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Evaluation of the potential effects of ingredients added to cigarettes. Part 4: subchronic inhalation toxicity☆

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Abstract

Mainstream smoke from blended research cigarettes with (test) and without (control) the addition of ingredients to the tobacco was assayed for inhalation toxicity. In total, 333 ingredients commonly used in cigarette manufacturing were assigned to three different groups. Each group of ingredients was introduced at a low and a high level to the test cigarettes. Male and female Sprague–Dawley rats were exposed nose-only either to fresh air (sham) or diluted mainstream smoke from the test, the control, or the Reference Cigarette 1R4F at a concentration of 150 µg total particulate matter/l for 90 days, 6 h/day, 7 days/week. A 42-day post-inhalation period was included to evaluate reversibility of possible findings. There were no remarkable differences in in-life observations or gross pathology between test and control groups. An increase in activity of liver enzymes, known to be due to the high smoke dose, revealed no toxicologically relevant differences between the test and control groups. No toxicological differences were seen between the test and control groups for smoke-related hematological changes, such as a decrease in total leukocyte count. The basic smoke-related histopathological effects, which were more pronounced in the upper respiratory tract than in the lower respiratory tract, were hyperplasia and squamous metaplasia of the respiratory epithelium, squamous metaplasia and atrophy of the olfactory epithelium, and accumulation of pigmented alveolar macrophages. There were no relevant qualitative or quantitative differences in findings in the respiratory tract of the rats exposed to the smoke from the control and test cigarettes. The data indicate that the addition of these 333 commonly used ingredients, added to cigarettes in three groups, did not increase the inhalation toxicity of the smoke, even at the exaggerated levels used.

Keywords: Tobacco; Ingredient; Flovor; Cigarette smoke; Boilogical activity; Subchronic inhalation

1. Introduction

Ingredients are used in cigarette manufacturing to smooth or intensify taste, odor, or pungency of the smoke (Triest, 1979). Cigarette manufacturers have used ingredients for many years, and a complete list of important ingredients used by the major US tobacco companies was published in 1994 (TR Staff Report, 1994). Individual ingredients are added to tobacco filler at relatively low levels, usually 2% or less; hence, their contribution to the composition of cigarette smoke is expected to be low. Some of these ingredients are volatile and may be transferred unchanged into cigarette smoke. Other less volatile ones may be subject to pyrolysis and combustion (Hoffman and Hoffman, 1997), and this has raised concern about potential biological activity in ingredient-supplemented cigarette products.

The effects of some of these ingredients as single ingredients or mixtures of ingredients in cigarette mainstream smoke (MS) have been evaluated (Gaworski et al., 1997); however, the effects of many of the ingredients were not thoroughly evaluated in an inhalation study, and the effects that might result from the addition of these ingredients remain unpredictable. The

Abbreviations: CMH, Cochran–Mantel–Haenszel test; CO, carbon monoxide; GSD, geometrical standard deviation; HbCO, carboxyhemoglobin; HE, hematoxylin–eosin; IARC, International Agency for Research on Cancer; IG, ingredient group; ISO, International Organization for Standardization; MMAD, mass median aerodynamic diameter; MS, mainstream smoke; OECD, Organisation for Economic Cooperation and Development; ppm, parts per million; RSD, relative standard deviation; SD, standard deviation; TPM, total particulate matter; 5′HCOT, 5′-hydroxycotinine; c3′HCOT, *cis*-3′-hydroxycotinine; t3′HCOT, *trans*-3′-hydroxycotinine; NCOT, norcotinine; COT, cotinine; NN′O, nicotine-*N*′-oxide; NNIC, nornicotine

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results of four inhalation studies conducted to evaluate the potential toxicity of 172 flavor ingredients have been reported by Gaworski et al. (1998), who concluded that the addition of flavor ingredients does not alter the severity of biological effects normally observed in inhalation studies on flavored tobacco smoke in rodents.

The objective of the present study was to confirm the results of previous ingredient studies by evaluating 333 ingredients in a subchronic inhalation study that complements extensive smoke chemistry analysis (Rustemeier et al., 2001), and in vitro evaluations (Roemer et al., 2001) conducted on the same ingredients. In the present study, a single smoke concentration (150 μg TPM/l) was tested in order to study the biological effects of the addition of ingredients to the tobacco filler. Three different groups of ingredients were added to tobacco filler, each at a low and high concentration. The low level of each ingredient in the groups approximated the typical use levels considered to be reflective of those used in modern cigarettes and the high levels were 1.5 or 3 times the low levels. The high level was introduced in order to enhance the possibility of detecting biological effects that might not be detected at normal use levels. The test cigarettes were designed to burn in a comparable manner, and this limitation prohibited the use of extremely high levels. The control cigarette had no added ingredients.

The respiratory tract has been identified as the primary site of histopathological changes in rats exposed to tobacco smoke (Dalbey et al., 1980; Wehner et al., 1981; Coggins et al., 1982). Coggins has shown that extending the smoke exposure period beyond 90 days in inhalation studies in rats does not produce any additional biologically significant histopathological findings or any progression of the respiratory tract lesions (Coggins et al., 1982, Coggins, 1998). Therefore, this evaluation focused primarily on histopathological evaluation of the respiratory tract in rats exposed to cigarette smoke for 90 days followed by a 42-day post-inhalation period. When there were findings in other organs, these were also evaluated.

The biological activity of the Reference Cigarette 1R4F from the University of Kentucky (Diana and Vaught, 1990) was determined in parallel to that of the control and the test cigarettes for comparison with our historical data. As the construction of the control cigarette is comparable to that of the 1R4F, comparable biological effects can be expected after exposure of rats to MS from these cigarettes.

2. Materials and methods

2.1. Experimental design

The biological parameters to be investigated were chosen according to OECD guideline 413 (OECD, 1981), with special emphasis on histopathological evaluation of the respiratory tract. The biological activity of diluted MS from cigarettes with one of three groups of ingredients added to the filler, each in a low and high concentration, was assayed and compared to that of a control cigarette with no ingredients added. Groups of male and female rats were exposed either to fresh air (sham) or to diluted MS from these cigarettes at a concentration of 150 μg/l for 6 h/day, 7 days/week, for 90 days. A reference group exposed to diluted MS from the Reference Cigarette 1R4F was also included. Each group contained 10 male and 10 female rats; the control group contained 14 male and 14 female rats. In order to assess the reversibility of ingredient-related effects on top of smoke effects, the sham group, the control group, the high ingredient level group, and the reference group each contained an additional 10 rats per sex, which were exposed with the other rats and evaluated after a 42-day post-inhalation period.

The study was conducted in compliance with the OECD (1997) Principles of Good Laboratory Practice.

2.1.1. Cigarettes

A total of 333 ingredients commonly used in the manufacturing of cigarettes were assigned to three different groups (Carmines, 2001). Ingredient Group 1 comprised casing materials, volatile top flavorings, and ingredients contained in reconstituted tobacco sheet; Group 2 comprised casing materials and volatile top flavorings; and Group 3 comprised casing materials and menthol. Each ingredient was introduced at a low and a high level. The low levels approximate the typical use levels considered to be reflective of those used in modern cigarettes and the high levels were either 1.5 or 3 times the low levels. The amount of menthol was the same in both levels of cigarette 3 because no more material could physically be incorporated into the cigarettes. The test cigarettes were constructed as much as possible to retain the same burning characteristics as the control cigarette with respect to puff count, puff volume, and burning time. The ingredients replaced 7–15% of the tobacco, so the total weight of the filler was the same for the cigarettes with and without the addition of ingredients. A cigarette of identical construction and made with the same tobacco blend as the test cigarettes but without the addition of ingredients was used as the control cigarette. The Reference Cigarette 1R4F was obtained from the University of Kentucky Tobacco and Health Institute (Diana and Vaught, 1990). All cigarettes were conditioned according to ISO standard 3402 (ISO, 1991a) at 22±1 °C and 60±1% relative humidity before being smoked.

2.2. Smoke generation

Cigarette smoke was generated on an INBIFO 30-port carousel smoking machine (modified type SM85)

designed for continuous smoke generation over several hours (Reininghaus and Hackenberg, 1977). The machine operates in conformity with the ISO 3308 (ISO, 1991b) standard smoking protocol. The key smoke generation parameters are a puff volume of 35 ml, a 2-sec puff duration, and a puff frequency of one puff every 60 s. The carousel rotates in steps at a speed of approximately 1 rotation per min, resulting in the generation of a constant stream of mainstream smoke. Only mainstream smoke was conducted into the exposure chambers, sidestream smoke generated during the non-puffing interval for each cigarette was directed into the exhaust air stream. Some minor deviations from the ISO 3308 protocol, such as rectangular puff profile, free (open-end) smoking, and air velocity, were necessary for technical reasons, but these did not affect the composition of the mainstream smoke.

During smoking, the machine is enclosed in a pressurized cabinet. The pressure difference across the puffing port is controlled so that the desired mean puff volume is achieved whenever a cigarette is positioned in front of the puffing port. This technique does not require the use of a pump in the MS stream. At the puffing port, cigarettes are lit with a halogen spot lamp; butts are ejected at a mean butt length of approximately 35 mm. Near the smoking position, the continuously generated MS is mixed with filtered conditioned fresh air at a dilution rate of 1:150 to 1:170 (v/v) depending on the yield of total particulate matter (TPM). The dilution system consists of two concentric glass tubes that minimize particle losses resulting from the redistribution of particles from the particulate phase of cigarette smoke to the gas/vapor phase. At this dilution rate, particle losses of 30–40% are typically observed because condensed volatiles in the undiluted mainstream TPM fraction are redistributed in the gas/vapor phase of the diluted smoke.

2.3. Analytical characterization of the test atmospheres

Several smoke constituents were determined at designated time intervals. Smoke samples were collected from within the exposure chambers at the breathing zone of the rats. The analytical methods used to determine TPM, CO, aldehydes (formaldehyde, acetaldehyde and acrolein), nicotine, nitrogen oxides and particle size distribution as well as those for temperature and relative humidity in the exposure chambers were performed as previously described (Haussmann et al., 1998).

2.4. Animals and animal care

Care and use of the animals was in conformity with the American Association for Laboratory Animal Science Policy on the Humane Care and Use of Laboratory Animals (AAALAC, 1991). Outbred male and female Sprague–Dawley rats (Crl: CD (SD) BR), bred under specific pathogen-free conditions, were obtained from Charles River Deutschland (Germany). Sprague–Dawley rats were chosen because of the large amount of data published on the strain (e.g. IARC, 1986; Coggins et al., 1989a). Animals were approximately 5 weeks old on arrival and were acclimatized for 8 days prior to exposure. Necropsy performed on five rats per sex on the day after arrival revealed no histopathological findings in the respiratory tract or in other organs. Blood samples taken from nine rats per sex for serological screening (Haussmann et al., 1998) revealed no interfering microbiological results. At the end of the inhalation and post-inhalation periods, serological screening on nine rats per sex was repeated and no antibodies against organisms that might affect the results of the study were found.

The rats were barrier-maintained in an animal laboratory unit under controlled hygienic conditions, and frequent bacteriological examinations were made of the laboratory air and surfaces and the diet and drinking water. The laboratory air was filtered conditioned fresh air. Room temperature and relative humidity were maintained at 21±1 °C and 58±4% (means±S.D.), respectively; the light/dark cycle was 12 h/12h.

Two rats of the same sex were housed together in polycarbonate cages supplied with sterilized softwood granulate bedding material (Braun and Co., Battenberg, Germany). A sterilized, fortified pellet diet (Altromin, standard rodent diet, Germany) from cage lid racks and heat-treated tap water (>68 °C for at least 10 min) from water bottles with sterilized sipper tubes was available ad lib. except during exposure. Chemical analysis of food, water and bedding material confirmed that no contaminants were present (aflatoxins, selected pesticides, heavy metals and polychlorinated biphenyls; diet also for nitrate, nitrite, nitrosamines and antioxidants; bedding material also for pentachlorophenol) that could adversely affect the outcome of the study. The rats were individually identified by means of subcutaneous transponders. The mean body weight of the male and the female rats at the beginning of the study was 188 g (RSD: 4%) and 150 g (RSD: 4%), respectively. The mean group body weight of all male and all the female rats at the beginning of the exposure period was equal.

2.5. Exposure

The rats were exposed nose-only in type IC88 exposure chambers (Haussmann et al., 1998) in glass tubes matching their size. The position of the rats was changed on a daily basis according to a predetermined rotation scheme. Sham rats were exposed in the same manner as the smoke-exposed rats, but to filtered, conditioned fresh air. The relative humidity in the sham exposure chamber was 50±4% (mean±SD); this was

considered to be representative for the other exposure groups. The temperature within the exposure chambers ranged from 21.6 to 23.3 °C (S.D. < 0.8 °C). These conditions are in compliance with the exposure conditions specified by the OECD (1981).

The inhalation period began with a 3-day dose and tube adaptation period; that is, 0.25, 0.5 and 0.75 of the final daily exposure duration on days 1 (1.5 h exposure), 2 (3 h exposure), and 3 (4.5 h exposure), respectively.

2.6. Biological parameters

2.6.1. Biomonitoring

Respiratory frequency, tidal volume, and respiratory minute volume were determined on 10 rats/sex/group by whole-body plethysmography to provide an estimate of the inhaled dose of cigarette smoke (Haussmann et al., 1998). Steady-state concentrations of blood carboxyhemoglobin (HbCO) were determined in five rats/sex/group according to the method of Klimisch et al. (1974), once during the inhalation period. Nicotine metabolites were determined by HPLC after derivatization with 1,3-diethyl-2-thiobarbituric acid as previously described (Rustemeier et al., 1993; Haussmann et al., 1998) in urine collected over 24 h during and following smoke exposure, once during the inhalation period.

2.6.2. Body weight, food consumption and in-life observations

Individual body weight and group food consumption were determined at least once per week. Rats were checked at least once each day for mortality/moribundity. Detailed checks of the general condition and behavior of the rats were performed on two to four rats per group per day, shortly after the daily exposure.

2.6.3. Ophthalmology

Ophthalmological examinations, namely direct ophthalmoscopy and split lamp examinations, were performed on all rats before the start of the inhalation period and on all rats in the sham, control, high ingredient level, and 1R4F groups at the end of the inhalation period.

2.6.4. Hematology and clinical chemistry

The parameters determined were those prescribed by OECD guideline (OECD, 1981). At necropsy, animals were anesthetized with an ip injection of sodium pentobarbital and blood samples were collected from the retro-orbital venous plexus for hematology and from the abdominal aorta for clinical chemistry evaluation. Clinical chemistry evaluations were performed on rats without prior fasting. Hematological parameters were determined according to standard methods with an automated hematology-analyser; differential white blood cell counts were determined from blood smears. Clinical chemistry parameters were determined according to standard laboratory methods.

2.6.5. Necropsy, gross pathology, organ weights and histopathology

At necropsy, the rats were killed by exsanguination after pentobarbital anesthesia. An external examination of the carcass was conducted to determine the presence of gross lesions. All rats were then completely dissected and examined for gross lesions of the internal tissues and organs. Weights of lungs with larynx and trachea, liver, heart, adrenal glands and kidneys were recorded. Lungs with larynx and trachea were removed together and fixed with EAFS (ethanol–acetic acid–formaldehyde–saline solution) solution by instillation at 20 cm water pressure. Testes were fixed in Bouin's solution, sternum in Schaffer's solution, eyes in Davidson's solution, and all other organs in 10% buffered formaldehyde solution.

Histological sections were prepared for the nose according to the method of Young (1981), and for the larynx according to Lewis (1981). The trachea was cut transversally (near the larynx) as well as frontally (at the bifurcation). For the left lung, one frontal section passing through the main bronchus was prepared, and for the right lung, one frontal section passing through a maximum number of lobes was prepared. All sections were stained with hematoxylin–eosin (HE). Sections from nose level 1, tracheal ring and bifurcation, and lungs were additionally stained with Alcian blue/Periodic acid Schiff's reagent and liver sections with oil-red orange stain. The laryngeal epithelial thickness at the floor of the larynx was also determined morphometrically. A veterinary pathologist with experience in cigarette smoke-related changes in the respiratory tract of rodents read the slides in a blind manner. Histopathological findings were scored according to a defined severity scale from 0 to 5. Mean severity scores were calculated based on all rats in a group.

2.6.6. Evaluation and statistics

For the results of the analytical assays and determinations, a number of values (mean values and a measure of variance) were calculated. For each ingredient group and both sexes, the results of the control, the low ingredient level and the high ingredient level were compared at the end of the inhalation period. At the end of the post-inhalation period, only the control and the high ingredient level groups were compared. The evaluation of the post-inhalation data was limited to those endpoints for which statistically significant treatment-related changes were seen at the end of the inhalation period.

For continuous data, the one-way analysis of variance was used for the overall comparison followed by a pairwise comparison (Duncan), or the *t*-test modified

according to Welch (after the post-inhalation period). For ordinal data the generalized Cochran–Mantel–Haenszel test (CMH) was used for overall comparison followed by a pairwise comparison (see above) where appropriate.

Results were evaluated for statistical significance at $P \leq 0.05$ without adjustment for multiple testing. Statistically significant results were considered as explorative indicators rather than confirmatory evidence of an effect except in cases where the parameters evaluated revealed a consistent, treatment-related response that was biologically meaningful.

3. Results

3.1. Mainstream smoke composition

Analysis of the test atmospheres throughout the inhalation period indicated that the MS was reproducibly generated and delivered to the exposure chambers with relative standard deviations of less than 10% for TPM, CO and nicotine (Table 1). The concentrations of the smoke constituents determined in the diluted MS were similar (within 10%) for the test cigarettes. Slight differences were observed with respect to the concentrations of formaldehyde, nicotine, nitric oxide and nitrogen oxides (Table 1). Compared to the control test atmosphere, the concentrations of these four smoke constituents were lower in the test atmospheres of Ingredient Group 2 cigarettes, while the formaldehyde concentration in the test atmosphere of Group 3 cigarettes was generally higher. Corresponding differences in these smoke constituents were also found in undiluted smoke (Rustemeier et al., 2001), indicating that diluting the smoke for inhalation purposes does not disproportionately affect the amounts of the smoke constituents.

The particle size distribution measurements indicated that particles were equally respirable in all MS-exposed groups [average median mass aerodynamic diameter (MMAD) of 0.48 μm and geometrical standard deviation (GSD) of 1.7 in all test atmospheres].

3.2. Biomonitoring

3.2.1. Respiratory physiology

Compared to the sham-exposed group, the respiratory rate of the smoke exposed-rats was up to 20% lower, while the tidal volume was unaffected (Table 2). The respiratory rates and tidal volumes obtained for the smoke-exposed rats were the same for both test and control groups; the respiratory minute volumes were at least as high in the test groups as in the control group. A more pronounced depression of the respiratory rate and the respiratory minute volume has been observed by others at higher MS concentrations (Coggins et al., 1989a). This effect has been shown to be a useful parameter in assessing the irritative potency of various airborne chemicals (Alarie, 1981).

3.2.2. Carboxyhemoglobin

The steady-state HbCO concentrations in the blood of the smoke-exposed rats were in agreement with predicted values based on the CO concentrations in the various test atmospheres (Table 2) and fit well with our historical controls (e.g. for male rats: $[\%HbCO] = [CO_{ppm}] \times 0.125 + 0.238$, $r^2 = 0.97$, $n = 121$). Smoke from test cigarettes supplemented with low and high levels of the three groups of ingredients had no

Table 1
Concentration of selected smoke components in the diluted mainstream smoke

Parameter	Unit	N	Exposure groups								
			Sham	Control	IG 1 (Test)		IG 2 (Test)		IG 3 (Test)		1R4F (Ref.)
					Low	High	Low	High	Low	High	
TPM	μg/l	93	< d.l	151±6	151±5	149±6	149±5	149±5	149±6	150±6	151±8
Carbon monoxide	ppm	93	< d.l.	172±7	162±7	163±8	162±7	178±7	158±7	169±8	183±11
Nicotine	μg/l	16–18	< d.l.	10.1±0.8	9.9±0.8	10.4±1.0	9.6±0.8	8.6±0.6	10.3±0.8	10.0±0.7	10.6±1.0
Formaldehyde	ppm	11–12	n.d.	0.33±0.02	0.31±0.02	0.35±0.03	0.28±0.02	0.27±0.02	0.40±0.04	0.53±0.04	0.39±0.04
Acetaldehyde	ppm	11–12	n.d	7.66±0.38	7.28±0.46	7.20±0.38	7.28±0.44	7.48±0.33	7.05±0.51	8.23±0.50	8.38±0.58
Acrolein	ppm	11–12	n.d	0.51±0.03	0.53±0.03	0.53±0.03	0.54±0.03	0.55±0.03	0.48±0.04	0.56±0.04	0.56±0.04
Nitric oxide	ppm	10–12	n.d	4.4±0.2	4.4±0.3	4.1±0.2	3.9±0.2	3.8±0.2	4.1±0.1	4.1±0.2	5.5±0.4
Nitrogen oxides	ppm	10–12	n.d	4.6±0.3	4.6±0.3	4.3±0.3	4.1±0.2	4.0±0.1	4.2±0.2	4.3±0.3	5.7±0.5
Particle size distribution		2	n.d								
MMAD	μm			0.49	0.49	0.48	0.48	0.48	0.48	0.48	0.48
GSD	–			1.69	1.66	1.65	1.68	1.63	1.69	1.66	1.67

Data are given as mean±standard deviation. Low and high: level of ingredient group added; <d.l.: below detection limit; n.d.: not determined. Detection limit: TPM 0.9 μg/l, CO 1.5 ppm, nicotine 0.58 μg/l.

Table 2
Biomonitoring parameters

Parameter	Unit	N	Exposure groups								
			Sham	Control	IG 1 (Test)		IG 2 (Test)		IG 3 (Test)		1R4F (Ref.)
					Low	High	Low	High	Low	High	
Male rats											
Resp. rate	1/min	9–10	119±4	109±4	107±5	103±4	111±5	105±3	106±4	114±3	102±4
Tidal volume	ml	9–10	1.5±0.1	1.3±0.1	1.5±0.1	1.3±0.1	1.4±0.1	1.4±0.1	1.4±0.1	1.3±0.1	1.3±0.1
Resp. minute volume	ml/min	9–10	186±8	137±9	161±11	138±10	156±6	145±10	155±7	149±8	133±8
HbCO proportion	%	4–5	0.3±0.0	24.1±0.7	25.2±0.6	25.7±0.6	21.5±0.9	23.5±0.5	23.8±0.5	25.5±0.6	28.5±0.4
Relative distribution of nicotine metabolites											
5′HCOT	%	8	n.d	19.2±0.9	19.2±0.9	19.0±1.1	18.8±1.3	18.7±1.4	21.2±1.6	21.6±1.5	18.0±1.1
c3′HCOT	%	8	n.d	0.2±0.1	0.3±0.0	0.3±0.1	0.3±0.1	0.3±0.1	0.2±0.0	0.2±0.1	0.2±0.0
t3′HCOT	%	8	n.d	4.7±0.3	4.4±0.2	4.4±0.2	4.3±0.4	3.8±0.4	4.3±0.3	3.9±0.4	4.7±0.3
NCOT	%	8	n.d	10.2±0.9	9.7±0.6	9.7±0.6	8.9±0.8	8.2±1.2	7.9±0.6	8.7±0.9	10.0±0.7
COT	%	8	n.d	26.7±1.4	28.9±1.3	28.9±1.3	25.1±1.5	25.9±1.8	25.6±0.9	26.6±1.4	25.7±0.8
NN′O	%	8	n.d	32.6±2.8	31.7±1.8	31.7±1.8	36.2±2.4	37.1±4.0	34.8±1.7	32.9±2.9	35.3±1.7
NNIC	%	8	n.d	6.5±0.4	5.8±0.4	5.8±0.4	6.5±0.5	6.0±0.4	6.1±0.4	6.1±0.5	6.2±0.3
Female rats											
Resp. rate	1/min	9–10	109±10	92±4	82±3	91±3	81±4	91±5	98±3	95±4	89±3
Tidal volume	ml	9–10	1.3±0.1	1.4±0.1	1.3±0.1	1.4±0.1	1.6±0.1	1.4±0.1	1.4±0.1	1.5±0.1	1.2±0.1
Resp. minute volume	ml/min	9—10	141±9	128±10	109±8	125±7	128±9	129±7	132±6	139±7	112±11
HbCO proportion	%	4—5	0.3±0.0	27.8±0.8	25.5±0.8	24.1±0.6	23.9±0.6	26.1±0.4	24.9±0.5	26.2±0.7	25.8±1.7
Relative distribution of nicotine metabolites											
5′HCOT	%	8	n.d	16.8±0.4	20.3±1.6	21.1±1.2	17.3±0.8	19.0±1.1	17.5±0.7	20.0±0.9	18.8±0.8
c3′HCOT	%	8	n.d	0.2±0.0	0.3±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.4±0.1	0.2±0.0
t3′HCOT	%	8	n.d	2.4±0.2	3.2±0.2	3.1±0.3	2.8±0.2	3.4±0.5	2.6±0.2	3.0±0.3	2.9±0.1
NCOT	%	8	n.d	8.8±0.5	10.4±0.4	8.4±0.9	9.0±0.9	9.3±1.0	8.3±0.6	9.9±0.8	8.9±0.8
COT	%	8	n.d	26.0±0.8	29.8±1.4	28.1±2.5	29.2±2.1	26.7±1.7	26.4±1.3	28.8±1.6	28.0±1.9
NN′O	%	8	n.d	39.7±1.3	29.7±1.5	32.9±4.2	34.9±3.4	35.5±3.6	38.4±1.4	32.1±2.4	34.8±3.3
NNIC	%	8	n.d	6.1±0.3	6.3±0.4	6.2±0.3	6.6±0.4	5.9±0.4	6.6±0.3	5.8±0.4	6.5±0.5

Data are given as mean±standard error. Low and High: level of ingredient group added. No statistically significant differences. 5′HCOT, 5′-hydroxycotinine; c3′HCOT, *cis*-3′-hydroxycotinine; t3′HCOT, *trans*-3′-hydroxycotinine; NCOT, norcotinine; COT, cotinine; NN′O, nicotine-*N*′-oxide; NNIC, nornicotine.

effect on steady-state carboxyhemoglobin concentrations in male and female rats compared to smoke-exposed controls.

3.2.3. Urinary nicotine metabolites

The urinary nicotine metabolite profiles were nearly the same for all groups (Table 2); however, the excretion of *trans*-3′-hydroxycotinine was slightly higher in male rats than in female rats (4.4 and 2.9%, respectively, mean of all groups). The amount of nicotine metabolites excreted represents up to 98% of the calculated nicotine uptake, which is calculated from the measured respiratory minute volume, the nicotine concentration in the test atmosphere, and the daily exposure duration. This is significantly higher than the nicotine metabolite recovery after iv administration of nicotine, which is reported to be approximately 50% of the administered iv dose (Schepers et al., 1993). These data indicate that nicotine uptake was also achieved through other exposure routes, such as oral ingestion during grooming following the inhalation exposure. Therefore, while the amount of nicotine metabolites excreted is a good biomarker of total nicotine uptake, the calculation of the recovery may not be meaningful as a measure for the absolute particulate uptake in inhalation studies. The biomonitoring parameters indicate no significant changes in smoke uptake in rats exposed to smoke from test cigarettes with added ingredients compared to the control and 1R4F groups.

These data suggest that smoke sensory irritancy, blood carboxyhemoglobin concentrations and nicotine metabolism were not affected by either the addition of elevated levels of ingredients, or by the different groups of ingredients in the test cigarettes.

3.2.4. Body weight gain and food consumption

The body weight of both male and female rats in all groups increased throughout the inhalation period

(Fig. 1). By the end of the inhalation period, the body weight gain for all male smoke-exposed rats was reduced by 20–28% compared to the sham group (Table 3). This reduction is considered to be related to MS toxicity and irritation-dependent stress. Both nicotine (Chowdhury et al., 1989; Chowdhury, 1990) and acrolein (Bouley et al., 1975; Feron et al., 1978) have been shown to be responsible for this effect. The absence of a body weight effect in smoke-exposed female rats, and the body weight gain reduction in male rats have already been reported by Coggins et al. (1989b, 1993).

Body weight gain for male rats was less affected in all test groups compared to the control group. The only statistically significant difference observed was for rats exposed to smoke from Ingredient Group 2 high level cigarettes (body weight gain reduction of 28±1% in

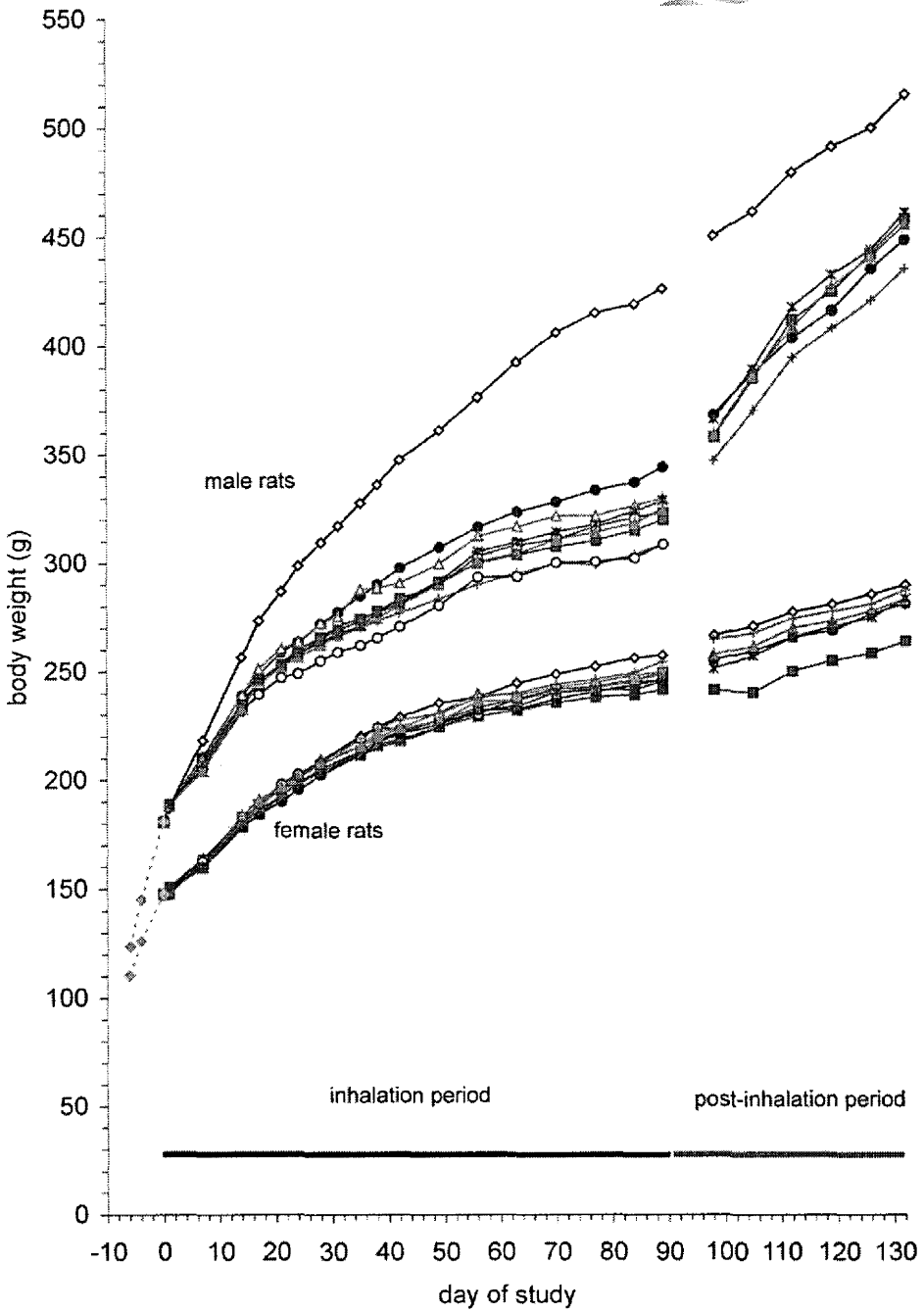

Fig. 1. Mean body weights of male and female rats exposed to fresh air (sham) and MS of the control, test and 1R4F cigarettes (RSD% < 13% for male rats and 9% for female rats). (◇): sham; (+): control; (□,■): ingredient group 1, low and high level; (○,●): ingredient group 2, low and high level; (△,▲): ingredient group 3, low and high level; (*): 1R4F.

control vs 20±2% in ingredient group 2, high level, means±S.E.). The fact that the body weight gain is less affected in the male rats in this group might be related to the lower concentration of nicotine in the diluted mainstream smoke. However, as this effect was not seen in female rats, it suggests that it is either a chance finding or that the potential effect of the lower nicotine concentration is mitigated by the lower body weight gain of the female rats. During the post-inhalation period, all groups of male rats demonstrated an increased rate of body weight gain; yet at the end of the post-inhalation period, the body weight of the male smoke-exposed rats was still statistically significantly lower compared to sham-exposed rats.

The absolute food consumption was statistically significantly higher in the male sham group than in the male smoke-exposed groups. Normalized to body weight, food consumption was equal in all male groups. In the female rats, there was no difference in food consumption between the smoke-exposed and sham rats, or between control and test groups (Table 3). Thus, the addition of groups of ingredients to the cigarettes did not affect the food consumption of the rats.

3.2.5. In-life observations

There was no smoke-related mortality.

Exposure and smoke-related findings were increased irritability, decreased reflexes, Harderian gland secretion, and moist or wet fur. These were comparable to findings seen in earlier cigarette smoke inhalation studies (Haussmann et al., 1998). No additional ingredient-related signs of systemic toxicity were seen in any of the test groups.

3.2.6. Ophthalmological investigations

At the end of the inhalation period, the ophthalmological examination did not reveal any ingredient-related effect or alteration in eyelids with conjunctivae, cornea, iris, lens, vitreous body or retina.

3.2.7. Clinical chemistry

At the end of the inhalation period, a twofold smoke-related increase in enzyme activity was observed for alkaline phosphatase, alanine aminotransferase, and aspartate aminotransferase (mainly females) (Table 4). The increase in activity of liver enzymes is interpreted as a reaction to the high dose of cigarette smoke, and may reflect increased lipid peroxidation in the liver (Watanabe et al., 1995). The addition of ingredients to the cigarettes did not affect the smoke-induced effects.

For the smoke-exposed rats, the γ-glutamyltransferase activity was higher in most of the female rats in the test groups compared to the control group. Statistically significant differences were found for the female rats exposed to smoke from Ingredient Group 2 cigarettes. Hepatic and renal injury induce elevated γ-glutamyltransferase activity; the increase in γ-glutamyltransferase observed here was not correlated by any other change in renal parameters—kidney weight (Table 6) or histological kidney changes—and was too low to indicate hepatic or renal injury (Crespo et al., 1999).

The serum concentrations of total cholesterol and glucose were decreased in all smoke-exposed groups by up to 40% (Table 4). The concentrations of triglycerides were lower in the female smoke-exposed groups. These changes are interpreted as being a consequence of the slight difference in the nutritional status of the smoke-

Table 3
Relative body weight gain reduction (relative to sham) and food consumption following 90-day inhalation

Parameter	Unit	Exposure groups								
		Sham	Control	IG 1 (Test)		IG 2 (Test)		IG 3 (Test)		1R4F (Ref.)
				Low	High	Low	High	Low	High	
Male rats										
Body gain weight reduction	%	0	27.5±1.4	24.4±2.0	25.2±1.9	28.4±2.5	20.2±1.9*	23.2±2.4	24.4±1.9	23.5±1.5
Food consumption										
Absolute	g/(rat×day)	28.5±0.5	24.9±0.3	25.4±0.5	25.6±0.5	25.1±0.3	25.7±0.4	25.7±0.3	25.3±0.4	25.0±0.3
Relative to body weight	g/(100 g rat×day)	8.6±0.4	9.2±0.2	9.3±0.3	9.3±0.2	9.4±0.3	9.0±0.3	9.0±0.3	9.2±0.3	9.1±0.3
Female rats										
Body weight gain reduction	%	0	1.3±1.2	3.7±2.4	4.9±1.3	3.6±1.7	3.8±1.1	3.0±1.9	3.2±1.7	4.8±1.5
Food consumption										
Absolute	g/(rat×day)	21.4±0.3	22.6±0.6	22.2±0.5	22.2±0.5	23.6±0.6	22.7±0.6	23.2±0.6	22.4±0.8	22.9±0.6
elative to body weight	g/(100 g rat×day)	9.8±0.4	10.5±0.2	10.4±0.3	10.6±0.2	11.0±0.3	10.8±0.2	10.8±0.2	10.7±0.2	10.8±0.2

Relative body weight gain reduction is given as mean±standard error, $N=10$–20 for sham, test and 1R4F groups, $N=24$ for control group; food consumption: $N=11$–12. Low and High: level of ingredient group added.

*Statistically significantly different from control.

Table 4
Clinical chemistry parameters following to 90-day inhalation period to fresh air (sham) and MS of the control, test (ingredient groups 1 to 3) and 1R4F cigarettes

Parameter	Unit	Exposure groups								
		Sham	Control	IG 1 (Test)		IG 2 (Test)		IG 3 (Test)		1R4F (Ref.)
				Low	High	Low	High	Low	High	
Male rats										
Alkaline phosphatase	U/l	138.5±6.3	226.7±16.1	201.3±15.2	227.0±15.2	262.3±26.6	201.9±18.2	222.6±15.7	205.0±13.3	247.2±24.2
Aspartate aminotransferase	U/l	51.55±3.26	60.99±2.64	59.25±3.42	58.87±3.60	62.68± 2.91	61.61±2.57	66.84±6.60	59.22±1.70	66.36±7.09
Alanine aminotransferase	U/l	43.72±3.26	59.88±3.06	56.93±4.85	53.16±3.71	58.11±2.97	52.33±2.61	63.43±5.33	58.88±2.90	64.67±6.35
γ-Glutamyltransf.	U/l	0.70±0.07	0.58±0.05	0.53±0.06	0.61±0.12	0.66±0.10	0.58±0.06	0.58±0.06	0.59±0.07	0.69±0.11
Total cholesterol	mmol/l	1.73±0.07	1.25±0.06	1.36±0.09	1.33±0.08	1.49±0.06*	1.40±0.08	1.41±0.06*	1.43±0.04*	1.38±0.11
Triglycerides	mmol/l	0.65±0.10	0.68±0.10	0.59±0.08	0.59±0.05	0.64±0.13	0.80±0.12	0.55±0.06	0.61±0.08	0.81±0.15
Glucose	mmol/l	14.32±0.79	11.66±0.65	12.86±1.90	11.45±0.59	13.01±2.03	13.46±2.44	11.54±0.36	12.46±0.68	11.11±0.56
Urea	mmol/l	6.87±0.30	9.03±0.42	7.53±0.48	8.31±0.62	8.07±0.39	8.16±0.53	7.88±0.26*	7.45±0.44*	7.99±0.38
Female rats										
Alkaline phosphatase	U/l	102.72±6.86	191.70±13.55	201.89±22.46	188.91±17.83	215.78±18.76	229.61±11.55	201.86±22.15	183.88 ±14.95	148.56±7.48
Aspartate aminotransferase	U/l	51.22±3.37	64.94±2.40	65.51±2.69	62.13±3.30	65.84±4.94	73.23±3.27	65.00±4.95	59.00±2.29	44.53±2.65
Alanine aminotransferase	U/l	44.70±2.67	69.16±4.19	67.00±4.64	63.26±5.07	68.21±2.83	79.62±4.69	62.11±3.93	60.23±3.40	42.36±2.65
γ-Glutamyltransf.	U/l	0.78±0.07	0.88±0.06	1.27±0.18	0.95±0.12	1.24±0.07*	1.34±0.10*	1.07±0.11	0.98±0.06	1.05±0.11
Total cholesterol	mmol/l	1.96±0.13	1.29±0.12	1.36±0.07	1.33±0.09	1.26±0.09	1.37±0.08	1.47±0.16	1.31±0.08	1.73±0.11
Triglycerides	mmol/l	0.69±0.09	0.42±0.03	0.44±0.05	0.42±0.07	0.53±0.06	0.42±0.06	0.43±0.05	0.45±0.06	0.83±0.08
Glucose	mmol/l	13.80±0.71	13.09±1.11	12.15±0.83	10.44±0.60	10.29±0.62	11.51±0.44	11.44±0.51	12.70±1.70	12.57±0.35
Urea	mmol/l	7.64±0.46	6.77±0.24	8.20±0.50	7.39±0.39	7.77±0.49*	8.35±0.23*	8.11±0.68	7.52±0.53	7.17±0.31

Cinical chemistry parameters are given as mean±standard error, $N = 8$–10 for sham, test and 1R4F groups, $N = 14$ for control group; Low and High: level of ingredient group added.
*Statistically significantly different from control.

exposed rats and have been observed by others; (Latha et al., 1988; Maida and Howlett, 1990; Gaworski et al., 1997). The cholesterol concentrations were higher in almost all test groups than in the control, resulting in concentrations between that of the control and the sham (statistically significant in three male groups only, Table 4) indicating that the addition of ingredients does not increase the smoke exposure-related effects.

With respect to the serum urea concentration, a general trend in smoke exposure-related increase was seen in all groups of male rats, whereas for the female rats, the effect was variable (increases and decreases). Statistically significant differences were observed between some of the groups exposed to smoke from the test cigarettes and the control cigarette (Table 4). In male and female rats, the effect of the exposure to smoke from test cigarettes caused the urea concentrations to be close to those of the sham-exposed rats and thus did not add to the effects caused by smoke. Almost all effects seen after smoke exposure were transient—exceptions are partial reversal of cholesterol concentration in male rats, lower in the control and Group 2 high level than in sham, and γ-glutamyltransferase activity in female rats, higher in Group 2 high level than in sham, results not shown—and remained within the range of reported physiological values (Lang, 1993). Because the differences between the test and control groups were inconsistently spread over both sexes and over the different exposure groups, they are considered to be incidental.

3.2.8. Hematology

At the end of the inhalation period, the following smoke-exposure related findings were observed (Table 5). There was a statistically significant reduction in the total leukocyte count for all smoke-exposed groups (up to 47%). In the majority of the smoke-exposed groups, the differential counts of segmented neutrophils were higher (up to 175%) and those of the lymphocytes were lower (up to 60%). Exposure to cigarette smoke promotes the release of neutrophils from bone marrow into the peripheral blood (Terashima et al., 1997, 1999) and, in human subjects, is associated with increased neutrophilic chemotactic activity (Anderson et al., 1991). This potentially proinflammatory change had no histological correlate. Detailed investigations of bronchoalveolar lavage fluid (not performed in the present study) have been reported to show inflammatory changes in the lungs (Hunninghake and Crystal, 1983).

Counts of circulating platelets were lower in most of the male rats (up to 16%). This effect has been reported by others and may be associated with enhanced platelet aggregation (Huang et al., 1988).

No statistically significant differences were observed between the test and control groups for any of the hematological parameters at the end of the inhalation period, except for a higher differential lymphocyte count in male rats exposed to smoke from Ingredient Group 1 (low level) cigarettes and for a lower differential neutrophil count in the group of female rats exposed to smoke from Ingredient Group 3 (high level) cigarettes. These differences, however, are considered to be isolated findings without biological significance.

3.2.9. Pathology of non-respiratory-tract organs

At the end of the inhalation period, smoke-exposure related changes were yellow discoloration of the fur, incidental occurrence of decubital ulcers on the paws [related to exposure mode in tubes (Dalbey et al., 1980)], the presence of brown pigmented macrophages in the mediastinal and bronchial lymph nodes, and atrophy of the lymphatic tissue of the thymus. The cortex appeared to display a thickness reduction, which is probably associated with a reduction in the number of cells; however, no morphometric evaluations were performed to confirm this finding. The thymic atrophy was associated with a decreased thymus weight (Table 6) and may be attributed to repeated exposure to aldehydes (Warholm et al., 1984). For the thymus, statistically significant differences in weight were seen for the males only.

Both the absolute and relative weight of the adrenals (Table 3) was higher in the smoke-exposed groups than in the sham. This increase can be attributed both to stress [tube restriction and MS-exposure (Huber et al., 1981)] and to the effects of nicotine (Boelsterli et al., 1984). Other changes in organ weight, for example of the liver (Table 3), can be attributed to body weight differences between sham and smoke-exposed rats.

No significant differences for either absolute or relative weight were seen between the test and control groups, with the exception of the thymus. Male rats in the test groups exhibited a less pronounced decrease in thymus weight than those in the control group. At the end of the post-inhalation period, thymus atrophy was no longer observed in any of the rats (thymus weight and size equal to sham).

3.2.10. Pathology of the respiratory tract

At the end of the inhalation period, the absolute and relative weight of lungs with larynx and trachea (Table 6) for all rats was statistically significantly higher in the smoke-exposed groups than in the sham. This smoke effect has also been reported by others (Gaworski et al., 1997, 1998), and is equal for the control and the test cigarette smoke-exposed groups (male and female).

The histopathological changes in smoke-exposed rats were almost completely limited to the epithelia of the respiratory tract. In the anterior-most nose section (level 1, Table 7), reserve cell (basal cells of the respiratory epithelium) hyperplasia, goblet cell hyperplasia and

Table 5
Hematology parameters following 90-day inhalation period to fresh air (sham) and MS of the control, test (ingredient groups 1 to 3) and 1R4F cigarettes

Parameter	Unit	Exposure groups								
		Sham	Control	IG 1 (Test)		IG 2 (Test)		IG 3 (Test)		1R4F (Ref.)
				Low	High	Low	High	Low	High	
Male rats										
Erythrocyte count	10^{12}/l	8.29± 0.18	8.10±0.10	8.14±0.15	8.11±0.10	8.28±0.10	8.23±0.08	8.29±0.10	8.27±0.12	8.09±0.13
Hematocrit	–	0.446±0.009	0.440±0.005	0.444±0.007	0.454±0.007	0477±0.006	0.448±0.005	0.453±0.005	0.445±0.005	0.445±0.008
Hemoglobin	g/l	162.7±2.5	159.9±1.7	159.8±2.0	163.8±2.1	162.2±1.7	161.6±1.1	164.2±1.8	164.0±1.2	163.6±2.0
Platelet count	10^9/l	889.5±28.7	774.1±24.4	770.5±24.3	750.5±18.6	794.5±30.4	784.0±24.5	762.8±21.0	814.0±16.4	730.2±41.0
Leukocyte count	10^9/l	9.16±0.83	5.17±0.43	6.14±0.29	5.46±0.43	5.69±0.65	6.32±0.55	5.89±0.54	5.48±0.25	5.97±0.35
Differential counts										
Neutrophils	10^9/l	1.26±0.14	1.56±0.20	1.37±0.13	1.63±0.25	1.75±0.52	1.36±0.20	1.44±0.18	1.14±0.12	1.59±0.18
Lymphocytes	10^9/l	7.68±0.74	3.47±0.31	4.66±0.29*	3.70±0.29	3.83±0.49	4.86±0.46	4.43±0.44	4.20±0,23	4.25±0.34
Female rats										
Erythrocyte count	10^{12}/l	7.68±0.10	7.81±0.13	7.79±0.07	7.99±0.09	7.93±0.07	8.05±0.13	8.12±0.16	7.84±0.10	7.67±0.12
Hematocrit	–	0.422±0.005	0.437±0.008	0.434±0.004	0.477±0.006	0.436±0.006	0.455±0.006	0.455±0.008	0.437±0.006	0.424±0.007
Hemoglobin	g/l	154.2±1.9	159.5±2.8	158.2±1.3	161.2±2.0	159.4±1.9	164.0±2.2	164.0±2.8	158.1±2.0	155.8±2.9
Platelet count	10^9/l	886.4±34.4	743.7±30.4	815.3±34.0	774.1±33.3	822.4±41.8	735.7±30.3	801.0±42.8	808.3±22.1	801.4±32.6
Leukocyte count	10^9/l	9.03±0.64	5.85±0.34	4.72±0.52	5.52±0.44	5.66±0.38	4.78±0.45	5.35±0.33	5.74±0.52	6.39±0.59
Differential counts	–									
Neutrophils	10^9/l	0.81±0.08	1.41±0.13	1.25±0.18	1.26±0.20	1.38±0.12	1.42±0.26	1.15±0.16	0.85±0.12*	1.74±0.25
Lymphocytes	10^9/l	8.03±0.60	4.33±0.25	3.37±0.43	4.14±0.35	4.15±0.35	3.25±0.39	4.10±0.38	4.79±0.51	4.51±0.49

Hematology parameters are given as mean±standard error, $N=8$–10 for sham, test and 1R4F groups, $N=14$ for control group; Low and High: level of ingredient group added.
*Statistically significantly different from control.

squamous metaplasia were seen. Reserve cell hyperplasia of the respiratory epithelium was also seen in section level 2, but it was less pronounced. Atrophy and squamous metaplasia of the olfactory epithelium were seen in the three posterior nose sections (levels 2, 3 and 4).

In the larynx (Table 8) of the smoke-exposed rats, hyperplasia of the squamous epithelium was most pronounced at the base of the epiglottis, pronounced at the lower medial region of the vocal cords, and less pronounced at the vocal folds. Squamous metaplasia of the pseudostratified epithelium was most pronounced at the base of the epiglottis, pronounced at the floor of the larynx, and less pronounced in the upper medial region of the vocal cords and at the vocal folds. Hyperplasia and squamous metaplasia of the cuboidal epithelium at the ventral depression was slight. The laryngeal epithelium at the floor of the larynx was generally thicker in the smoke-exposed rats than in the sham (Table 9).

In the trachea, occasional reserve cell hyperplasia and goblet cell hyperplasia of the respiratory epithelium was found in all smoke-exposed rats (results not shown). At the tracheal bifurcation, where a quantitative assessment was possible, the severity of goblet cell hyperplasia of the respiratory epithelium was similar for all smoke-exposed groups (Table 10).

Goblet cell hyperplasia of the respiratory epithelium was also found in the lungs of all smoke-exposed rats (Table 10); it was more pronounced in the female rats

Table 6
Absolute and relative organ weights following 90-day inhalation period to fresh air (sham) and MS of the control, test (ingredient groups 1 to 3), and 1R4F cigarettes

Parameter	Exposure groups								
	Sham	Control	IG 1 (Test)		IG 2 (Test)		IG 3 (Test)		1R4F (Ref.)
			Low	High	Low	High	Low	High	
Male rats									
Absolute weights (g)									
Thymus	0.27±0.03	0.08±0.01	0.12±0.02*	0.12±0.01*	0.10±0.02	0.15±0.02*	0.11±0.01*	0.12±0.01*	0.08±0.01
Liver	13.68±0.57	10.66±0.45	10.88±0.47	10.66±0.43	10.57±0.40	12.02±0.36*	10.80±0.20	11.38±0.63	11.07±0.54
Adrenals	0.024±0.002	0.029±0.002	0.032±0.003	0.032±0.001	0.032±0.002	0.031±0.002	0.031±0.002	0.029±0.002	0.029±0.002
Lungs with trachea and larynx	1.47±0.04	1.38±0.03	1.45±0.04	1.46±0.03	1.44±0.04	1.53±0.04*	1.48±0.04	1.46±0.05	1.45±0.05
Kidneys	1.30±0.04	1.05±0.04	1.07±0.03	1.11±0.03	1.05±0.02	1.13±0.03	1.07±0.04	1.05±0.04	1.09±0.03
Relative weight (1×E-4)									
Thymus	6.91±0.79	2.59±0.22	3.79±0.48*	3.83±0.43*	3.40±0.48	4.65±0.45*	3.64±0.22*	3.95±0.32*	2.62±0.25
Liver	345.1±6.3	369.7±11.4	359.5±7.9	366.7±12.2	371.0±11.6	370.1±14.3	356.3±12.3	378.0±13.2	364.2±13.0
Adrenals	0.60±0.04	1.00±0.06	1.07±0.08	1.11±0.10	1.11±0.08	0.94±0.07	1.00±0.07	0.97±0.07	0.96±0.06
Lungs with trachea and larynx	37.2±0.7	47.9±0.6	48.1±1.1	50.5±2.4	50.9±2.2	47.0±1.3	48.7±1.4	48.1±0.7	48.0±1.7
Kidneys	32.8±0.9	36.3±1.3	35.7±0.73	38.1±2.2	37.1±1.6	34.6±1.3	35.1±1.4	34.9±1.1	36.1±1.0
Female rats									
Absolute weights (g)									
Thymus	0.25±0.02	0.10±0.01	0.08±0.01	0.11±0.01	0.10±0.00	0.10±0.01	0.10±0.01	0.13±0.01 *	0.09±0.01
Liver	9.03±0.51	9.42±0.42	9.41±0.37	9.23±0.41	10.13±0.51	8.93±0.47	9.20±0.41	9.07±0.38	9.84±0.44
Adrenals	0.025±0.002	0.037±0.003	0.040±0.002	0.035±0.002	0.044±0.002	0.040±0.003	0.037±0.002	0.036±0.002	0.038±0.002
Lungs with trachea and larynx	1.11±0.02	1.28±0.02	1.32±0.04	1.32±0.03	1.31±0.03	1.24±0.04	1.29±0.03	1.31±0.02	1.31±0.03
Kidneys	0.79±0.02	0.85±0.02	0.86±0.02	0.83±0.03	0.86±0.02	0.86±0.03	0.84±0.02	0.84±0.04	0.82±0.02
Relative weight (1×E-4)									
Thymus	10.31±0.68	4.56±0.31	3.92±0.32	5.11±0.49	4.52±0.18	4.63±0.27	4.55±0.33	5.84±0.47*	4.08±0.42
Liver	375.5±13.8	406.3±9.5	416.6±12.7	412.3±14.8	446.6±15.6*	399.1±14.0	405.9±11.0	400.6±17.9	437.5±14.8
Adrenals	1.02±0.05	1.58±0.07	1.77±0.09	1.59±0.12	1.92±0.08*	1.76±0.09*	1.59±0.07	1.62±0.10	1.70±0.08
Lungs with trachea and larynx	46.6±0.9	56.4±0.9	58.4±1.0	59.1±0.8	58.1±1.2	57.0±0.5	57.2±1.1	58.4±1.3	58.7±1.8
Kidneys	32.9±0.6	37.1±0.7	38.0±1.1	37.1±1.1	37.8±1.0	38.2±0.7	36.9±0.7	37.4±1.3	36.8±0.8

Organ weights are given as mean±standard error, $N=8$–10 for sham, test and 1R4F groups, $N=14$ for control group; Low and High: level of ingredient group added.

*Statistically significantly different from control.

Table 7
Histopathological findings in the nose of male and female rats following 90-day inhalation period to fresh air (sham) and MS of the control, test (ingredient groups 1 to 3) and 1R4F cigarettes

Parameter		Exposure groups								
		Sham	Control	IG 1 (Test)		IG 2 (Test)		IG 3 (Test)		1R4F (Ref.)
				Low	High	Low	High	Low	High	
Male rats										
Nasal cavity, level 1	Respiratory epithelium									
	Reserve-cell hyperplasia	0.0±0.0	3.9±0.1	3.4±0.3	3.4±0.4	3.7±0.2	3.4±0.3	3.8±0.2	3.9±0.1	3.1±0.4
		0/10	14/14	10/10	10/10	10/10	10/10	10/10	10/10	8/8
	Squamous metaplasia	0.0±0.0	2.7±0.3	2.8±0.4	2.9±0.4	2.3±0.4	2.2±0.4	2.4±0.5	2.1±0.5	2.3±0.6
		0/10	12/14	9/10	9/10	9/10	8/10	8/10	7/10	6/8
	Goblet-cell hyperplasia	0.0±0.0	1.6±0.2	1.3±0.3	1.3±0.3	1.3±0.3	1.5±0.3	1.2±0.3	1.4±0.3	1.9±0.3
		0/10	13/14	8/10	8/10	8/10	9/10	8/10	8/10	8/8
	Loss of goblet-cells	0.0±0.0	0.6±0.2	1.2±0.2	1.3±0.3	1.3±0.3	0.6±0.3	0.4±0.2	0.7±0.2	0.5±0.2
		0/10	7/14	8/10	7/10	8/10	4/10	4/10	6/10	4/8
Nasal cavity, level 2	Respiratory epithelium									
	Reserve-cell hyperplasia	0.0±0.0	0.8±0.2	0.6±0.2	0.7±0.2	0.5±0.2	1.1±0.4	0.6±0.3	0.8±0.3	0.0±0.0
		0/10	8/14	5/10	6/10	5/10	5/10	4/10	5/10	0/8
	Olfactory epithelium									
	Atrophy	0.0±0.0	4.0±0.1	3.1±0.5	3.8±0.3	3.5±0.3	2.2± 0.5*	3.0±0.4	3.5±0.3	4.1±0.1
		0/10	14/14	9/10	10/10	10/10	9/10	10/10	10/10	8/8
	Squamous metaplasia	0.0±0.0	1.0±0.3	0.7±0.3	1.2±0.4	0.8±0.2	1.2±0.4	1.1±0.4	0.5±0.2	1.3±0.3
		0/10	9/14	5/10	7/10	7/10	5/10	5/10	5/10	7/8
Nasal cavity, level 3	Olfactory epithelium									
	Atrophy	0.0±0.0	3.9±0.2	3.2±0.4	3.7±0.3	3.3±0.5	2.3±0.6	2.9±0.5	3.3±0.5	4.1±0.1
		0/10	14/14	9/10	10/10	9/10	7/10	9/10	9/10	8/8
	Squamous metaplasia	0.0±0.0	1.6±0.2	1.4±0.3	1.5±0.2	1.0±0.3	0.8±0.3	1.1±0.2	1.5±0.4	1.9±0.3
		0/10	12/14	8/10	9/10	7/10	5/10	8/10	8/10	7/8
Nasal cavity, level 4	Olfactory epithelium									
	Atrophy	0.0±0.0	1.4±0.2	1.3±0.3	1.7±0.2	1.5±0.3	1.0±0.4	1.5±0.4	1.9±0.3	1.8±0.2
		0/10	12/14	7/10	9/10	8/10	6/10	8/10	9/10	8/8
	Squamous metaplasia	0.0±0.0	1.1±0.3	0.7±0.3	1.0±0.3	1.0±0.3	0.6±0.3	0.8±0.2	1.0±0.3	1.5±0.3
		0/10	8/14	5/10	7/10	6/10	4/10	6/10	6/10	7/8
Female rats										
Nasal cavity, level 1	Respiratory epithelium									
	Reserve-cell hyperplasia	0.0±0.0	3.1±0.2	3.4±0.2	3.8±0.1*	3.5±0.2	3.6±0.2	3.6±0.2	3.2±0.2	3.4±0.2
		0/10	14/14	9/9	10/10	8/8	9/9	10/10	10/10	10/10
	Squamous metaplasia	0.0±0.0	2.1±0.3	2.0±0.2	2.2±0.3	1.9±0.4	1.6±0.3	2.2±0.3	2.8±0.2	1.9±0.3
		0/10	13/14	9/9	9/10	8/8	7/9	9/10	10/10	8/10
	Goblet-cell hyperplasia	0.0±0.0	1.1±0.2	1.3±0.2	1.4±0.2	1.1±0.1	1.3±0.2	1.2±0.2	1.5±0.2	1.2±0.2
		0/10	12/14	8/9	10/10	8/8	8/9	9/10	9/10	9/10
	Loss of goblet-cells	0.0±0.0	1.2±0.2	1.2±0.2	0.9±0.2	0.8±0.2	0.9±0.2	0.8±0.2	0.7±0.2	1.3±0.2

(continued on next page)

Table 7 (*continued*)

Parameter		Exposure groups								
		Sham	Control	IG 1 (Test)		IG 2 (Test)		IG 3 (Test)		1R4F (Ref.)
				Low	High	Low	High	Low	High	
		0/10	13/14	8/9	8/10	6/8	7/9	6/10	6/10	10/10
Nasal cavity, level 2	Respiratory epithelium									
	Reserve-cell hyperplasia	0.1±0.1	1.2±0.2	1.2±0.2	1.4±0.2	1.4±0.3	1.6±0.2	1.3±0.2	1.6±0.2	1.3±0.2
		1/10	11/14	8/9	9/10	7/8	9/9	9/10	10/10	9/10
	Olfactory epithelium									
	Atrophy	0.0±0.0	2.7±0.3	3.4±0.3	3.3±0.4	3.8±0.3*	3.9±0.1*	3.2±0.4	2.8±0.4	3.9±0.1
		0/10	14/14	9/9	10/10	8/8	9/9	10/10	10/10	10/10
	Squamous metaplasia	0.0±0.0	1.1±0.3	1.0±0.2	1.1±0.3	1.5±0.3	1.7±0.2	1.4±0.4	1.1±0.3	1.4±0.3
		0/10	10/14	7/9	7/10	7/8	9/9	7/10	7/10	9/10
Nasal cavity, level 3	Olfactory epithelium									
	Atrophy	0.0±0.0	2.6±0.5	3.8±0.2	3.4±0.4	4.0±0.0*	4.0±0.0*	3.2±0.5	2.9±0.5	4.0±0.0
		0/10	12/14	9/9	9/10	8/8	9/9	8/10	9/10	10/10
	Squamous metaplasia	0.0±0.0	1.2±0.3	2.0±0.2	1.6±0.2	1.6±0.2	2.1±0.1*	1.6±0.3	1.4±0.3	1.9±0.1
		0/10	9/14	9/9	9/10	8/8	9/9	8/10	7/10	10/10
Nasal cavity, level 4	Olfactory epithelium									
	Atrophy	0.0±0.0	1.4±0.3	2.3±0.3	1.6±0.2	2.0±0.3	1.7±0.2	1.5±0.3	1.4±0.3	1.8±0.1
		0/10	9/14	9/9	9/10	8/8	8/9	8/10	7/10	10/10
	Squamous metaplasia	0.0±0.0	1.1±0.3	1.7±0.2	1.3±0.3	1.1±0.4	1.6±0.2	1.5±0.3	1.3±0.3	1.2±0.2
		0/10	8/14	8/9	8/10	5/8	8/9	8/10	7/10	8/10

Histopathological findings are given as mean score±standard error and incidence. Low and High: level of ingredient group added.
statistically significantly different from control.

than in the males (also in 1R4F groups). Pigmented alveolar macrophages were seen in the lungs of all smoke-exposed rats. The changes observed in the respiratory tract organs were analogous to those reported in the literature for subchronic smoke inhalation studies, Coggins et al., 1980, 1989a; Gaworski et al., 1997).

Throughout the respiratory tract, a consistent spectrum of histopathological changes was observed in all smoke-exposed groups. With respect to the severity of these changes, some minor numerical differences were seen. In the nasal epithelia of the test groups, there was a general trend towards a lower severity of histopathological findings compared to the control group, while in the female rats, the trend was in the opposite direction. Moreover, the severity of findings in the 1R4F group (male rats only) was comparable to that of the control and thus slightly higher (approx. 20%) than for the test groups. In the female rats, the severity of histopathological findings in the 1R4F group was higher (up to 30%) than in the control group, but comparable to that of the test groups.

In the female rats exposed to smoke from Ingredient Group 1 high level cigarettes, the reserve cell hyperplasia in the nasal respiratory epithelium at section level 1 was statistically significantly higher than in the control group. This effect is considered to be incidental since it was not seen at level 2 or paralleled by any other more pronounced histopathological effects in this group compared to the control group. Moreover this effect was not seen in male rats.

The overall severity of histopathological findings in the nasal epithelia of the male rats exposed to smoke from Group 2 high level cigarettes was slightly lower compared to the control group. The only statistically significant difference observed was for the atrophy of the olfactory epithelium in the section at level 2. In contrast, in the female rats, the histopathological effects in the nasal epithelia seen for Ingredient Group 2 were generally more pronounced than in the control. Statistically significant differences were observed for atrophy in the nose sections at the levels 2 and 3 (equal for both ingredient levels) and squamous metaplasia in the nose section level 4 (high ingredient level). These statistically significant differences are unlikely to be relevant since the severity of histopatho-

Table 8
Histopathological findings in the larynx of male and female rats following 90-day inhalation period to fresh air (sham) and MS of the control, test (ingredient groups 1 to 3) and 1R4F cigarettes

Parameter		Exposure groups								
		Sham	Control	IG 1 (Test)		IG 2 (Test)		IG 3 (Test)		1R4F (Ref.)
				Low	High	Low	High	Low	High	
Male rats										
Larynx										
Base of epiglottis	Pseudostratified epithelium Squamous metaplasia	0.0±0.0 0/10	3.9±0.4 13/14	4.2±0.3 10/10	4.4±0.3 10/10	4.8±0.2 10/10	4.6±0.3 10/10	4.3±0.3 10/10	4.2±0.3 10/10	4.6±0.3 8/8
	Squamous epithelium Hyperplasia	0.0±0.0 0/10	4.4±0.4 13/14	4.6±0.3 10/10	5.0±0.0 10/10	5.0±0.0 10/10	5.0±0.0 10/10	4.8±0.2 10/10	4.4±0.3 10/10	4.8±0.3 8/8
Arythenoid projections										
Floor of the larynx	Pseudostratified epithelium Squamous metaplasia	0.0±0.0 0/10	3.6±0.4 14/14	3.8±0.3 10/10	3.6±0.4 10/10	4.1±0.4 9/9	4.1±0.4 10/10	4.1±0.2 10/10	3.4±0.5 10/10	3.8±0.4 8/8
Vocal cords, Lower medial	Squamous epithelium Hyperplasia	0.0±0.0 0/10	2.6±0.2 14/14	1.7±0.3 10/10	2.7±0.4 10/10	2.6±0.3 9/9	2.4±0.2 10/10	2.5±0.2 10/10	2.1±0.3 10/10	3.3±0.2 8/8
Vocal cords, Upper medial	Pseudostratified epithelium Squamous metaplasia	0.0±0.0 0/10	1.3±0.3 8/14	0.9±0.2 8/10	1.7±0.4 8/10	1.6±0.5 6/9	1.6±0.4 8/10	1.2±0.5 6/9	1.4±0.4 7/10	2.1±0.5 6/8
Vocal folds	Squamous epithelium Hyperplasia	0.0±0.0 0/10	1.9±0.2 14/14	1.5±0.3 10/10	1.4±0.3 9/10	1.6±0.3 8/10	1.8±0.4 8/10	1.7±0.4 9/10	1.7±0.4 8/10	2.3±0.3 7/7
Female rats										
Larynx										
Base of epiglottis	Pseudostratified epithelium Squamous metaplasia	0.0±0.0 0/10	5.0±0.0 14/14	4.6±0.3 9/9	4.3±0.5 9/10	5.0±0.0 8/8	5.0±0.0 9/9	4.5±0.3 10/10	4.5±0.3 10/10	4.8±0.2 10/10
	Squamous epithelium Hyperplasia	0.0±0.0 0/10	5.0±0.0 14/14	5.0±0.0 9/9	4.3±0.5 9/10	5.0±0.0 8/8	5.0±0.0 9/9	5.0±0.0 10/10	4.8±0.2 10/10	4.8±0.2 10/10
Arythenoid projections										
Floor of the larynx	Pseudostratified epithelium Squamous metaplasia	0.0±0.0 0/10	4.9±0.1 14/14	4.1±0.5 9/9	4.2±0.3 10/10	4.3±0.4 8/8	4.3±0.3 9/9	4.1±0.5 9/10	3.7±0.4* 10/10	3.5±0.6 9/10
Vocal cords, Lower medial	Squamous epithelium Hyperplasia	0.0±0.0 0/10	3.2±0.3 14/14	3.1±0.3 9/9	2.1±0.3 10/10	2.8±0.3 8/8	2.7±0.4 9/9	2.5±0.4 10/10	2.4±0.2 10/10	3.1±0.2 10/10
Vocal cords, Upper medial	Pseudostratified epithelium Squamous metaplasia	0.0±0.0 0/10	2.4±0.4 13/14	2.3±0.5 7/9	1.8±0.3 10/10	2.4±0.5 8/8	2.7±0.6 9/9	3.2±0.5 10/10	2.6±0.6 10/10	1.8±0.5 8/10
Vocal folds	Squamous epithelium Hyperplasia	0.0±0.0 0/10	2.5±0.3 14/14	2.1±0.3 9/9	2.0±0.3 9/10	2.0 ±0.4 7/8	1.6±0.3 7/9	2.2±0.3 9/10	2.2±0.2 10/10	2.4±0.4 10/10

Histopathological findings are given as mean score±standard error and incidence. Low and High: level of ingredient group added. *Statistically significantly different from control.

logical findings at these sites in the control appears to be unusually low.

In the male rats exposed to smoke from Group 3 low level cigarettes, the epithelial thickness at the floor of the larynx was statistically significantly greater than in the control. In the female rats in Group 3 (low and high levels), the epithelial thickness was smaller than in the control groups. This was paralleled by a lower degree of squamous metaplasia of the pseudostratified epithelium at this site.

Table 9
Thickness of the laryngeal epithelium following 90-day inhalation period to fresh air (sham) and MS of the control, test (ingredient group 1 to 3) and 1R4F cigarettes

Parameter	Unit	Exposure groups								
		Sham	Control	IG 1 (Test)		IG 2 (Test)		IG 3 (Test)		1R4F (Ref.)
				Low	High	Low	High	Low	High	
Male rats										
Floor of the larynx	μm	9.8±0.6	16.6±0.9	16.8±1.2	16.8±1.3	19.0±1.5	19.0±1.4	19.9±1.3*	15.9±1.1	19.8±2.4
Female rats										
Floor of the larynx	μm	9.3±0.4	20.5±1.1	19.7±1.5	18.2±1.5	20.2±1.9	20.5±1.6	16.4±0.8*	15.5±1.1*	19.9±2.4

Laryngeal epithelial thickness is given as mean±standard error, $N=8$–10 for sham, test and 1R4F groups, $N=14$ for control group. Low and High: level of ingredient group added.
*Statistically significantly different from control.

Table 10
Histopathological findings in the trachea, bronchi and lungs of male and female rats following to 90-day inhalation period to fresh air (sham) and MS of the control, test (ingredient groups 1 to 3) and 1R4F cigarettes

Parameter		Exposure groups								
		Sham	Control	IG 1 (Test)		IG 2 (Test)		IG 3 (Test)		1R4F (Ref.)
				Low	High	Low	High	Low	High	
Male rats										
Tracheal										
Respiratory	Goblet cell	0.0±0.0	1.1±0.2	0.7±0.3	1.3±0.3	2.0±0.5	1.2±0.3	1.0±0.3	1.1±0.2	1.6±0.3
		0/10	11/14	5/10	9/10	9/10	7/10	6/10	8/10	8/8
Right Lung										
Alveoli	Alveolar	0.0±0.0	0.6±0.2	0.8±0.1	0.5±0.2	0.8±0.1	0.4±0.2	0.7±0.2	1.0±0.3	0.8±0.3
		0/10	7/14	8/10	5/10	8/10	4/10	6/10	7/10	5/8
Left Lung										
Respiratory	Goblet-cell	0.1±0.1	2.4±0.5	2.2±0.6	2.4±0.5	2.7±0.5	4.0±0.3	2.2±0.6	2.8±0.6	2.9±0.5
		1/10	10/14	8/10	8/10	9/10	10/10	8/10	9/10	8/8
Alveoli	Alveolar	0.0±0.0	0.7±0.2	0.8±0.1	0.6±0.2	1.0±0.1	0.4±0.2	0.9±0.2	1.0±0.2	0.9±0.2
		0/10	9/14	8/10	6/10	9/10	4/10	8/10	8/10	6/8
Female rats										
Tracheal										
Respiratory	Goblet cell	0.1±0.1	1.7±0.4	1.4±0.3	1.5±0.4	1.6±0.5	1.6±0.4	1.1±0.3	0.9±0.4	1.8±0.4
		1/10	12/14	8/9	7/10	6/8	9/9	7/10	5/10	9/10
Right lung										
Bronchi										
Respiratory epithelium	Goblet-cell	0.0±0.0	1.9±0.5	1.7±0.7	0.7±0.5	3.5±0.7	1.7±0.5	1.2±0.6	2.8±0.6	2.5±0.7
		0/10	9/14	5/9	3/10	8/8	6/9	5/10	9/10	6/10
Alveoli	Alveolar	0.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0	0.9±0.1	1.0±0.0	1.1±0.1	0.9±0.1
		0/10	14/14	9/9	10/10	8/8	8/9	10/10	10/10	9/10
Left lung										
Respiratory	Goblet-cell	0.5±0.5	4.4±0.2	4.2±0.6	4.0±0.3	4.6±0.3	3.9±0.5	3.9±0.4	3.6±0.6	3.8±0.4
		1/10	14/14	9/9	10/10	8/8	9/9	10/10	10/10	10/10
Alveoli	Alveolar	0.0±0.0	1.0±0.0	1.1±0.1	1.0±0.0	1.0±0.0	1.0±0.0	1.1±0.1	1.1±0.1	1.0±0.0
		0/10	14/14	9/9	10/10	8/8	9/9	10/10	10/10	10/10

Histopathological findings are given as mean score±standard error and incidence. Low and High: level of ingredient group added.
*Statistically significantly different from control.

4. Discussion

This subchronic inhalation study was performed with MS from cigarettes with and without the addition of groups of ingredients in order to determine the potential biological effects resulting from the addition of ingredients. Mainstream smoke from control cigarettes with the same tobacco blend as the test cigarettes was used for comparison; mainstream smoke from the Reference Cigarette 1R4F was used for comparison with our historical data. As the construction of the control cigarette and the 1R4F cigarettes is comparable, comparable biological effects could be expected.

In order to increase the sensitivity of the assays for each biological endpoint and improve the detection of possible ingredient-related changes, the rats received a smoke dose chosen to cause effects in the sensitive (dynamic) range of as many biological endpoints as possible. his choice was based on INBIFO historical data obtained with the Reference Cigarette 1R4F. Indeed, in the TPM concentration range of 100 to 200 μg TPM/l with an inhalation exposure regimen of 6 h/day, for 21 or 90 days, as many as 30 biological endpoints are reproducibly responsive, although not always in a dose-dependent manner. The data presented here also confirm that most of the parameters investigated are responsive to MS and in the dynamic range at the TPM dose applied. In contrast to most of the published inhalation studies where rats were exposed for 1 h/day at very high smoke concentrations of up to 1200 μg TPM/l (Coggins et al., 1980, 1989a; Gaworski et al., 1997; Coggins, 1998), an exposure regimen of 6 h/day at a much lower smoke exposure concentration (150 μg TPM/l) was chosen.

Administration of a similar cumulative TPM dose (TPM concentration×exposure time) spread over a 6-h exposure interval, however, does not produce any additional effects compared to 1 h exposure (R.J. Reynolds Tobacco Company, 1988). At the same time, effects that can be considered as artefacts of the excessively high TPM exposure regimen for a short duration, such as cardiomegaly induced by exposure to high concentrations of CO (Ayres et al., 1989; Gaworski et al., 1997) and massive depression of the respiratory minute volume (up to 60%) (Coggins et al., 1989b), are prevented in the low concentration/long duration exposure regimen. The low concentration/long duration exposure regimen has successfully been used by INBIFO/CRC in the past.

Throughout this inhalation study, the results of the determination of constituents in diluted MS, indicated relatively constant smoke generation and achievement of target smoke concentrations in all smoke-exposed groups.

Furthermore, the biomonitoring parameters showed that there was comparable smoke exposure in the groups exposed to the different test atmospheres. As indicated by the high steady-state carboxyhemoglobin concentrations found in the rats (22–28%), the smoke dose used in the present study far exceeds the dose taken up by a typical human smoker [up to 11% carboxyhemoglobin peak concentration (IARC, 1986)], exaggerating the estimated exposure in humans.

A number of smoke-induced changes were seen in all smoke-exposed rats. Despite some minor differences, similar effects were seen with respect to body weight gain reduction, food consumption, changes in clinical chemical and hematological parameters, organ weight and gross pathology in all rats exposed to smoke from any of the cigarettes (see Results). As expected from previously performed subchronic inhalation studies on cigarette smoke and from published data (Dalbey et al., 1980; Wehner et al., 1981; Coggins et al., 1982), the respiratory tract was clearly identified as the primary site of response to smoke inhalation. The principal histopathological changes were most pronounced in the respiratory and olfactory epithelia in the nasal cavity and in the epithelium of the larynx. The epithelial hyperplasia, atrophy and squamous metaplasia observed are considered to be adaptive in nature and to have resulted from airway irritation (Burger et al., 1989). In the lungs, accumulation of pigmented alveolar macrophages was found, suggesting a clearance process of inhaled smoke particles (Gaworski et al., 1998).

Comparison of endpoints in rats exposed to smoke from the test cigarettes and the control revealed a few minor differences. Of a total of 23 smoke-responsive endpoints in the respiratory tract, only two statistically significant differences were found between the test and control groups for the male rats; and nine statistically significant differences were found for the female rats. These differences do not follow a clear pattern within any group and are spread over different groups and there was no consistency between the sexes. Moreover, only in the female rat control group is the severity of the findings in the respiratory tract inexplicably low. Consequently, these differences are considered to be due to statistical inference.

It is therefore concluded that the addition of the groups of ingredients did not affect the inhalation toxicity of the smoke. The smoke chemistry analysis of the cigarettes containing the different groups of ingredients (Rustemeier et al., 2001) revealed several differences in smoke composition, both towards higher and lower yields; however, these differences in smoke chemistry appeared not to affect the biological endpoints investigated in this inhalation study. In addition, the results of the in vitro toxicity evaluations (Roemer et al., 2001) are consistent with the conclusion that can be drawn from this inhalation study; that is, that the addition of these 333 commonly used ingredients added to cigarettes in three groups did not increase the biological

activity of the mainstream smoke, even at the exaggerated levels used.

Uncited references

NTP, 1992 is not cited in the text.

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