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

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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.

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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.