

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

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Abbreviations: ANOVA-analysis of variance; B0.2M-treatment groups include group, dose, gender (*e.g.*, B0.2M); B-tobacco blend; C-negative control; E-tobacco extract; F-females; GLP-Good Laboratory Practices; LSRO-Life Sciences Research Office; M-males; NCI-National Cancer Institute; NTP-National Toxicology Program; NT-positive control-nicotine tartrate; PFC-pair-fed control; RJRT-R.J. Reynolds Tobacco Company; TK-toxicokinetics.

### **Abstract**

Several studies evaluated the effects of feeding a smokeless tobacco blend and an aqueous extract of that blend to rodents. Positive control (nicotine tartrate) and treatment groups were targeted to match a range of nicotine levels. Target doses selected for 14-day studies spanned 0.2-40 (rats) and 0.2-400 mg nicotine/kg/day (mice). Endpoints measured in 14-day studies included body weights, feed consumption, and clinical observations. Based on 14-day study results, doses selected for the 28-day studies spanned 0.2-20 (rats) and 2-200 mg nicotine/kg/day (mice). Endpoints measured in 28-day studies included body weights, feed consumption, clinical observations, plasma nicotine and cotinine, functional observational endpoints, and clinical and gross anatomic pathology. Statistically significant effects in rats and mice occurred at the highest doses of positive control and test articles (e.g., reductions in body and absolute organ weights, arousal and rectal temperature). The effects of blend, extract, and nicotine tartrate positive control were generally similar.

## **1. Introduction**

The purpose of these new, 14- and 28-day studies was to evaluate the palatability and short-term toxicological effects of feeding a smokeless tobacco blend and an aqueous extract of that tobacco blend to rodents. The objectives were to broaden the understanding of diet palatability, species and gender effect differences, and toxicokinetics. These studies are important for several reasons. First, various organizations (e.g., Life Sciences Research Office, LSRO, 2008) have reviewed the effects of smokeless tobacco and pointed out the need to add to the weight of scientific evidence. Second, these are the first known studies that directly compare the effects of ingesting tobacco with the effects of ingesting 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 and remove the tobacco from the mouth. Third, these studies corroborate previous findings on the key effects of ingesting tobacco in Sprague-Dawley rats (Krautter et al., 2008) using two additional rodent models (Wistar Hannover rats and CD-1 mice). Fourth, these studies provide key data to inform the scientific dialog on tobacco regulation and the role of smokeless tobacco in tobacco harm reduction.

Results from five studies are presented in this paper: three 14-day studies (one in rats and two in mice) and two 28-day studies (one in rats and one in mice). The doses used in each of these studies are shown in Table 1. Initially, in the 14-day studies, the rats and mice were studied at the same doses; however, because mice were less sensitive than rats, a second mouse study was conducted at higher doses.

## **2. Materials and methods**

### **2.1. Test articles, controls, and diets**

The test articles used in diets were: 1) a smokeless tobacco blend (26 mg nicotine/g tobacco) and 2) a water extract of that tobacco blend (23 mg nicotine/g tobacco). The smokeless tobacco blend consisted of leaf and stem tobaccos that are used in commercial US smokeless products. The blend did not contain any ingredients added to tobacco. The extract was produced by mixing 1 part tobacco blend with 8 parts potable water at 100°F for 1 hour. The 100°F extraction temperature was selected to mimic the normal oral temperature in humans. The extraction batch was then dewatered using a continuous decanter. The extract was filtered through a 10 micron filter and pumped to a vertical thin film evaporator which was operated at 100°F. The finished extract contained 38% total solids. Test articles were stored frozen ( $\leq 0^{\circ}\text{C}$ ).

The positive control used in diets was nicotine hydrogen tartrate salt (purity  $\geq 98\%$ ; Sigma-Aldrich Co., St. Louis, MO). The negative control was diet alone.

The abbreviations used are: negative control (C); pair-fed control (PFC); positive control-nicotine tartrate (NT), blend (B), extract (E); males (M), females (F); doses, *e.g.*, 0.2 mg nicotine/kg/day (0.2); treatment groups include group, dose, gender (*e.g.*, B0.2M).

The test articles were matched in terms of target dietary nicotine content 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 monitoring feed formulations and rodent exposures.

Test articles were characterized by analyzing various tobacco-specific compounds and microbial endpoints and determined to be stable under study storage conditions. NTP-

2000 powdered diet (Zeigler Bros., Inc., Gardners, PA, 14-day studies; Harlan Teklad, Inc., Madison, WI, 28-day studies) was mixed with test articles or positive control weekly (14-day studies) or monthly (28-day studies). Diets were analyzed for nicotine (Krautter *et al.*, 2008), were confirmed stable and homogeneous, and were stored at room temperature.

## **2.2. 14-day palatability studies: rats and mice**

The 14-day mouse and rat studies were designed to determine the palatability of diets and to define doses for 28-day studies. For the initial 14-day rat and mouse studies, the target dose groups were as indicated in Table 1. Numbers of animals/group were: 5 males/group (except sentinels: 10/group; mouse study 2 C: 10/group). Wistar Hannover rats and Swiss Webster/CD-1 mice (4-7 weeks old, Charles River Laboratories, Raleigh, NC or Portage, MI) were selected for 14-day studies (males) and 28-day studies (males, females) because they are generally accepted toxicological models. Rats and mice were individually housed (rats on stainless steel racks in polycarbonate cages with Alpha-Dri bedding; mice in stainless steel, wire-bottomed cages on stainless steel racks).

These studies evaluated moribundity and mortality (twice/day on weekdays and daily on weekends/holidays). Clinical observations data were collected daily excluding weekends (rats) or twice/week (mice). Detailed scheduled clinical observations occurred before exposure start, at group allocation, then twice/week. Individual non-fasted body weights were determined on the day after arrival, prior to group allocation, daily during the study, and at termination. Feed consumption was measured before study start, then daily during the study. There was no necropsy. These studies were conducted in-house.

Study animals (14- and 28-day studies) 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 reviewed the protocols. Animal care programs were

fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care.

### **2.3. 28-day studies: rats and mice**

The 28-day mouse and rat studies were designed to determine the short-term toxicity of diets. Animals were assigned to: 1) core or 2) toxicokinetics (TK) groups. The general experimental designs (target dose groups) are shown in Table 1. The numbers of animals/group were: rat and mouse core: 10/gender/group; rat TK: 6/gender/group (except pair-fed controls, PFC); mouse TK: 5-C or 23/gender/group-NT, B, E. In the rat study (only) PFC groups (to high dose) were also included to determine if NT, B, or E induced additional body weight-related effects when matched for the same feed intake. Animals were fed the diets *ad libitum* (except for necropsy and PF groups).

Rats were individually housed in wire-bottomed cages. Male mice were individually housed and female mice were housed up to 4/cage in polycarbonate cages with hardwood bedding (except during the functional observational test battery, FOB, when these animals were individually housed).

Endpoints included moribundity and mortality checks (twice/day) and detailed clinical examinations (core animals) prior to exposure start, once/week during the study, and at necropsy. Body weights (core animals) were recorded before exposure, then twice/week, and at necropsy. Feed consumption (core animals) was measured twice/week. The exception was the rat study in which feed consumption was determined daily in the high dose treatment groups and then the mean quantity of control feed was administered to matching PFC groups.

The FOB was performed on 5 animals/gender/group randomly selected from each core group (exception: rats, PF groups) approximately mid-study. The FOB measured several responses (Table 2). Then, animals were returned to their cages.

To confirm exposures, plasma nicotine and cotinine concentrations were measured by liquid chromatography-mass spectrometry (Krautter *et al.*, 2008). The TK study had 2 phases. Phase 1 involved estimation of  $C_{\max}$  and  $T_{\max}$  (time-course analyses about mid-study). Phase 2 involved measuring plasma nicotine and cotinine at the time point determined in Phase 1. In Phase 1, the TK rats (6/gender/group) were randomly subdivided in 2 subgroups spanning 6 time points (target blood collections: 10 p.m., 12 a.m., 4 a.m., 10 a.m., 6 a.m., 12 p.m.). In Phase 1, excluding the 5 mice/gender/group (C), the treated TK mice (18/gender/group) were randomly subdivided in 6 subgroups spanning 6 time points (target blood collections: 10 p.m., 2 a.m., 6 a.m., 10 a.m., 2 p.m., 6 p.m.).

These studies evaluated clinical chemistry, hematology, coagulation, and gross pathology endpoints (including typical organs and tissues) for core animals at necropsy. Clinical chemistry parameters evaluated included aspartate aminotransferase, bilirubin, gamma glutamyl transferase, albumin, albumin/globulin ratio, alkaline phosphatase, glucose, triglycerides, cholesterol, creatinine, total protein, urea nitrogen, calcium, chloride, phosphorus, potassium, and sodium. Hematologic parameters evaluated 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 effects were measured by prothrombin time (rats only).

Scheduled necropsies were conducted under the supervision of a board-certified pathologist. Gross parameters evaluated included: examination of external body surface

and all orifices, the cranial, thoracic, abdominal and pelvic cavities and their contents, and collection of tissues. Organs and tissues evaluated grossly were: adrenals, bone and marrow (femur), brain, clitoral gland, epididymis, esophagus, pharynx, eyes, gross lesions, Harderian glands, heart, large intestine (cecum, colon, rectum), small intestine (duodenum, jejunum, ileum), kidneys, liver (median lobe, left lateral lobe), lungs with bronchi, lymph node (mesenteric), mammary gland (females only), nasal cavity and turbinates, ovaries (without oviduct), oral cavity, pancreas, pituitary, preputial glands, prostate, salivary gland (mandibular), sciatic nerve, seminal vesicles, skeletal muscle (biceps femoris), skin, spinal cord (cervical, thoracic, lumbar), spleen, sternum with bone marrow, stomach (fore-stomach and glandular), testes, thymus, thyroid (with parathyroids, if present, rat only), tongue, urinary bladder, uterus, vagina, and Zymbal glands. Tissues, if present, were placed in 10% neutral buffered formalin (NBF) except for testes that were preserved in Bouin's fixative and then transferred to 70% ethanol and eyes with optic nerve fixed in Davidson's fixative and then transferred to 10% NBF.

Organ weights (absolute), organ-to-body weight, and organ-to-brain weight ratios were recorded on core animals. Organs evaluated included: adrenals (rats only), brain, epididymis, heart, kidneys, liver (with gall bladder and bile drained in mice), spleen, ovaries (without oviduct, rats only), testes (without epididymis), thymus, salivary glands (mandibular) and uterus (with cervix).

These studies were conducted at Battelle, Columbus, OH. The studies were compliant with Good Laboratory Practices.

## **2.4. Statistical analyses**

Except for FOB, data were analyzed using analysis of variance 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 two sample t-test was performed. For categorical FOB data, the CATMOD procedure and chi-square tests were applied. For continuous response FOB data, ANOVA, the MIXED procedure, and F-tests were applied, followed by pairwise comparisons. Statistical tests were carried out to 0.05. Group comparisons included: C vs. NT; C vs. B, E; NT vs. high dose B, E; B vs. E (corresponding doses), and PFC vs. high dose NT, B, E.

### **3. Results**

#### **3.1. 14-day studies**

Key changes measured in these studies were related to body weights. In the 14-day rat study, 20 and 40 mg nicotine/kg/day NT, B, E induced dose-responsive body weight reductions with time vs. C (Figure 1). After exposing mice and rats to the same doses and seeing no differences in body weights in mice, the repeat 14-day mouse study indicated that higher doses (than in rats) were necessary to induce body weight changes in mice (rats were more sensitive than mice). In this second mouse study (Figure 2), the 240 and 400 mg nicotine/kg/day B, E, NT groups were returned to the C diet within week 1 (body weights decreased >20%).

Rat feed consumption vs. C was generally decreased at 20 and 40 mg nicotine/kg/day (except for E20). Mouse feed consumption was generally similar among study groups. There were neither treatment-related deaths nor clinical signs.

Based on the 14-day studies, the doses selected for the 28-day studies spanned 0.2-20 mg nicotine/kg/day (rats) and 2-200 mg nicotine/kg/day (mice).

#### **3.2. 28-day studies**

In the rat core study, all animals survived except for one animal eliminated (B20F; non-treatment-related). In the mouse core study, unscheduled terminations occurred in high

dose groups (entire B200M, B200F, E200M; 6/10 E200F and 4/10 NT200M). In the rat study, clinical signs (e.g., rough hair coat, tremors) were fewer than in the mouse study. Clinical signs tended to occur in the highest dose groups and may have been treatment- and/or palatability-related.

Body weights (Figures 3, 4) and body weight gains were dose-responsive. In rats, body weight gains were lower in the high dose groups than in the matched PFC groups, indicating that other effects (possibly nicotine-related) may have occurred beyond reduced palatability.

In both studies, high dose group feed consumption (Table 3) tended to be lower than in C, indicating reduced palatability. As expected, mice data were more variable.

From the Phase 1 TK study (e.g., Figure 5), the time points chosen for Phase 2 TK were 12:00 a.m. (rats) and 10:00 a.m. (mice). For both rats and mice, doses administered in the diet were generally consistent with plasma nicotine and cotinine (Figures 5, 6). Thus, as expected, target exposures were achieved, plasma nicotine and cotinine levels were dose-dependent, and cotinine levels were substantially greater than nicotine levels. In general, TK mouse data were more variable and male  $C_{\max}$  tended to be higher than female  $C_{\max}$  (at higher doses).

The FOB evaluation indicated minimal treatment-related changes (mainly in arousal and rectal temperature). For example, high dose rat groups generally exhibited slightly reduced arousal vs. C. Mean rectal temperatures of NT, B, E vs. C differed in rats (e.g., NT20F, B20F, E20F<CF) and in mice (e.g., NT200M, B80M, E20M, E80M<CM).

In the rat study, decreases (vs. C) were noted in all absolute organ weights (Tables 4-7) at high doses (mostly at 8 and 20 mg nicotine/kg/day). Statistically significant differences in organ/body weight ratios (vs. C) were noted: decreases in thymus, adrenals

(F), spleen (F), uterus, and ovaries (mostly at 20 mg nicotine/kg/day); increases in brain, testes, epididymis, kidney, liver, and salivary glands (mostly at 8 and 20 mg nicotine/kg/day). The reduced organ weights were likely related to the reduced body weights.

In the mouse study, changes (vs. C) in most absolute organ weights (Tables 8, 9) were noted at high doses (mostly 80 and 200 mg nicotine/kg/day). Some statistically significant differences in organ/body weight ratios (vs. C) were noted (mostly at 200 mg nicotine/kg/day): decreases in spleen (M), thymus (M), kidney (F), and uterus; increases in brain.

In the rat study, there were some statistically significant differences in hematologic endpoints at the high dose (e.g., increased mean corpuscular hemoglobin concentration, M; decreased mean corpuscular volume, hematocrit, platelet count, reticulocytes). Statistically significant changes at the high dose in clinical chemistry data included decreased total protein, glucose and increased alkaline phosphatase (F), bilirubin (F), blood urea nitrogen, albumin/globulin ratio (M), triglycerides, and cholesterol (F). These changes were typically of modest magnitude. The decreased total protein in the high dose groups (NT, B, E) may indicate malnutrition and reduced palatability. In addition, PFC data indicated that high dose group changes were not just due to decreased feed consumption.

In the mouse study, most of hematology and serum chemistry changes were not statistically significantly different from C and were likely stress-mediated.

#### **4. Discussion**

The Life Sciences Research Office (LSRO, 2008) has reviewed the scientific evidence available for smokeless tobacco products and has suggested the conduct of

additional studies to further characterize the effects of smokeless tobacco. These studies are consistent with LSRO recommendations.

Feeding was chosen over gavage because it resembles intended smokeless tobacco use in humans (e.g., exposes the entire gastrointestinal tract, has similar pharmacokinetics).

Human exposure to smokeless tobacco can lead to ~10-50 ng/ml plasma nicotine, with typical steady state levels ~30 ng/ml (LSRO, 2008, NCI, 1992). Based on plasma nicotine, exposures to 0.2-2 mg nicotine/kg/day (rats) and 2-20 mg nicotine/kg/day (mice) are relevant to smokeless tobacco consumers (typical use).

The results of these studies are consistent with results from a 90-day Sprague-Dawley rat study (Krautter *et al.*, 2008). Rats were fed diets containing powdered tobacco pellet or NT at 0, 1.8, 5.4, and 9 mg nicotine/kg/day and were assessed for clinical, hematological, macroscopic, and histopathologic changes. There were dose-dependent differences in body weights and feed consumption vs. C (no histopathologic changes). At the end of that study, mean absolute body weight reductions vs. C for the 9 mg nicotine/kg/day groups were 13-15% (NT, tobacco pellet groups) while in our 28-day rat study, the reductions for the 8 mg nicotine/kg/day groups were 7-17% (B, E).

In our 14- and 28-day studies, the key effects were reductions in body weights that were dose-responsive (with associated organ weight reductions). B, E, and NT generally induced similar effects at comparable doses. In addition, nicotine may have acted as an appetite suppressant and limited the tobacco amount incorporated into diets without leading to significant toxicity.

## 5. References

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**Table 1. Study designs**

14-day studies				28-day studies			
#	Group	Target dose (mg nicotine/kg bw/day)		#	Group	Target dose (mg nicotine/kg bw/day)	
		Rat, Mouse 1	Mouse 2			Rat	Mouse
1	Control	0	0	1	Control	0	0
2	Blend	0.2	40	2	Nicotine tartrate	20	200
3	Blend	2	80	3	Control diet pair-fed to nicotine tartrate	0	-
4	Blend	4	160	4	Blend	0.2	2
5	Blend	8	240	5	Blend	2	20
6	Blend	20	400	6	Blend	8	80
7	Blend	40	-	7	Blend	20	200
8	Extract	0.2	40	8	Control diet pair-fed to blend	0	-
9	Extract	2	80	9	Extract	0.2	2
10	Extract	4	160	10	Extract	2	20
11	Extract	8	240	11	Extract	8	80
12	Extract	20	400	12	Extract	20	200
13	Extract	40	-	13	Control diet pair-fed to extract	0	-
14	Nicotine tartrate	2	40	14	Sentinels	0	0
15	Nicotine tartrate	8	80				
16	Nicotine tartrate	20	160				
17	Nicotine tartrate	40	240				
18	Nicotine tartrate	-	400				
19	Sentinels	0	0				

Blend, Extract, and Nicotine Tartrate designations in this table represent the diets incorporating test articles (tobacco blend or aqueous extract of tobacco blend) or positive control (nicotine tartrate) at the indicated target doses (expressed in mg nicotine/kg body weight/day) for the 14- and 28-day studies. These groups represent the core study groups. Separate groups were used for toxicokinetics (28-day studies).

**Table 2. 28-Day studies: neurobehavioral endpoints measured**

Test	Parameter	Type of Measurement <sup>1</sup>
<b>Home cage</b>		
1	Posture	Score
2	Tremor activity	Score
3	Convulsive activity	Score
4	Lethargy/arousal	Score
5	Eyelid closure	Score
<b>Handling rodents</b>		
6	Ease of removing	Score
7	Ease of handling	Score
8	Hand to hand	Score
9	General condition/appearance	Score
10	Urine stain	Score
11	Fecal stain	Score
12	Salivation	Score
13	Piloerection	Score
14	Fur appearance	Score
15	Lacrimation	Score
16	Palpebral reflex responsivity	Score
17	Pupillary diameter test	Score
18	Pupil response	Score
19	Vocalizations	Yes/No
<b>Open field</b>		
20	Number of lines crossed	Continuous
21	Number of rearings	Continuous
22	Number of urine pools	Continuous
23	Number of fecal boluses	Continuous
24	Posture	Score
25	Tremor activity	Score
26	Convulsive activity	Score
27	Gait	Score
28	Gait score	Score
29	Stereotypy	Score
30	Bizarre behavior	Score
31	Vocalizations-spontaneous	Yes/No
<b>Reflexes</b>		
32	Approach response	Score
33	Touch response	Score
34	Startle response	Score
35	Tail pinch response	Score
36	Placing of paws on grid	Score
37	Righting reflex air	Score
38	Righting reflex surface	Score
39	Rectal temperature (°F)	Continuous
46	Grip strength	Score

<sup>1</sup> Score variables: response represented by a categorical score. Yes/No variables: response given by "Yes" or "No". Continuous variables: response represents a numeric reading on a continuous scale.

**Table 3. 28-Day grand mean feed consumption (g/day  $\pm$  sd)**

<i>Rats</i>		
	<i>Males</i>	<i>Females</i>
C	23.7 $\pm$ 2.8	16.1 $\pm$ 0.6
NT20	14.6 $\pm$ 1.9	11.6 $\pm$ 3.2
B0.2	23.3 $\pm$ 2	15.6 $\pm$ 0.6
B2	21.6 $\pm$ 2.3	15.4 $\pm$ 1
B8	19.3 $\pm$ 1.3	14.5 $\pm$ 2.6
B20	14.3 $\pm$ 2.1	11.3 $\pm$ 2.3
E0.2	25.5 $\pm$ 1.5	17.5 $\pm$ 0.9
E2	23.4 $\pm$ 0.8	15.2 $\pm$ 0.6
E8	20.2 $\pm$ 1.3	15.5 $\pm$ 1.3
E20	13.9 $\pm$ 3.1	12.2 $\pm$ 3
<i>Mice</i>		
	<i>Males</i>	<i>Females</i>
C	4.8 $\pm$ 0.2	4.4 $\pm$ 0.6
NT200	2.7 $\pm$ 0.9	3.1 $\pm$ 0.9
B2	4.7 $\pm$ 0.2	4.0 $\pm$ 0.5
B20	4.7 $\pm$ 0.2	4.1 $\pm$ 0.3
B80	4.0 $\pm$ 0.5	3.6 $\pm$ 0.7
B200	2.4 $\pm$ 2.1	4.3 $\pm$ 3.7
E2	5.2 $\pm$ 0.2	4.1 $\pm$ 0.8
E20	4.9 $\pm$ 0.4	4.4 $\pm$ 0.6
E80	4.4 $\pm$ 0.9	3.9 $\pm$ 0.5
E200	3.4 $\pm$ 1.8	3.3 $\pm$ 1.7



**Table 4. 28-Day Rat Study: Group Mean Absolute Organ Weights (g) – Males**

Group		Adrenal Glands	Brain	Epididymides	Heart	Kidneys	Liver	Salivary Gland	Spleen	Testes	Thymus
CM	Mean	0.067	1.938	0.9945	0.977	1.956	7.882	0.619	0.534	3.362	0.627
	SD	0.006	0.070	0.1191	0.092	0.131	0.825	0.043	0.067	0.345	0.083
	N	10	10	10	10	10	10	10	10	10	10
NT20M	Mean	0.045 <sup>A</sup>	1.839 <sup>A</sup>	0.8699 <sup>A</sup>	0.710 <sup>A</sup>	1.613 <sup>A</sup>	6.176 <sup>a</sup>	0.544 <sup>A</sup>	0.418 <sup>A</sup>	3.228	0.349 <sup>A</sup>
	SD	0.006	0.066	0.0735	0.064	0.126	0.358	0.060	0.049	0.286	0.065
	N	10	10	10	10	10	10	10	10	10	10
B0.2M	Mean	0.061	1.927	0.9670	0.902	1.916	7.006	0.569	0.551	3.266	0.566
	SD	0.007	0.073	0.0782	0.103	0.162	0.607	0.058	0.086	0.170	0.095
	N	10	10	10	10	10	10	10	10	10	10
B2M	Mean	0.065	1.932	0.9554	0.877 <sup>B</sup>	1.858	6.723 <sup>B</sup>	0.613	0.523	3.313	0.473 <sup>B</sup>
	SD	0.010	0.082	0.0623	0.086	0.218	0.919	0.080	0.079	0.146	0.080
	N	10	10	10	10	10	10	10	10	10	10
B8M	Mean	0.049 <sup>B</sup>	1.843 <sup>B</sup>	0.8928 <sup>B</sup>	0.763 <sup>B</sup>	1.678 <sup>B</sup>	6.264 <sup>B</sup>	0.549	0.412 <sup>B</sup>	3.139	0.427 <sup>B</sup>
	SD	0.008	0.083	0.0741	0.075	0.207	0.908	0.043	0.092	0.144	0.090
	N	10	10	10	10	10	10	10	10	10	10
B20M	Mean	0.047 <sup>B</sup>	1.758 <sup>B,C</sup>	0.8282 <sup>B</sup>	0.653 <sup>B</sup>	1.520 <sup>B</sup>	5.895 <sup>B</sup>	0.521 <sup>B</sup>	0.380 <sup>B</sup>	3.107	0.297 <sup>B</sup>
	SD	0.005	0.050	0.0508	0.065	0.143	0.644	0.084	0.037	0.188	0.084
	N	10	10	10	10	10	10	10	10	10	10
E0.2M	Mean	0.061	1.923	0.9337	0.948	2.001	7.498	0.596	0.536	3.221	0.565
	SD	0.012	0.065	0.1133	0.095	0.110	0.618	0.079	0.071	0.286	0.113
	N	10	10	10	10	10	10	10	10	10	10
E2M	Mean	0.061	1.932	0.9136	0.921	1.904	7.057	0.627	0.518	3.201	0.521
	SD	0.011	0.072	0.1012	0.124	0.215	0.946	0.068	0.074	0.317	0.105
	N	10	10	10	10	10	10	10	10	10	10
E8M	Mean	0.056 <sup>B</sup>	1.869	0.9206	0.810 <sup>B</sup>	1.660 <sup>B</sup>	6.356 <sup>B</sup>	0.577	0.469	3.267	0.507 <sup>B</sup>
	SD	0.006	0.046	0.1129	0.059	0.103	0.589	0.048	0.063	0.246	0.093
	N	10	10	10	10	10	10	10	10	10	10
E20M	Mean	0.044 <sup>B</sup>	1.793 <sup>B</sup>	0.7962 <sup>B,C</sup>	0.648 <sup>B</sup>	1.463 <sup>B,C</sup>	6.037 <sup>B</sup>	0.500 <sup>B</sup>	0.372 <sup>B</sup>	2.995 <sup>B</sup>	0.249 <sup>B,C</sup>
	SD	0.007	0.071	0.0784	0.061	0.146	0.621	0.064	0.063	0.278	0.073
	N	10	10	10	10	10	10	10	10	10	10

Multiple comparisons: significantly different ( $p \leq 0.05$ )-capitals-Dunnett's test; lower cases-Modified T test. A = CM vs. NT20M; B = CM vs. B0.2M, B2M, B8M, B20M, E0.2M, E2M, E8M, E20M; C = NT20M vs. B20M, E20M; D = B0.2M vs. E0.2M; E = B2M vs. E2M; F = B8M vs. E8M; G = B20M vs. E20M.

**Table 5. 28-Day Rat Study: Group Mean Absolute Organ Weights (g) – Females**

Group		Adrenal Glands	Brain	Heart	Kidneys	Liver	Ovaries	Salivary Gland	Spleen	Thymus	Uterus
CF	Mean	0.072	1.796	0.625	1.263	4.672	0.108	0.426	0.406	0.440	0.631
	SD	0.007	0.051	0.049	0.102	0.279	0.025	0.048	0.044	0.073	0.200
	N	10	10	10	10	10	10	10	10	10	10
NT20F	Mean	0.043 <sup>A</sup>	1.665 <sup>A</sup>	0.481 <sup>A</sup>	0.985 <sup>a</sup>	4.419	0.055 <sup>A</sup>	0.375 <sup>A</sup>	0.296 <sup>A</sup>	0.278 <sup>A</sup>	0.161 <sup>a</sup>
	SD	0.005	0.062	0.037	0.046	0.340	0.013	0.033	0.034	0.070	0.051
	N	10	10	10	10	10	10	10	10	10	10
B0.2F	Mean	0.066	1.767	0.628	1.250	4.540	0.103	0.406	0.379	0.425	0.523
	SD	0.006	0.053	0.062	0.083	0.299	0.028	0.035	0.059	0.069	0.164
	N	10	10	10	10	10	10	10	10	10	10
B2F	Mean	0.072	1.818	0.614	1.195	4.369	0.101	0.457	0.395	0.411	0.484
	SD	0.010	0.065	0.045	0.074	0.306	0.020	0.052	0.044	0.066	0.098
	N	10	10	10	10	10	10	10	10	10	10
B8F	Mean	0.059 <sup>B</sup>	1.742	0.515 <sup>B</sup>	1.125 <sup>B</sup>	4.292 <sup>B</sup>	0.091	0.450	0.348 <sup>B</sup>	0.377	0.496
	SD	0.009	0.081	0.033	0.081	0.245	0.029	0.028	0.039	0.041	0.214
	N	10	10	10	10	10	10	10	10	10	10
B20F	Mean	0.045 <sup>B</sup>	1.691 <sup>B</sup>	0.461 <sup>B</sup>	0.985 <sup>B</sup>	4.044 <sup>B,C</sup>	0.055 <sup>b</sup>	0.345 <sup>B</sup>	0.230 <sup>B,C</sup>	0.200 <sup>B,C</sup>	0.143 <sup>b</sup>
	SD	0.006	0.060	0.033	0.046	0.247	0.009	0.030	0.042	0.044	0.022
	N	9	9	9	9	9	9	9	9	9	9
E0.2F	Mean	0.078 <sup>D</sup>	1.760	0.665	1.279	4.649	0.099	0.437	0.421	0.416	0.641
	SD	0.007	0.054	0.040	0.068	0.334	0.022	0.058	0.060	0.065	0.172
	N	10	10	10	10	10	10	10	10	10	10
E2F	Mean	0.078	1.778	0.593	1.227	4.308 <sup>B</sup>	0.101	0.446	0.393	0.408	0.567
	SD	0.019	0.037	0.049	0.083	0.367	0.018	0.060	0.029	0.073	0.212
	N	10	10	10	10	10	10	10	10	10	10
E8F	Mean	0.058 <sup>b</sup>	1.749	0.558 <sup>B,F</sup>	1.175	4.410	0.082 <sup>B</sup>	0.444	0.397 <sup>F</sup>	0.376	0.524
	SD	0.011	0.062	0.052	0.092	0.348	0.016	0.035	0.056	0.062	0.238
	N	10	10	10	10	10	10	10	10	10	10
E20F	Mean	0.041 <sup>b</sup>	1.661 <sup>B</sup>	0.464 <sup>B</sup>	1.000 <sup>B</sup>	4.021 <sup>B,C</sup>	0.057 <sup>B</sup>	0.359 <sup>B</sup>	0.245 <sup>B,C</sup>	0.210 <sup>B,C</sup>	0.120 <sup>b,c,G</sup>
	SD	0.008	0.074	0.031	0.061	0.237	0.011	0.033	0.059	0.057	0.020
	N	10	10	10	10	10	10	10	10	10	10

Multiple comparisons: significantly different ( $p \leq 0.05$ )-capitals-Dunnett's test; lower cases-Modified T test. A = CF vs. NT20F; B = CF vs. B0.2F, B2F, B8F, B20F, E0.2F, E2F, E8F, E20F; C = NT20F vs. B20F, E20F; D = B0.2F vs. E0.2F; E = B2F vs. E2F; F = B8F vs. E8F; G = B20F vs. E20F.

**Table 6. 28-Day Rat Study: Pairfed Group Mean Absolute Organ Weights (g) – Males**

Group		Adrenal Glands	Brain	Epididymides	Heart	Kidneys	Liver	Salivary Gland	Spleen	Testes	Thymus
NT20M	Mean	0.045 <sup>A</sup>	1.839	0.8699 <sup>A</sup>	0.710	1.613	6.176	0.544 <sup>A</sup>	0.418	3.228	0.349
	SD	0.006	0.066	0.0735	0.064	0.126	0.358	0.060	0.049	0.286	0.065
	N	10	10	10	10	10	10	10	10	10	10
PFCNTM	Mean	0.050	1.870	0.9724	0.740	1.579	6.298	0.599	0.400	3.267	0.397
	SD	0.006	0.077	0.0769	0.042	0.105	0.505	0.037	0.034	0.184	0.058
	N	10	10	10	10	10	10	10	10	10	10
B20M	Mean	0.047	1.758 <sup>b</sup>	0.8282 <sup>B</sup>	0.653 <sup>B</sup>	1.520	5.895	0.521 <sup>B</sup>	0.380	3.107	0.297 <sup>B</sup>
	SD	0.005	0.050	0.0508	0.065	0.143	0.644	0.084	0.037	0.188	0.084
	N	10	10	10	10	10	10	10	10	10	10
PFCBM	Mean	0.050	1.877	0.9265	0.764	1.620	6.409	0.595	0.429	3.253	0.432
	SD	0.006	0.115	0.0636	0.061	0.151	0.607	0.068	0.064	0.220	0.096
	N	10	10	10	10	10	10	10	10	10	10
E20M	Mean	0.044 <sup>C</sup>	1.793	0.7962 <sup>C</sup>	0.648 <sup>C</sup>	1.463 <sup>C</sup>	6.037 <sup>C</sup>	0.500 <sup>C</sup>	0.372 <sup>C</sup>	2.995	0.249 <sup>C</sup>
	SD	0.007	0.071	0.0784	0.061	0.146	0.621	0.064	0.063	0.278	0.073
	N	10	10	10	10	10	10	10	10	10	10
PFCEM	Mean	0.053	1.844	0.9507	0.782	1.644	6.794	0.606	0.489	3.247	0.431
	SD	0.009	0.085	0.0716	0.038	0.097	0.515	0.056	0.061	0.259	0.099
	N	10	10	10	10	10	10	10	10	10	10

Multiple comparisons: significantly different ( $p \leq 0.05$ )-capitals-Dunnett's test; lower cases-Modified T test. A = PFCNTM vs. NT20M; B = PFCBM vs. B20M; C = PFCEM vs. E20M.

**Table 7. 28-Day Rat Study: Pairfed Group Mean Absolute Organ Weights (g) – Females**

Group		Adrenal Glands	Brain	Heart	Kidneys	Liver	Ovaries	Salivary Gland	Spleen	Thymus	Uterus
NT20F	Mean	0.043 <sup>a</sup>	1.665	0.481 <sup>A</sup>	0.985 <sup>A</sup>	4.419	0.055 <sup>A</sup>	0.375 <sup>A</sup>	0.296 <sup>a</sup>	0.278 <sup>A</sup>	0.161 <sup>a</sup>
	SD	0.005	0.062	0.037	0.046	0.340	0.013	0.033	0.034	0.070	0.051
	N	10	10	10	10	10	10	10	10	10	10
PFCNTF	Mean	0.062	1.730	0.583	1.143	4.412	0.084	0.420	0.398	0.393	0.491
	SD	0.010	0.078	0.046	0.080	0.410	0.010	0.047	0.077	0.068	0.339
	N	10	10	10	10	10	10	10	10	10	10
B20F	Mean	0.045 <sup>B</sup>	1.691	0.461 <sup>B</sup>	0.985 <sup>B</sup>	4.044	0.055 <sup>B</sup>	0.345 <sup>B</sup>	0.230 <sup>B</sup>	0.200 <sup>b</sup>	0.143 <sup>b</sup>
	SD	0.006	0.060	0.033	0.046	0.247	0.009	0.030	0.042	0.044	0.022
	N	9	9	9	9	9	9	9	9	9	9
PFCBF	Mean	0.065	1.747	0.600	1.107	4.334	0.088	0.424	0.391	0.452	0.559
	SD	0.011	0.085	0.052	0.078	0.372	0.017	0.040	0.034	0.109	0.369
	N	10	10	10	10	10	10	10	10	10	10
E20F	Mean	0.041 <sup>C</sup>	1.661 <sup>C</sup>	0.464 <sup>C</sup>	1.000 <sup>C</sup>	4.021 <sup>C</sup>	0.057 <sup>C</sup>	0.359 <sup>C</sup>	0.245 <sup>C</sup>	0.210 <sup>C</sup>	0.120 <sup>c</sup>
	SD	0.008	0.074	0.031	0.061	0.237	0.011	0.033	0.059	0.057	0.020
	N	10	10	10	10	10	10	10	10	10	10
PFCEF	Mean	0.058	1.728	0.596	1.152	4.359	0.084	0.445	0.376	0.425	0.506
	SD	0.007	0.067	0.035	0.059	0.246	0.009	0.048	0.039	0.111	0.211
	N	10	10	10	10	10	10	10	10	10	10

Multiple comparisons: significantly different ( $p \leq 0.05$ )-capitals-Dunnett's test; lower cases-Modified T test. A = PFCNTF vs. NT20F; B = PFCBF vs. B20F; C = PFCEF vs. E20F.

**Table 8. 28-Day Mouse Study: Group Mean Absolute Organ Weights (g) – Males**

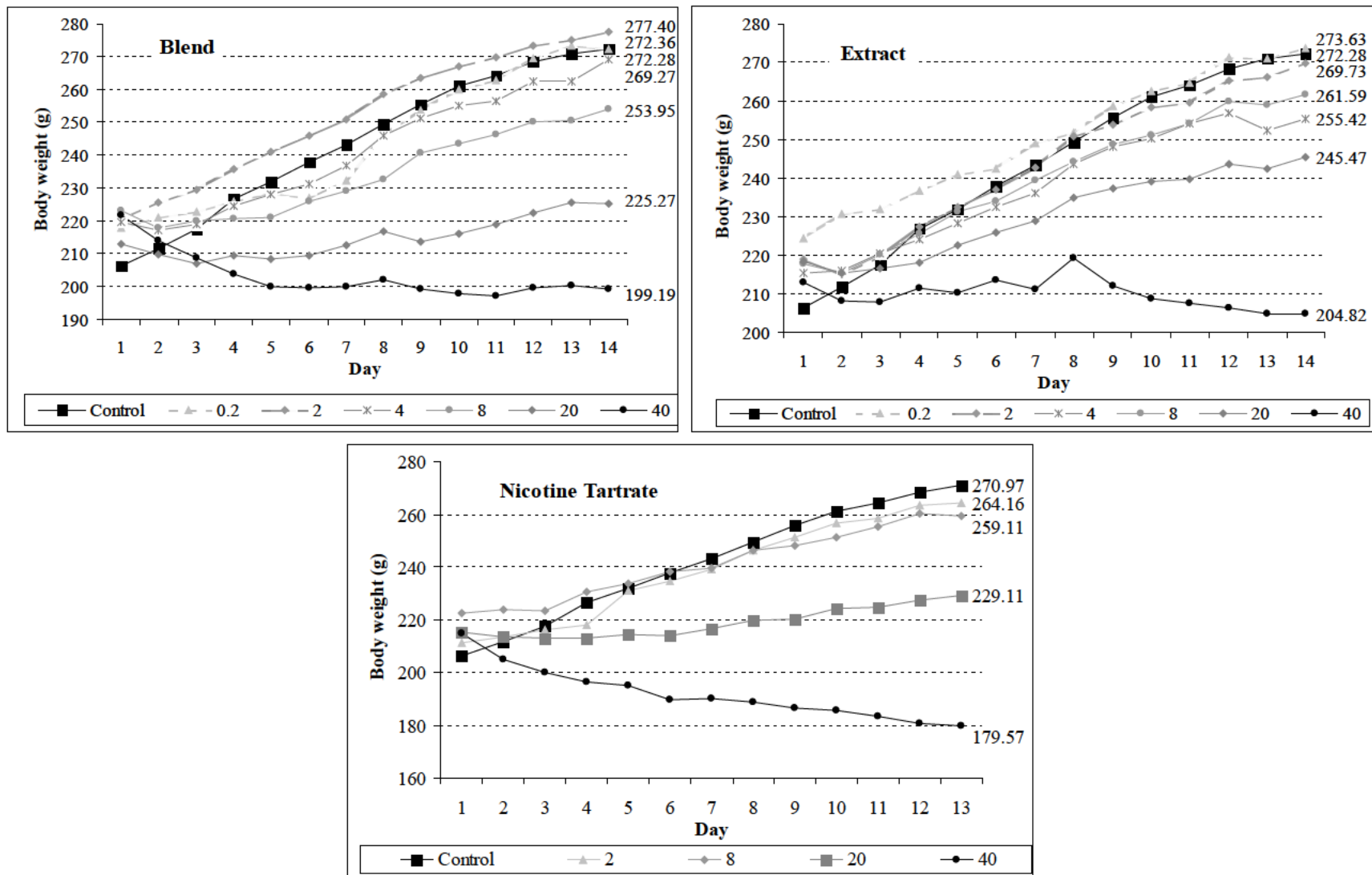
Group		Brain	Epididymides	Heart	Kidneys	Liver	Salivary Gland	Spleen	Testes	Thymus
CM	Mean	0.493	0.1031	0.205	0.496	1.241	0.226	0.086	0.253	0.045
	SD	0.011	0.0176	0.046	0.063	0.118	0.028	0.009	0.035	0.009
	N	10	10	10	10	10	10	10	10	10
NT200M	Mean	0.465 <sup>a</sup>	0.0671 <sup>A</sup>	0.149 <sup>A</sup>	0.335 <sup>A</sup>	0.964 <sup>A</sup>	0.146 <sup>A</sup>	0.042 <sup>A</sup>	0.163 <sup>A</sup>	0.024 <sup>A</sup>
	SD	0.024	0.0182	0.028	0.073	0.229	0.038	0.017	0.042	0.015
	N	6	6	6	6	6	6	6	6	6
B2M	Mean	0.503	0.0970	0.198	0.476	1.212	0.211	0.091	0.238	0.044
	SD	0.025	0.0105	0.022	0.035	0.085	0.023	0.013	0.034	0.009
	N	10	10	10	10	10	10	10	10	10
B20M	Mean	0.481	0.0957	0.192	0.497	1.231	0.218	0.085	0.232	0.048
	SD	0.014	0.0137	0.033	0.056	0.125	0.024	0.010	0.024	0.010
	N	10	10	10	10	10	10	10	10	10
B80M	Mean	0.472 <sup>B</sup>	0.1026	0.163 <sup>B</sup>	0.448	1.178	0.197	0.076	0.232	0.046
	SD	0.014	0.0167	0.027	0.069	0.118	0.028	0.014	0.020	0.007
	N	10	10	10	10	10	10	10	10	10
E2M	Mean	0.486	0.0990	0.188	0.494	1.176	0.200	0.083	0.224	0.043
	SD	0.016	0.0158	0.018	0.049	0.075	0.029	0.009	0.045	0.010
	N	10	10	10	10	10	10	10	10	10
E20M	Mean	0.494	0.0990	0.199	0.488	1.209	0.213	0.095	0.236	0.041
	SD	0.021	0.0139	0.031	0.061	0.088	0.023	0.014	0.038	0.011
	N	10	10	10	10	10	10	10	10	10
E80M	Mean	0.475	0.0889 <sup>F</sup>	0.184	0.428 <sup>B</sup>	1.267	0.188 <sup>B</sup>	0.078	0.231	0.043
	SD	0.015	0.0088	0.038	0.057	0.244	0.022	0.018	0.017	0.016
	N	10	10	10	10	10	10	10	10	10

Multiple comparisons: significantly different ( $p \leq 0.05$ )-capitals-Dunnett's test; lower cases-Modified T test. A = CM vs. NT200M; B = CM vs. B2M, B20M, B80M, B200M, E2M, E20M, E80M, E200M; C = NT200M vs. B200M, E200M; D = B2M vs. E2M; E = B20M vs. E20M; F = B80M vs. E80M; G = B200M vs. E200M.

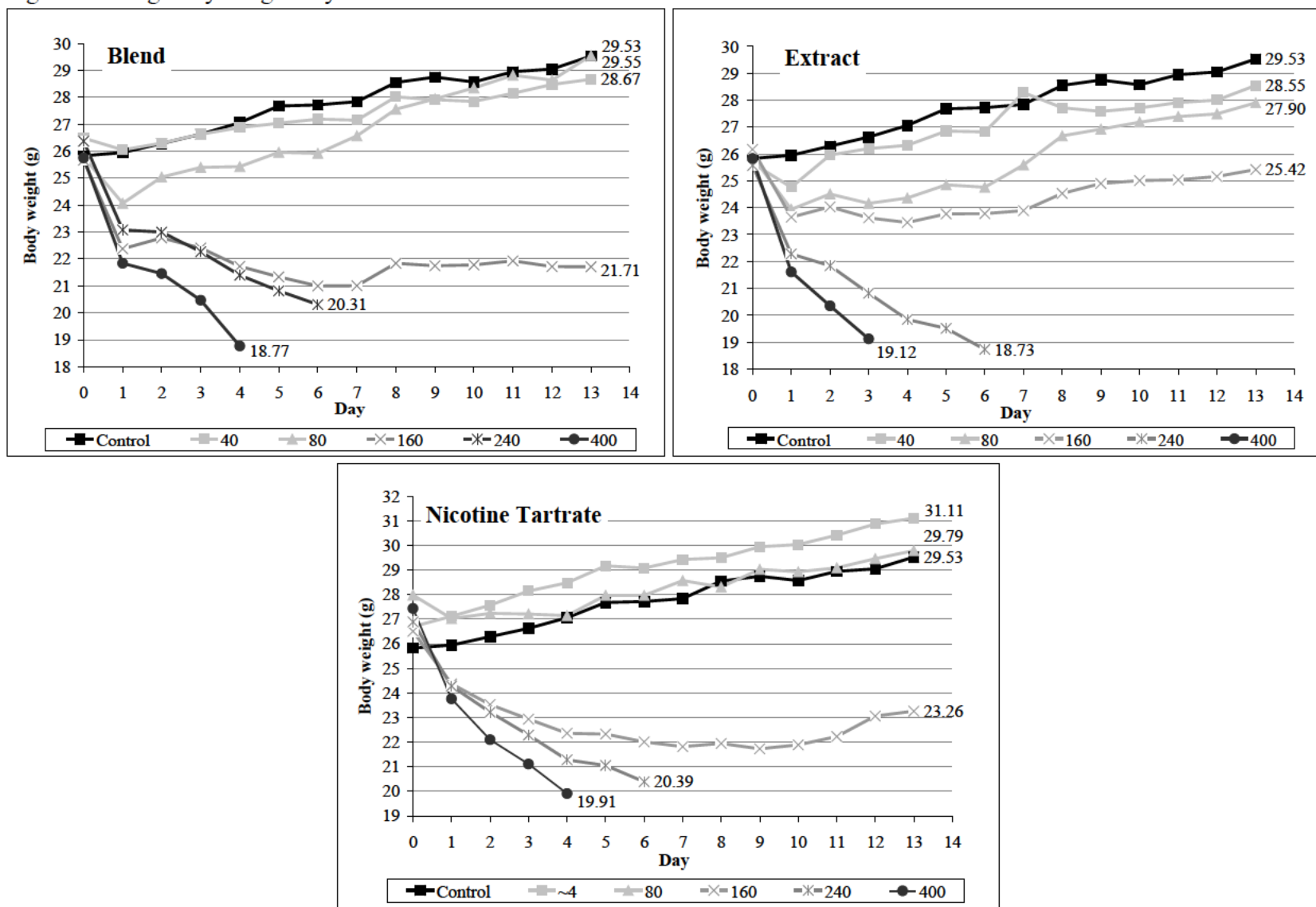
**Table 9. 28-Day Mouse Study: Group Mean Absolute Organ Weights (g) – Females**

Group		Brain	Heart	Kidneys	Liver	Salivary Gland	Spleen	Thymus	Uterus
CF	Mean	0.491	0.151	0.333	0.981	0.142	0.090	0.047	0.142
	SD	0.025	0.031	0.033	0.116	0.028	0.013	0.010	0.037
	N	10	10	10	10	10	10	10	10
NT200F	Mean	0.442 <sup>A</sup>	0.113 <sup>A</sup>	0.244 <sup>A</sup>	0.857	0.101 <sup>A</sup>	0.065 <sup>A</sup>	0.041	0.067 <sup>A</sup>
	SD	0.030	0.024	0.041	0.154	0.024	0.021	0.019	0.027
	N	10	10	10	10	10	10	10	10
B2F	Mean	0.486	0.161	0.334	1.120 <sup>B</sup>	0.149	0.101	0.057	0.162
	SD	0.034	0.025	0.040	0.141	0.022	0.021	0.013	0.052
	N	10	10	10	10	10	10	10	10
B20F	Mean	0.485	0.154	0.312	0.955	0.136	0.087	0.046	0.204
	SD	0.017	0.021	0.042	0.101	0.021	0.017	0.013	0.061
	N	10	10	10	10	10	10	10	10
B80F	Mean	0.483	0.142	0.298	0.957	0.141	0.085	0.052	0.162
	SD	0.016	0.022	0.024	0.088	0.016	0.016	0.010	0.074
	N	10	10	10	10	10	10	10	10
E2F	Mean	0.489	0.178	0.310	0.968 <sup>D</sup>	0.145	0.081 <sup>D</sup>	0.046	0.150
	SD	0.021	0.026	0.032	0.106	0.024	0.018	0.014	0.079
	N	10	10	10	10	10	10	10	10
E20F	Mean	0.488	0.153	0.298	0.946	0.145	0.080	0.051	0.148 <sup>E</sup>
	SD	0.016	0.030	0.025	0.120	0.014	0.024	0.008	0.052
	N	10	10	10	10	10	10	10	10
E80F	Mean	0.487	0.143	0.309	0.992	0.129	0.087	0.050	0.157
	SD	0.028	0.026	0.025	0.095	0.021	0.011	0.016	0.064
	N	10	10	10	10	10	10	10	10
E200F	Mean	0.451 <sup>B</sup>	0.125	0.269 <sup>B</sup>	0.838	0.125	0.062	0.035	0.128
	SD	0.015	0.029	0.027	0.144	0.028	0.030	0.015	0.102
	N	4	4	4	4	4	4	4	4

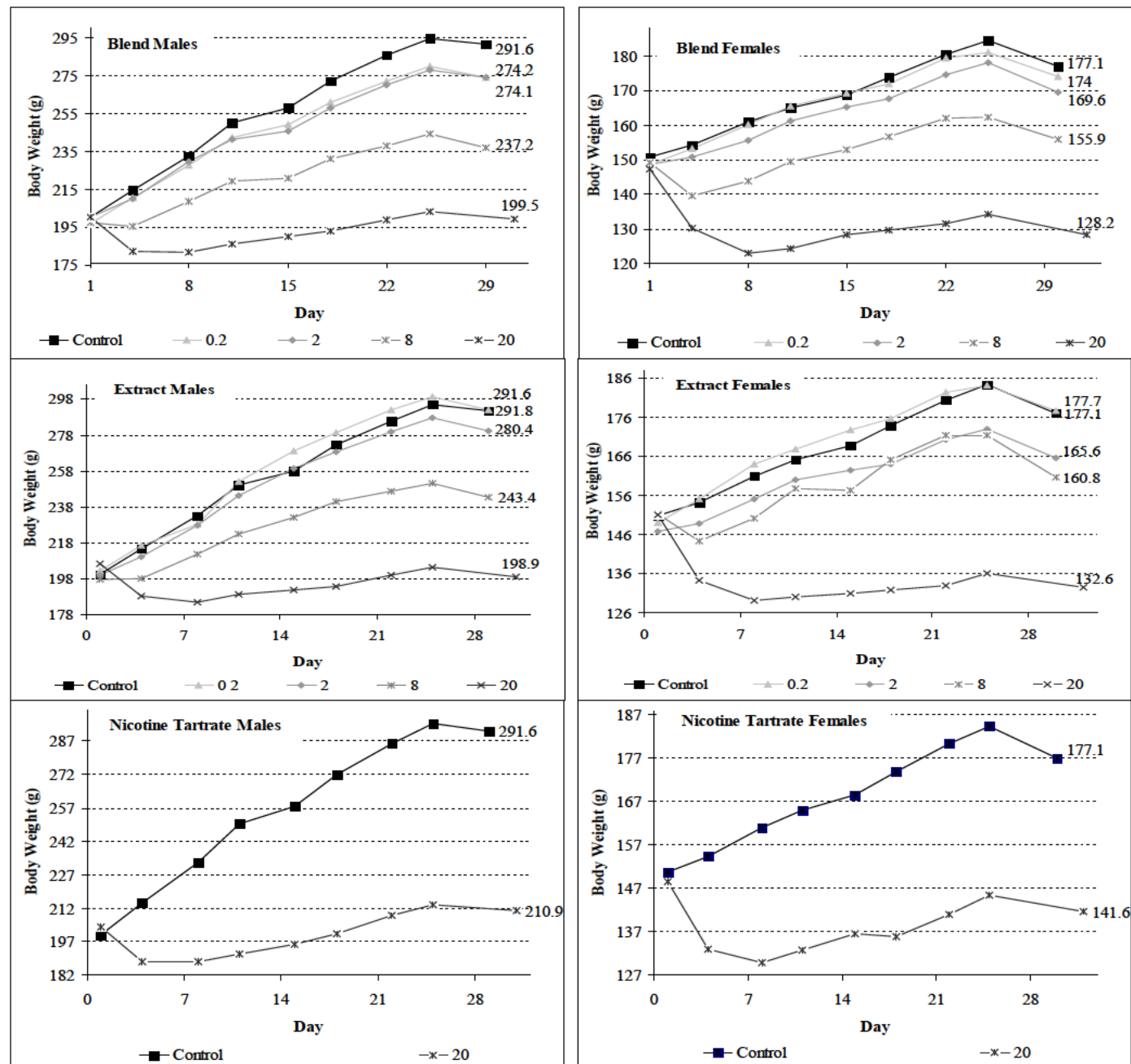
Multiple comparisons: significantly different ( $p \leq 0.05$ )-capitals-Dunnett's test; lower cases-Modified T test. A = CF vs. NT200F; B = CF vs. B2F, B20F, B80F, B200F, E2F, E20F, E80F, E200F; C = NT200F vs. B200F, E200F; D = B2F vs. E2F; E = B20F vs. E20F; F = B80F vs. E80F; G = B200F vs. E200F.

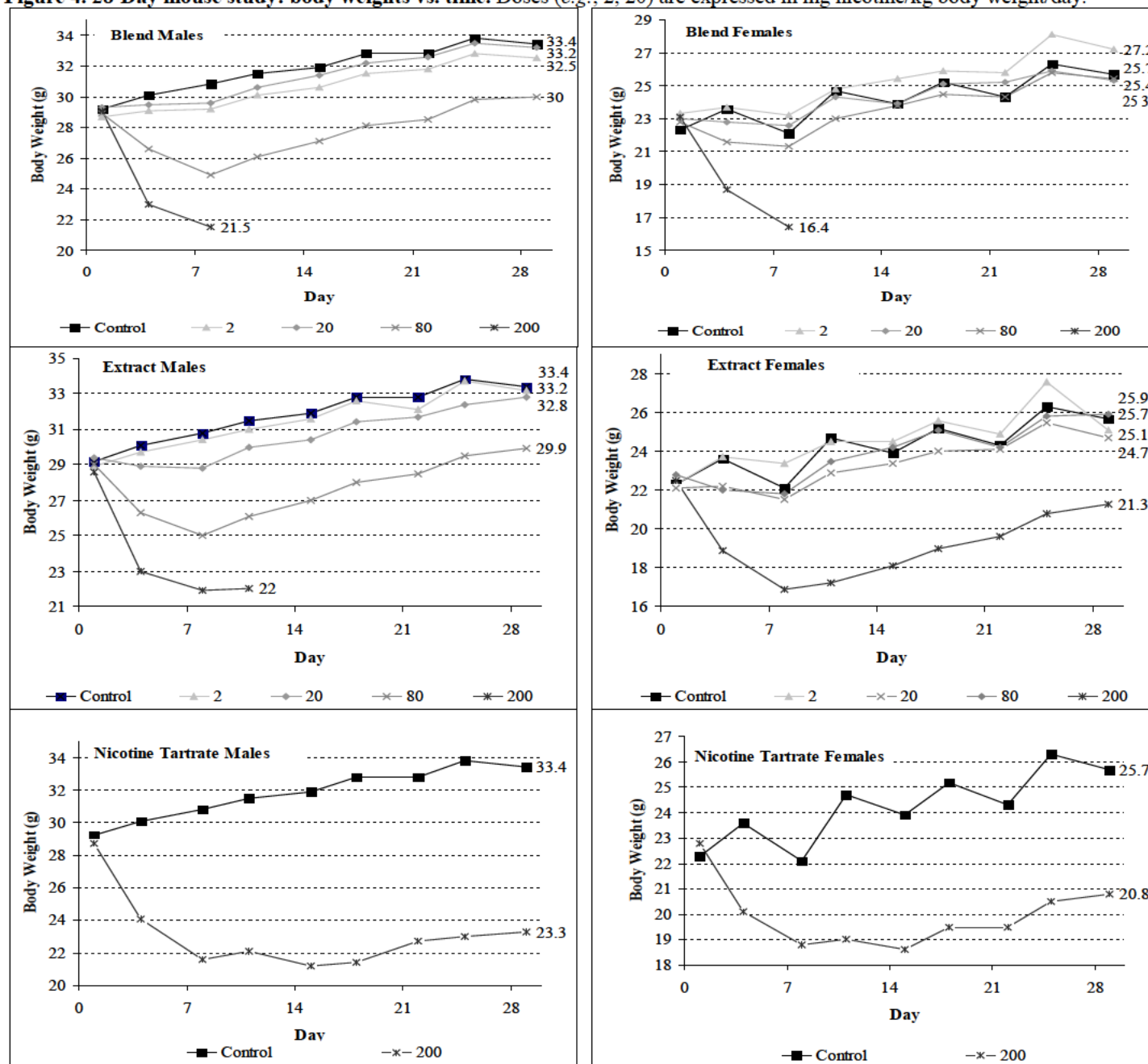
**Figure 1. 14-day rat study: body weights vs. time.** Doses (e.g., 0.2, 2) are expressed in mg nicotine/kg body weight/day.

**Figure 2. 14-Day mouse (repeat) study (with higher doses than for rat): body weights vs. time.** Doses (e.g., 40, 80) are expressed in mg nicotine/kg body weight/day.

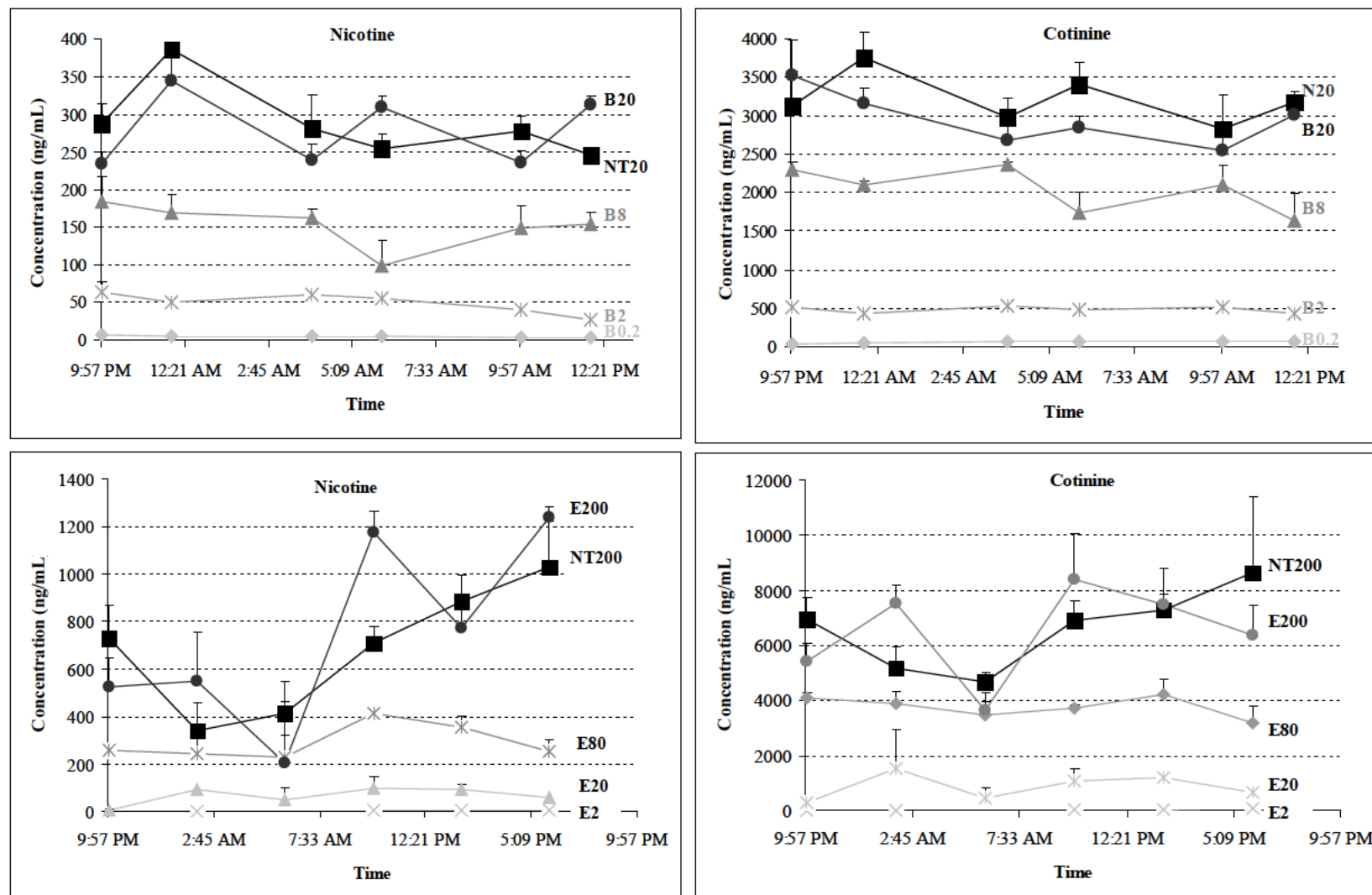




**Figure 3. 28-Day rat study: body weights vs. time.** Doses (e.g., 0.2, 2) are expressed in mg nicotine/kg body weight/day.

**Figure 4. 28-Day mouse study: body weights vs. time.** Doses (e.g., 2, 20) are expressed in mg nicotine/kg body weight/day.

**Figure 5: 28-Day studies (midpoint): plasma nicotine and cotinine, time course.** Examples (females): upper graphs-rats, Blend (B), Nicotine Tartrate (NT); lower graphs-mice, Extract (E), NT. Doses (*e.g.*, 0.2) are expressed in mg nicotine/kg body weight/day.



**Figure 6: 28-Day rat study (end of study): plasma nicotine and cotinine.**