	0.002	0.205	0.080	0.215	0.069	0.233	0.075	0.005
B6M	Mean	325.7[a]	0.053	2.003	1.2335	0.959[A]	1.959[A]	7.419[A]	2.918	0.013	1.038	0.661	1.079[A]	0.555	3.692	0.351	0.027
	SD	28.8	0.007	0.074	0.1156	0.080	0.160	0.880	0.561	0.002	0.182	0.088	0.231	0.069	0.261	0.068	0.006
E0.3M	Mean	370.2	0.057	2.023	1.2747	1.077	2.101	8.347	3.018	0.012	1.163	0.656	1.200	0.622	3.657	0.403	0.030
	SD	30.4	0.009	0.077	0.1376	0.108	0.118	0.830	0.576	0.002	0.139	0.087	0.152	0.081	0.306	0.077	0.007
E3M	Mean	365.3	0.055	2.057	1.3062	1.028[A]	2.130	8.292	3.066	0.012	1.203	0.668	1.263	0.628	3.746	0.381	0.032
	SD	33.3	0.008	0.073	0.1445	0.080	0.235	1.234	0.612	0.002	0.188	0.080	0.244	0.069	0.245	0.073	0.006
E6M	Mean	344.8[a,b,c]	0.055	2.023	1.2085	0.978[A]	2.055	7.861[A]	2.990	0.010[A,C]	1.032	0.647	1.088[A]	0.562	3.517	0.350	0.026[A,B]
	SD	12.6	0.010	0.074	0.1135	0.110	0.196	0.882	0.492	0.003	0.183	0.050	0.149	0.057	0.718	0.078	0.006

Multiple comparisons were made according to the letters listed below. Capital letters indicate significant difference ($p \leq 0.05$, Dunnett's test). Lower case letters indicate significant difference ($p \leq 0.05$, modified t test). A = CM vs. NT6M, B0.3M, B3M, B6M, E0.3M, E3M, E6M. B = NT6M vs. B6M, E6M. C = corresponding blend vs. extract dose groups (B0.3M vs. E0.3M, B3M vs. E3M, B6M vs. E6M). N (number of animals/group) = 20 except B6M seminal vesicles (N = 19). SD = standard deviation.

Table 8
Rats: group mean terminal body weights (g) and group mean absolute organ weights (g)—females.

Group	Mean SD	Terminal body weight	Adrenal glands	Brain	Heart	Kidneys	Liver	Lungs	Ovaries	Pituitary gland	Salivary gland	Spleen	Thymus	Thyroid glands	Uterus
CF	Mean	214.1	0.064	1.884	0.734	1.372	5.321	2.129	0.094	0.017	0.443	0.444	0.315	0.026	0.807
	SD	15.7	0.010	0.074	0.057	0.122	0.396	0.356	0.015	0.002	0.044	0.052	0.049	0.005	0.498
NT6F	Mean	190.5[A]	0.056[A]	1.844	0.656[A]	1.210[A]	4.871[A]	2.044	0.089	0.015[A]	0.442	0.417	0.280	0.024	0.651
	SD	12.2	0.007	0.089	0.063	0.111	0.511	0.408	0.012	0.002	0.043	0.055	0.063	0.005	0.292
B0.3F	Mean	212.3	0.065	1.857	0.734	1.381	5.396	2.217	0.103	0.017	0.457	0.457	0.328	0.027	0.531[a]
	SD	11.5	0.009	0.094	0.055	0.124	0.332	0.326	0.018	0.002	0.036	0.067	0.035	0.005	0.125
B3F	Mean	196.9[A]	0.064	1.869	0.700	1.306	5.107	2.285	0.094	0.017	0.456	0.428	0.291	0.025	0.701
	SD	14.7	0.009	0.087	0.059	0.119	0.406	0.361	0.014	0.002	0.044	0.049	0.057	0.004	0.312
B6F	Mean	187.1[A]	0.054[A]	1.839	0.633[A]	1.233[A]	4.946	1.915	0.085	0.015[A]	0.444	0.416	0.277	0.025	0.828
	SD	17.2	0.008	0.095	0.060	0.131	0.513	0.360	0.016	0.002	0.057	0.046	0.060	0.005	0.468
E0.3F	Mean	212.3	0.065	1.871	0.706	1.340	5.261	2.192	0.096	0.017	0.445	0.444	0.326	0.027	0.660
	SD	14.0	0.008	0.078	0.050	0.106	0.416	0.403	0.016	0.002	0.045	0.044	0.073	0.004	0.277
E3F	Mean	204.9	0.065	1.914	0.721	1.366	5.368	2.362	0.095	0.017	0.455	0.438	0.279	0.027	0.862
	SD	13.6	0.012	0.069	0.050	0.097	0.641	0.505	0.019	0.003	0.043	0.071	0.061	0.004	0.419
E6F	Mean	188.6[A]	0.055[A]	1.837	0.624[A]	1.212[A]	4.932[A]	1.889	0.082[b]	0.015[A]	0.448	0.399[A]	0.281	0.025	0.772
	SD	9.0	0.007	0.078	0.043	0.090	0.426	0.327	0.009	0.002	0.043	0.039	0.052	0.005	0.365

Multiple comparisons were made according to the letters listed below. Capital letters indicate significant difference ($p \leq 0.05$, Dunnett's test). Lower case letters indicate significant difference ($p \leq 0.05$, modified t test). A = CF vs. NT6F, B0.3F, B3F, B6F, E0.3F, E3F, E6F. B = NT6F vs. B6F, E6F. C = corresponding blend vs. extract dose groups (B0.3F vs. E0.3F, B3F vs. E3F, B6F vs. E6F). N (number of animals/group) = 20 except B3F Days 77, 84, 91 (N = 19). SD = standard deviation.

Table 9
Mice: group mean terminal body weights (g) and group mean absolute organ weights (g)—males.

Group	Mean SD	Terminal body weight	Brain	Epididymides	Heart	Kidneys	Liver	Lungs	Prostate	Salivary gland	Spleen	Testes	Thymus
CM	Mean	35.9	0.502	0.1034	0.266	0.610	1.424	0.426	0.079	0.274	0.081	0.256	0.026
	SD	2.8	0.029	0.0141	0.048	0.076	0.160	0.099	0.025	0.042	0.024	0.035	0.008
NT120M	Mean	30.0[A]	0.479[a]	0.0918[a]	0.207[A]	0.488[A]	1.201[A]	0.395	0.069	0.204[A]	0.070	0.246	0.021
	SD	2.2	0.023	0.0151	0.048	0.063	0.156	0.114	0.018	0.032	0.026	0.027	0.006
B6M	Mean	36.1	0.494	0.0942[a]	0.251	0.613	1.398	0.421	0.073	0.268	0.076	0.228	0.026
	SD	2.5	0.025	0.0076	0.044	0.073	0.103	0.095	0.019	0.034	0.017	0.036	0.008
B60M	Mean	32.9[A]	0.497	0.1001	0.221[A]	0.513[A]	1.256[A]	0.413	0.062[a]	0.227[A]	0.068	0.248	0.022
	SD	3.4	0.038	0.0160	0.044	0.077	0.178	0.086	0.021	0.028	0.019	0.031	0.007
B120M	Mean	29.7[A]	0.485[a]	0.0888[a]	0.204[A]	0.454[A]	1.234[A]	0.359	0.066	0.203[A]	0.063[a]	0.238	0.024
	SD	2.6	0.017	0.0088	0.039	0.050	0.132	0.102	0.014	0.029	0.017	0.031	0.007
E6M	Mean	35.8	0.502	0.1068[c]	0.263	0.606	1.402	0.424	0.080	0.251	0.078	0.247	0.029
	SD	2.9	0.024	0.0146	0.036	0.085	0.153	0.068	0.029	0.026	0.017	0.035	0.011
E60M	Mean	32.6[A]	0.492	0.0947[a]	0.231[A]	0.521[A]	1.295[A]	0.409	0.069	0.224[A]	0.066[a]	0.229	0.023
	SD	2.5	0.024	0.0122	0.032	0.060	0.150	0.097	0.028	0.025	0.013	0.048	0.009
E120M	Mean	29.7[A]	0.482[a]	0.0910[a]	0.206[A]	0.468[A]	1.199[A]	0.386	0.060[a]	0.207[A]	0.063[a]	0.235	0.025
	SD	2.2	0.024	0.0107	0.035	0.059	0.102	0.071	0.014	0.041	0.012	0.030	0.009

Multiple comparisons were made according to the letters listed below. Capital letters indicate significant difference ($p \leq 0.05$, Dunnett's test). Lower case letters indicate significant difference ($p \leq 0.05$, modified t test). A = CM vs. NT120M, B6M, B60M, B120M, E6M, E60M, E120M. B = NT120M vs. B120M, E120M. C = corresponding blend vs. extract dose groups (B6M vs. E6M, B60M vs. E60M, B120M vs. E120M). Number of animals/group = 20. SD = standard deviation.

Table 10
Mice: group mean terminal body weights (g) and group mean absolute organ weights (g)—females.

Group	Mean SD	Terminal body weight	Brain	Heart	Kidneys	Liver	Lungs	Salivary gland	Spleen	Thymus	Uterus
CF	Mean	27.0	0.499	0.170	0.335	1.110	0.335	0.152	0.085	0.032	0.182
	SD	2.7	0.023	0.033	0.036	0.115	0.076	0.019	0.018	0.007	0.082
NT120F	Mean	24.2[A]	0.487	0.150	0.303[A]	0.941[A]	0.283[A]	0.135	0.073	0.028	0.223
	SD	2.0	0.024	0.025	0.030	0.101	0.057	0.024	0.017	0.007	0.065
B6F	Mean	26.3	0.501	0.168	0.315	1.054	0.315	0.146	0.078	0.034	0.191
	SD	2.4	0.025	0.031	0.031	0.109	0.050	0.023	0.012	0.010	0.061
B60F	Mean	24.7[A]	0.489	0.176	0.320	1.016	0.327	0.140	0.076	0.027	0.177
	SD	2.0	0.024	0.031	0.033	0.134	0.069	0.013	0.020	0.008	0.057
B120F	Mean	24.9[A]	0.496	0.173[B]	0.309	1.031[B]	0.299	0.134[A]	0.074	0.029	0.163[B]
	SD	1.9	0.029	0.034	0.041	0.151	0.047	0.024	0.015	0.007	0.045
E6F	Mean	26.4	0.499	0.167	0.327	1.102	0.319	0.142	0.075	0.030	0.177
	SD	1.6	0.025	0.030	0.032	0.115	0.075	0.015	0.016	0.008	0.079
E60F	Mean	25.3	0.486	0.169	0.314	1.013	0.316	0.146	0.087	0.029	0.187
	SD	1.8	0.031	0.032	0.039	0.094	0.069	0.021	0.023	0.006	0.086
E120F	Mean	24.2[A]	0.490	0.141[A,C]	0.297[A]	1.000[A]	0.286	0.130[A]	0.073	0.031	0.167[B]
	SD	1.6	0.023	0.024	0.021	0.096	0.045	0.019	0.016	0.009	0.070

Multiple comparisons were made according to the letters listed below. Capital letters indicate significant difference ($p \leq 0.05$, Dunnett's test). Lower case letters indicate significant difference ($p \leq 0.05$, modified t test). A = CF vs. NT120F, B6F, B60F, B120F, E6F, E60F, E120F. B = NT120F vs. B120F, E120F. C = corresponding blend vs. extract dose groups (B6F vs. E6F, B60F vs. E60F, B120F vs. E120F). N (number of animals/group) = 20 except E120F lungs (N = 19). SD = standard deviation.

Table 11
Rats: microscopic observations summary (incidence, average severity).

Males					Females				
Tissue/observation	Number observed per group				Tissue/observation	Number observed per group			
	CM	NT6M	B6M	E6M		CF	NT6F	B6F	E6F
Adrenal gland: cytoplasmic vacuolization, cortex	0	0	1	1	*Adrenal gland:* hypertrophy, cortex	0	0	1	0
Average severity	0.0	0.0	0.1	0.1	Average severity	0.0	0.0	0.1	0.0
Cecum: inflammation	0	3	0	0	*Cecum:* inflammation	2	0	1	1
Average severity	0.0	0.2	0.0	0.0	Average severity	0.1	0.0	0.1	0.1
Colon: hyperplasia, peyers patch	0	0	0	1	*Clitoral gland:* inflammation	1	1	0	2
Average severity	0.0	0.0	0.0	0.2	Average severity	0.1	0.1	0.0	0.2
Harderian gland: inflammation	1	0	0	2	*Harderian gland:* inflammation	0	1	0	0
Average severity	0.1	0.0	0.0	0.1	Average severity	0.0	0.1	0.0	0.0
Heart: cardiomyopathy	2	3	3	2	*Heart:* cardiomyopathy	4	0	1	2
Average severity	0.1	0.2	0.2	0.1	Average severity	0.2	0.0	0.1	0.1
Kidney: cyst(s), tubular	0	0	0	1	Fibrosis: endocardial	0	0	0	1
Average severity	0.0	0.0	0.0	0.1	Average severity	0.0	0.0	0.0	0.1
Hydronephrosis	1	3	0	3	*Kidney:* cyst(s), tubular	0	0	1	0
Average severity	0.1	0.2	0.0	0.3	Average severity	0.0	0.0	0.1	0.0
Inflammation	0	0	1	0	Hydronephrosis	1	1	1	0
Average severity	0.0	0.0	0.1	0.0	Average severity	0.1	0.1	0.1	0.0
Nephropathy	0	0	0	2	Nephropathy	1	2	2	2
Average severity	0.0	0.0	0.0	0.1	Average severity	0.1	0.1	0.1	0.1
Liver: inflammation	2	0	1	0	*Liver:* inflammation	0	0	1	0
Average severity	0.1	0.0	0.1	0.0	Average severity	0.0	0.0	0.1	0.0
Lung: alveolar macrophages, increased	6	2	5	3	*Lung:* alveolar macrophages, increased	3	3	2	1
Average severity	0.3	0.1	0.3	0.2	Average severity	0.2	0.2	0.1	0.1
Eosinophilic crystals	0	0	0	1	Eosinophilic crystals	0	0	0	1
Average severity	0.0	0.0	0.0	0.1	Average severity	0.0	0.0	0.0	0.1
Infiltrate, perivascular, mixed cell	5	1	2	2	Infiltrate, perivascular, mixed cell	1	1	3	3
Average severity	0.3	0.1	0.1	0.1	Average severity	0.1	0.1	0.2	0.2
Inflammation	4	4	2	9	Inflammation	2	1	2	7
Average severity	0.2	0.2	0.1	0.5	Average severity	0.1	0.1	0.1	0.4
Metaplasia, osseous	1	1	2	0	*Nose/turbinates:* inflammation	0	0	1	0
Average severity	0.1	0.1	0.1	0.0	Average severity	0.0	0.0	0.1	0.0
Mineralization	2	0	0	0	*Pharynx:* inflammation	0	0	0	1
Average severity	0.1	0.0	0.0	0.0	Average severity	0.0	0.0	0.0	0.1
Pancreas: atrophy, acinar	0	0	0	1	*Pituitary gland:* hyperplasia, pars distalis	0	2	0	0
Average severity	0.0	0.0	0.0	0.1	Average severity	0.0	0.1	0.0	0.0
Pituitary gland: hyperplasia, pars distalis	0	0	0	1	*Rectum:* inflammation	0	0	0	1
Average severity	0.0	0.0	0.0	0.1	Average severity	0.0	0.0	0.0	0.1
Preputial gland: inflammation	0	2	2	1	*Tongue:* myodegeneration	0	1	0	0
Average severity	0.0	0.2	0.1	0.1	Average severity	0.0	0.1	0.0	0.0
Prostate: inflammation	1	4	6	2	*Uterus:* inflammation	0	0	1	1
Average severity	0.1	0.3	0.6	0.2	Average severity	0.0	0.0	0.1	0.1
Rectum: lymphoid hyperplasia, peyers patch	0	0	1	0	Physiologic dilatation, horn(s)	10	8	7	9
Average severity	0.0	0.0	0.2	0.0	Average severity	0.8	0.7	0.6	0.9
Stomach: inflammation, glandular region	0	2	1	1					
Average severity	0.0	0.1	0.1	0.1					
Inflammation, non-glandular	0	0	0	1					
Average severity	0.0	0.0	0.0	0.1					
Testis: atrophy	0	0	0	1					
Average severity	0.0	0.0	0.0	0.2					
Urinary bladder: amyloid deposition, submucosa	0	1	0	0					
Average severity	0.0	0.2	0.0	0.0					

In summary, since lower doses were necessary to obtain measurable effects in rats compared to mice when ingesting feed containing B, E, or NT, rats were deemed more sensitive than mice. The key findings observed in rats and mice were lower body weights vs. C at the two higher B and E exposure levels and at the high NT level. The reductions in body weights of treated vs. C groups generally correlated with reduced food consumption for the high dose groups. Despite the small, exposure-related reductions in food consumption, for each test article, the administered doses and plasma nicotine and cotinine values showed corresponding dose-response increases. Also, TK studies indicated that administration of comparable nicotine doses for NT, B, and E lead to comparable systemic exposures. Changes in organ weights were secondary to decreased body weights vs. C, which were likely due to decreased palatability of the diets (and possibly nicotine appetite suppressant effects) and were not associated with any gross or microscopic findings. Consequently, based on these studies, the doses evaluated were confirmed to span the no observable adverse effect level, the lowest observable adverse effect level and the maximum tolerated dose.

Table 12
Mice: microscopic observations summary (incidence, average severity).

Males					Females[a]				
Tissue/observation	Number observed per group				Tissue/observation	Number observed per group			
	C	NT120	B120	E120		C	NT120	B120	E120
Epididymis: aspermia	1	0	0	0	*Clitoral gland:* pyogranuloma	0	1	1	0
Average severity	0.2	0.0	0.0	0.0	Average severity	0.0	0.1	0.2	0.0
Eye: cataract, unilateral	0	0	0	1	*Harderian gland:* atrophy	11	9	10	7
Average severity	0.0	0.0	0.0	0.2	Average severity	0.9	0.6	0.7	0.5
Unilateral rupture	0	0	1	0	Porphyrin pigment	0	0	1	0
Average severity	0.0	0.0	0.2	0.0	Average severity	0.0	0.0	0.1	0.0
Harderian gland: atrophy	1	1	0	3	Inflammation, chronic	1	3	0	1
Average severity	0.1	0.1	0.0	0.2	Average severity	0.1	0.2	0.0	0.1
Inflammation, chronic	0	0	2	1	*Heart:* cardiomyopathy	0	0	1	0
Average severity	0.0	0.0	0.1	0.1	Average severity	0.0	0.0	0.1	0.0
Heart: cardiomyopathy	0	1	0	0	Infiltration, mononuclear cells, epicardium	0	0	1	0
Average severity	0.0	0.1	0.0	0.0	Average severity	0.0	0.0	0.1	0.0
Kidney: granuloma	0	1	0	0	*Kidney:* hyperplasia, plasma cells	0	0	0	3
Average severity	0.0	0.1	0.0	0.0	Average severity	0.0	0.0	0.0	0.3
Infiltration, mononuclear cells, perirenal fat	0	1	0	0	Mineralization	1	0	0	0
Average severity	0.0	0.1	0.0	0.0	Average severity	0.1	0.0	0.0	0.0
Nephropathy	8	9	8	4	Nephropathy	15	7	13	6
Average severity	0.5	0.5	0.4	0.2	Average severity	0.8	0.4	0.7	0.4
Liver: infiltration, mononuclear cells	1	1	0	1	*Liver:* eosinophilic focus	0	0	0	1
Average severity	0.1	0.1	0.0	0.1	Average severity	0.0	0.0	0.0	0.1
Lung: hyperplasia, alveolar cells	1	0	0	0	Infiltration, mononuclear cells	5	6	3	7
Average severity	0.1	0.0	0.0	0.0	Average severity	0.3	0.3	0.2	0.4
Inflammation, subacute	0	0	1	0	*Lung:* hyperplasia, alveolar lining cells	0	0	0	2
Average severity	0.0	0.0	0.1	0.0	Average severity	0.0	0.0	0.0	0.1
Pancreas: necrosis	0	0	1	0	Lymph node, other hyperplasia, lymphoid	–	–	1	1
Average severity	0.0	0.0	0.1	0.0	Average severity	–	–	2.0	2.0
Preputial gland: atrophy	0	0	1	0	Pigment	–	–	1	1
Average severity	0.0	0.0	0.2	0.0	Average severity	–	–	2.0	2.0
Chronic inflammation	0	2	0	0	*Ovary:* cyst	3	4	5	2
Average severity	0.0	0.2	0.0	0.0	Average severity	0.3	0.3	0.3	0.2
Pyogranuloma, unilateral	1	1	1	2	*Salivary gland:* infiltration, mononuclear cells	0	2	1	0
Average severity	0.1	0.1	0.1	0.2	Average severity	0.0	0.1	0.1	0.0
Skin: hyperplasia, epidermis	1	0	0	0	*Sciatic nerve:* infiltration, perineural tissue, macrophages	0	1	0	0
Average severity	0.1	0.0	0.0	0.0	Average severity	0.0	0.1	0.0	0.0
Spinal cord: cyst	0	1	0	0	*Skin:* abscess	1	0	0	1
Average severity	0.0	0.1	0.0	0.0	Average severity	0.1	0.0	0.0	0.2
Spleen: apoptosis, lymphocytes	0	0	1	0	Chronic inflammation	0	0	2	3
Average severity	0.0	0.0	0.1	0.0	Average severity	0.0	0.0	0.2	0.3
Stomach: hyperplasia/dilation, epithelium, mucosa, glandular	2	3	4	3	Ulcer	0	0	0	2
Average severity	0.1	0.2	0.2	0.2	Average severity	0.0	0.0	0.0	0.2
Infiltration, tunica muscularis, lymphocytic	0	0	1	0	*Stomach:* hyperplasia/dilatation, epithelium, mucosa, glandular	0	1	1	1
Average severity	0.0	0.0	0.1	0.0	Average severity	0.0	0.1	0.1	0.1
Inflammation, chronic, tunica muscularis	1	0	0	0	*Uterus:* cystic endometrial hyperplasia	1	2	1	2
Average severity	0.1	0.0	0.0	0.0	Average severity	0.1	0.1	0.1	0.1
Testis: atrophy, germinal epithelium, bilateral	1	0	1	1					
Average severity	0.1	0.0	0.1	0.1					
Atrophy, germinal epithelium, unilateral	1	0	0	2					
Average severity	0.1	0.0	0.0	0.1					
Thymus: atrophy	0	0	1	0					
Average severity	0.0	0.0	0.1	0.0					
Apoptosis, lymphocytes	0	0	1	0					
Average severity	0.0	0.0	0.1	0.0					

[a] Also jejunum, diverticulum E6M, E60F: incidence 1, severity 2.

References

Krautter GR, Potts RJ, Mould AP. Comparative toxicity of nicotine and a tobacco pellet to Sprague–Dawley rats in a 90-day feeding study. In: American College of Toxicology annual meeting; 2008.

LSRO. Differentiating the health risks of categories of tobacco products. Bethesda, MD: Life Sciences Research Office Inc; 2008, www.lsro.org [accessed 3.24.10].

National Research Council. Guide for the Care and Use of Laboratory Animals;. Washington, DC: Institute of Laboratory Animal Resources, Commission on Life Sciences; 1996.

NCI. Monograph 2: Smokeless tobacco or health: an international perspective; 1992, http://cancercontrol.cancer.gov/TCRB/monographs/2/index.html [accessed 3.24.10].

Theophilus EH, Hayes JR, Potts RJ, Ayres PH, Williams CW, Garner CD, Hejtmancik MR, Fallacara DM, Singer AW. Toxicological evaluation of smokeless tobacco: 14- and 28-day rodent feeding studies. Abstracts of the 46th Congress of the European Societies of Toxicology (EUROTOX). Toxicol Lett 198S 2009: S130.