

Snuff-induced Carcinogenesis: Effect of Snuff in Rats Initiated with 4-Nitroquinoline *N*-Oxide¹

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ABSTRACT

A canal in the lower lip to function as a reservoir for snuff was surgically created in 150 male Sprague-Dawley rats. The animals were randomized into five groups of 30 each: Group I received snuff twice a day, 5 days a wk; Group II was painted with propylene glycol (solvent control) on the hard palate 3 times a wk during 4 wk; Group III underwent painting on the hard palate with 4-nitroquinoline *N*-oxide (4-NQO) dissolved in propylene glycol, 3 times a wk for 4 wk; Group IV received 4-NQO as in Group III followed by snuff application as in Group I; and Group V received a cotton pellet dipped in saline twice a day, 5 days a wk. Treatment continued for up to 108 wk. There was no significant difference in mean survival time between the groups. Squamous cell tumors of the lip, oral and nasal cavities, esophagus, and forestomach were seen only in Groups I, III, and IV. Nine tumors of these organs were found in Group I (six carcinomas and three papillomas), nine in Group III (seven carcinomas and two papillomas), and ten in Group IV (eight carcinomas and two papillomas). The difference between each of these groups and the control groups (II and V) with regard to tumor incidence is statistically significant ($P < 0.05$). In Group I, four oral cavity or lip carcinomas were found in 29 rats, a significant difference in relation to control rats ($P < 0.05$). In addition, hyperplastic lesions of the lip, palate, and forestomach were significantly more common in Groups I and IV compared with Groups II, III, and V. The study has shown that snuff and 4-NQO by themselves have the potential to induce malignant tumors. Initiation with 4-NQO followed by snuff did not significantly enhance tumor formation.

INTRODUCTION

The use of moist snuff has been increasing in western Europe and the United States during the last decade. Snuff dipping is a habit which is most common among Caucasian men. It is often falsely portrayed as a less health-threatening substitute for cigarette smoking, although the snuff habit has considerable health consequences. Epidemiological studies have demonstrated that both the general health and oral health are affected in snuff dippers (1-3). The placement of snuff results in characteristic mucosal lesions in both rats and humans. These lesions have been shown to be reversible in rats after discontinuation of snuff exposure (4).

The most serious complication associated with snuff dipping is the markedly increased risk of developing oral cancer, especially after a long-time exposure. Thus, Winn *et al.* (5) demonstrated that snuff exposure lasting for 4 decades or longer was associated with approximately 50 times increased risk of developing squamous carcinoma in the oral cavity. The International Agency on Research on Cancer and the NIH have stated that there is sufficient evidence to regard snuff as an oral carcinogenic agent when used as in North America and western

Europe (6, 7). It has been further stated that, in contrast to the human situation, sufficient evidence to support carcinogenicity of snuff in experimental animals is lacking (6). To some extent this may be related to the lack of a suitable animal model. Such a model, which allows long-time administration of snuff in a surgically created canal in the lower lip of the rat, has been developed (8). In this model oral tumors have been induced by administration of snuff alone (9, 10).

The development of cancer is regarded as a multistep process which can be divided into two major events, initiation and promotion (11). The findings of hyperplastic reversible lesions induced by snuff (4) may lend support to snuff functioning as a tumor promoter in the oral cavity. However, since snuff contains more than 3000 chemical substances, including some 20 tobacco-specific, volatile, and nonvolatile *N*-nitrosamines, some carcinogenic volatile aldehydes, polycyclic aromatic hydrocarbons, and Po-210, it is of considerable interest to evaluate the influence of snuff in both stages of cancer development (12, 13).

Attempts to chemically induce malignant tumors of the oral squamous epithelium in rats were unsuccessful until Fujino *et al.* (14) in 1965 introduced the water-soluble carcinogen 4-NQO.³ The carcinogenic potential of 4-NQO is now well documented, and it has been shown to induce both oral and squamous cell carcinomas as well as spindle cell sarcomas in various rodent species (15-17).

The aim of the present investigation was to evaluate the tumor-promoting effects of snuff in rats initiated with a subcarcinogenic dose of 4-NQO as well as to determine the effects of long-term administration of snuff in male Sprague-Dawley rats.

MATERIALS AND METHODS

Animals

One-hundred fifty, 6-wk-old male Sprague-Dawley rats (Charles River Company, Portage, MI) were used. The rats were kept in quarantine for 2 wk and were randomized into 5 groups of 30 rats. They were kept in plastic cages with hardwood bedding, 5 rats in each cage. They were fed a standard pelleted diet (Prolab 3000; Agway, Inc., St. Mary, OH) and tap water *ad libitum*. Temperature was kept constant between 21 and 23°C, and the relative humidity was 50 ± 20%. They were exposed to 12 h of light and 12 h of darkness. A canal was surgically created in the lower lip according to the method described by Hirsch and Thilander (8). The rats were operated on when they were 8 to 9 wk old and weighed 250 to 300 g. After 3 to 4 wk of healing, the rats were subjected to the experimental regimens.

Snuff

A commercially available United States brand purchased every other month on the open market in Omaha, NE, was used in the study. The snuff was kept at 4°C. The snuff was applied in the test canal, which

Received 12/12/88; revised 3/6/89; accepted 3/8/89.

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¹ This study was supported in part by funds from the Department of Pathology and Microbiology, University of Nebraska Medical Center; by NIH Research Grant CA36727; and by funds from the Smokeless Tobacco Research Council (Grant 0144-1).

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³ The abbreviations used are: 4-NQO, 4-nitroquinoline-*N*-oxide; NNN, *N*'-nitrosanornicotine; NAT, *N*'-nitrosoanatabine; NNK, *N*-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)butanone; NPYR, *N*-nitrosopyrrolidine; TSNA, tobacco-specific *N*-nitrosamines; GLC, gas-liquid chromatography; TEA, thermal energy analyzer.

was completely filled 2 times a day by use of a spatula. At least 100 mg were administered with each application. Immediately before the afternoon applications, the old snuff from the morning was removed. The average exposure time was 8 to 16 h daily (10).

4-NQO

In order to initiate the squamous epithelium of the hard palate, 4-NQO (Sigma Chemical Co., St. Louis, MO) was dissolved in propylene glycol to a concentration of 0.5%. At each application, approximately 0.13 mg of 4-NQO were applied to the palatal mucosa.

Analysis of Tobacco-specific *N*-Nitrosamines

The TSNA, NNN, NAT, and NNK, were gifts from Dr. J. D. Adams, Naylor Dana Institute for Disease Prevention, American Health Foundation, Valhalla, NY. Stock and standard solutions of the *N*-nitrosamines were prepared in chloroform. Dichloromethane (analytical grade) was obtained from Riedel de Haen AG (Seelze-Hannover, Federal Republic of Germany), and chloroform (high-performance liquid chromatography grade) was purchased from Fisons Scientific Apparatus (Loughborough, England). Kieselguhr (Extrelut; E. Merck AG, Darmstadt, Federal Republic of Germany) was dried overnight at 160°C prior to use and stored at the same temperature. The organic solvents were checked to ensure the absence of substances that could interfere with the analysis for *N*-nitrosamines. Three-g samples of snuff were suspended in 20 ml of dichloromethane in a flask. After allowing to stand for 30 min at room temperature, the mixture was placed on an Extrelut column (15 cm long, 2-cm inner diameter), and after 15 min the column was eluted with dichloromethane (4 x 25 ml). The eluate was concentrated to about 1 ml in a water bath at 55°C. The extract was transferred to a vial and diluted to 5.0 ml with chloroform.

Analyses were carried out using isothermal GLC (Model 2700; Varian, Palo Alto, CA), interfaced with a TEA (Model 502; Thermo Electron Corp., Waltham, MA). The furnace was removed from the TEA and connected to the GLC column via a 5.5-cm-long glass tube. For detection and quantification, 5.0-μl portions were analyzed against external standards by injection into a 1.8- x 1.9-mm (inside diameter) glass column containing 10% UCW-982 on Chromosorb W, AW-DMCS, 80/100 mesh. The GLC-TEA conditions were: column temperature, 200°C; injector temperature, 250°C; helium carrier-gas flow, ~28 ml/min (1.8-m column); furnace temperature, 475°C; oxygen flow, ~10 ml/min; and vacuum pressure, ~0.6 mm of Hg. A CRT gas stream filter (Thermo Electron Corp.) was used. The detection limits of the method were 0.01 to 0.02 mg of tobacco-specific *N*-nitrosamines/kg wet weight of snuff.

Analysis of Volatile *N*-Nitrosamines

The 5-ml extract from the analysis of TSNA above was concentrated to about 0.5 ml in a water bath at 70°C. The final volume was measured with a 1000-μl Hamilton syringe.

Analyses were carried out by injection of 5-μl portions into a GLC-TEA equipped with a 1.8-m x 1.9-mm (inner diameter) glass column containing 20% Carbowax 20M and 2% KOH on Chromosorb W, AW-DMCS, 80/100 mesh. The GLC-TEA conditions were: column temperature, 160°C; injector temperature, 200°C; helium carrier-gas flow, ~27 ml/min; furnace temperature, 475°C; oxygen flow, ~10 ml/min; vacuum pressure, ~0.7 mm of Hg. A CRT gas stream filter was used.

The detection limits of the method were 0.5 to 1 μg of volatile nitrosamines/kg wet weight of snuff.

Analysis was performed on every tenth box of snuff, and altogether 28 boxes were analyzed. The levels of tobacco-specific and volatile *N*-nitrosamines are given in Table 1.

Experimental Design

The rats were divided into 5 groups, each containing 30 rats. All rats were operated on as described above, and the experimental treatment began 1 mo after the canal in the lower lips was surgically created and the rats were 3 mo old (summarized in Table 2). This was considered time zero of the experiment. In 3 rats the canals were not suitable for

Table 1 Isolated amounts of TSNA and volatile *N*-nitrosamines in the snuff used for the long-term bioassay

The values are based on wet weight (moisture content about 50%).

TSNA content (mg/kg)		Volatile <i>N</i> -nitrosamine content (μg/kg)	
NNN	NAT	NNK	NPYR
5.14 ± 1.39*	5.09 ± 1.02	0.89 ± 0.12	22 ± 7.0

* Mean ± SD.

Table 2 Experimental design

Group	Treatment		No. of wk
I	Surgery	Snuff	104
II	Surgery	Propylene glycol	4
III	Surgery	4-NQO	4
IV	Surgery	4-NQO + snuff	4 + 104
V	Surgery	Cotton pellet	104

snuff application, and these rats were excluded from the experiment. The following groups were used in the study.

Group I (30 Rats). Snuff was applied in the experimental canal 2 times daily as described above beginning at wk 4 of the experiment. This was repeated 5 days a wk for up to 104 wk.

Group II (29 Rats). Propylene glycol was applied to the palate 3 times weekly with a sable hair brush (No. 2) with a one-stroke painting from the soft palate to papilla incisiva. No anesthesia was used. The treatment was applied for the first 4 wk of the experiment, followed by no further treatment for the remaining 104 wk of the experiment.

Group III (29 Rats). 4-NQO dissolved in propylene glycol was applied as described above for Group II.

Group IV (30 Rats). 4-NQO was applied identically to the rats of Group III for the first 4 wk, and the rats were then treated with snuff identically to the animals of Group I for the following 104 wk.

Group V (29 Rats). They were operated on and received a cotton pellet dipped in physiological saline twice a day, 5 days a wk for the last 104 wk of the experiment.

All remaining rats were terminated at 108 wk after the start of the different treatment regimens.

Morphological Methods

All animals underwent complete autopsy for the recording of tumors and other pathological lesions. Specimens from the lip, test canal, palate, oral and nasal cavities, lungs, heart, liver, esophagus, forestomach, glandular stomach, kidneys, urinary bladder, and other grossly abnormal tissues were taken for light microscopic examination. Tissue specimens were fixed in 4% neutral buffered formalin solution, embedded in paraffin, sectioned, and stained by hematoxylin-eosin. Immunohistochemical staining with antibodies against keratin (MAK-6) and vimentin was performed on selected cases.

Statistical Methods

Statistical significance was calculated by Fisher's exact test (18). A *P* value of < 0.05 was regarded as statistically significant.

RESULTS

Two rats in Group IV and one rat each in Groups I and II died during the experiment and were not suitable for histopathological evaluation due to severe autolysis and/or extensive cannibalism. The mean survival time in the different groups is shown in Table 3. There was no significant difference in survival time between the groups.

The average body weight of the rats at the beginning of the experimental regimens was approximately 375 g in all groups. The body weight curve of the different groups during the experiment is shown in Fig. 1. As shown in this figure, the snuff-treated groups had a slower weight gain than the groups which did not receive snuff. The difference in body weight

Table 3 Mean survival time in the different groups (wk)

Group	(n) ^a	Mean survival	
		Wk	Range
I	29	106	68-120
II	28	102	71-119
III	29	102	76-119
IV	28	97	53-120
V	29	100	56-119

^a Number of rats available for histopathological evaluation.

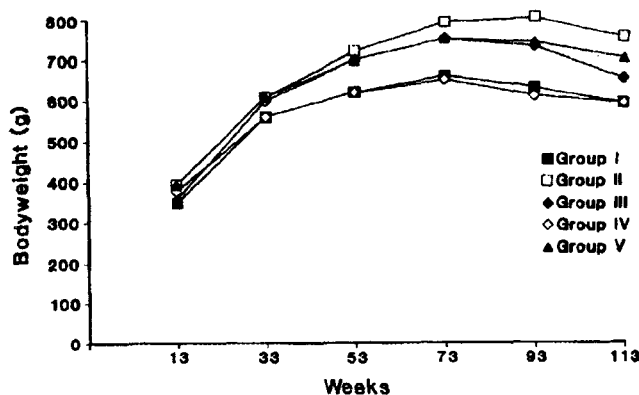


Fig. 1. The mean body weight of the different groups.

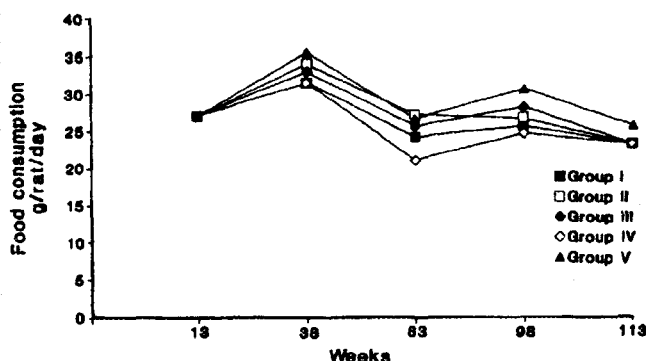


Fig. 2. Daily food consumption per rat in the different groups.

between the snuff-treated groups (I and IV) and the other groups (II, III, and V) was 100 g after 40 wk and remained at this level throughout the experiment (statistically significant at $P < 0.05$).

The food consumption during the experiment is given in Fig. 2. In the beginning of the experimental treatment, the food consumption was the same in all groups, on the average, 27 g per day. The differences in food consumption between the groups during the experiment were not statistically significant, but throughout the experiment, Groups I and IV consumed less food than the other groups. The average daily water consumption did not significantly differ between the groups, but after an initial decrease, it increased in all groups until the end of the experiment (Fig. 3).

The incidence of tumors and hyperplastic (possibly preneoplastic) lesions is given in Table 4. The tumor data are basically expressed per tumor-bearing animal. However, 2 rats in each of Groups I, III, and IV had 2 primary tumors, but only one of them (in Group III) had 2 squamous cell carcinomas (tongue and forestomach). Squamous tumors (papilloma or carcinoma) of the lip, oral cavity (tongue, hard palate), nasal cavity, esophagus, and forestomach were exclusively seen in rats treated with snuff only, 4-NQO only, or 4-NQO followed by snuff. In Group I (snuff only), there were 6 squamous cell carcinomas and 3

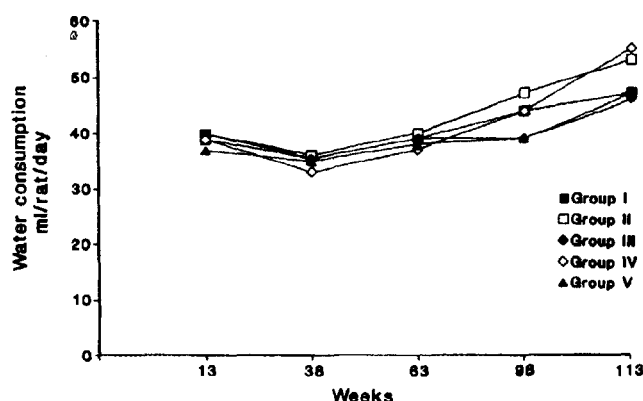


Fig. 3. Daily water consumption in the different groups.

papillomas; in Group III (4-NQO only), there were 7 carcinomas in 6 rats and 2 papillomas; and in Group IV (4-NQO followed by snuff), there were 8 carcinomas and 2 papillomas (Figs. 4 to 6). The differences in incidence of squamous cell tumors between Groups I, III, and IV versus Groups II and V are statistically significant ($P < 0.01$). Even if limited to malignant squamous cell tumors, the difference is statistically significant ($P < 0.05$). In addition, hyperplastic and/or dysplastic lesions of the squamous epithelium in the lip, hard palate, and forestomach were significantly more common in Groups I and IV (Table 4; Fig. 7). Furthermore, there were two spindle cell sarcomas of the lip in Group I and three in Group IV (Figs. 8 to 11). The tumor cells in these rats were negative for keratin and positive for vimentin by immunohistochemical staining. These tumors grew rapidly, and one metastasized to the lungs (Group IV). Two rats in Group I and one in Group IV had moderately well-differentiated hepatocellular carcinomas.

The overall tumor incidence was highest in Groups I and IV, 23 tumors in 21 rats being present in Group I and 22 in 20 rats in Group IV (Table 4). In the group of rats treated with 4-NQO only, 13 tumors in 11 rats were found; in the propylene glycol-treated group, 5 tumors; and in the control group, 3 tumors. The difference in total tumor incidence calculated per tumor-bearing animals between Groups I and IV as compared with groups II and V is statistically significant ($P < 0.01$), as well as the difference in total tumor incidence between Groups I and IV versus Group III ($P < 0.05$).

Marked inflammatory changes with foreign body giant cell reaction were seen in 92% of the rats of Groups I and IV (Fig. 12). In contrast, only 20 to 30% of the rats in Groups II, III, and V had inflammatory changes, and foreign body granulomas were virtually absent. Severe fibrosis of the lip was seen in almost all of the rats of Groups I and IV. Less pronounced fibrosis was seen in the lips of Groups II, III, and V.

DISCUSSION

Chronic snuff exposure, with or without preceding 4-NQO treatment, did not significantly influence the mean survival time of the rats (Table 3). The mean body weight of the rats (Fig. 1) was almost identical in all groups at the beginning of the experiment. However, after 40 wk of snuff administration, the body weight was approximately 100 g lower in the snuff-treated groups (I and IV) as compared to the other groups (Groups II, III, and V). From 40 wk on, this difference remained at the same level until the end of the experiment. The difference in weight between the snuff-exposed rats and controlled rats

SNUFF-INDUCED CARCINOGENESIS

Table 4 Incidence and distribution of tumors and preneoplastic lesions in the different groups of male Sprague-Dawley rats

Type of lesion and location	Group				
	I (29) ^a	II (28)	III (29)	IV (28)	V (29)
Squamous cell carcinoma					
Lip	1 ^b				
Hard palate	2		2 ^c	4 ^d	
Tongue			2 ^e	1 ^f	
Nasal cavity	1			1	
Esophagus			1		
Forestomach	1		2	2	
Squamous cell carcinoma <i>in situ</i>					
Lip				1	
Hard palate	1				
Squamous cell papilloma					
Lip	1				
Hard palate	1				
Tongue			2	1	
Nasal cavity	1				
Hepatoma	2 ^g			1	
Adenocarcinoma					
Breast					1
Kidney					
Renal pelvic tumor	1				
Wilms' tumor					1
Testis					
Leydig cell tumor				1	1
Pituitary gland					
Adenoma	1				
Malignant lymphoma	4		1	1	
Malignant fibrous histiocytoma					
Skin-subcutis	2	1	1		
Sarcoma undifferentiated					
Lip	2			3	
Skin			2	1	
Neurofibrosarcoma					
Skin-subcutis	1	1			
Neurofibroma					
Skin-subcutis		2			
Fibroma					
Skin	1			1	
Squamous cell hyperplasia					
Lip, lip canal	24	6	4	25	10
Hard palate	18	7	7	14	2
Forestomach	18	4	6	18	1
Squamous cell dysplasia					
Lip, lip canal	10		4	12	
Hard palate	5			7	

^a Numbers in parentheses, effective number of rats.

^b This rat had both lip papilloma and neurofibrosarcoma of skin.

^c One of these rats had both carcinoma of hard palate and skin sarcoma.

^d One of these rats had both carcinoma of hard palate and sarcoma of lip.

^e One of these rats had carcinoma of both tongue and forestomach.

^f One of these rats had carcinoma of tongue and malignant lymphoma.

^g This rat had both hepatoma and malignant fibrous histiocytoma of skin.

was similar to what we found in an earlier study (10), which is in accordance with the results by Hecht *et al.* (9) who reported weight differences lasting through wk 84 of their study. After that time, no difference was recorded. They administered snuff only 5 times/wk as compared to 10 times in our study, and another commercial brand of snuff was used.⁴ Ninety-two % of the rats in Groups I and IV had marked inflammatory changes with foreign body giant cell reaction, which may have been associated with soreness and pain in the lip preventing optimal

⁴ D. Hoffmann, personal communication.



Fig. 4. Polypoid invasive moderately differentiated squamous cell carcinoma occurring at the transition between the hard palate and the nasal cavity in a rat exposed to snuff. H & E, $\times 33$.

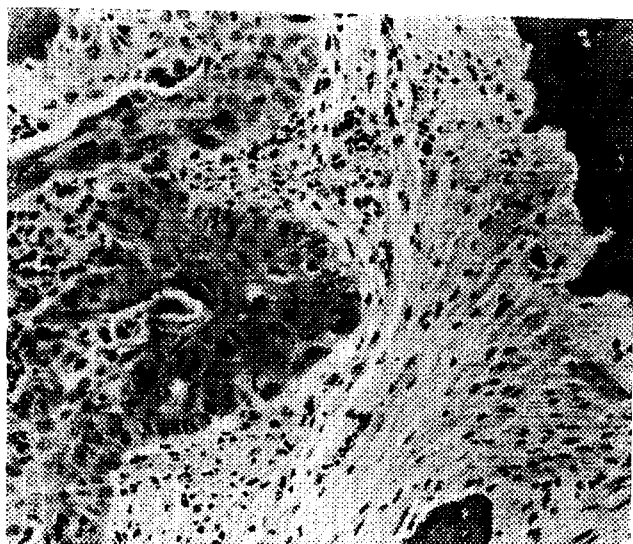


Fig. 5. Poorly differentiated squamous cell carcinoma of the hard palate invading bone in a Group I rat. H & E, $\times 132$.

food intake. This is supported by the lower food consumption in the rats treated with snuff (Fig. 2). It is also known that snuff-treated rats have high blood concentrations of nicotine (8), resulting in an increased level of metabolism which also might have contributed to the lower weight gain of these rats.

The water consumption did not differ significantly among the groups. However, there was an increased water consumption among all rats towards the end of the experiment which may be related to the pronounced rat nephrosis present in the majority of rats at the time of sacrifice (19).

Snuff administration resulted in a high number of tumors overall. There were 23 tumors in Group I and 22 in Group IV, respectively, which is significantly higher than in the control Groups II and V or rats treated with 4-NQO only (Group III) (see Table 3). Squamous cell tumors (carcinoma and papilloma) of the lip, oral cavity, nasal cavity, esophagus, and forestomach were exclusively seen in rats treated with snuff only (Group I, nine tumors), 4-NQO (Group III, nine tumors), and 4-NQO

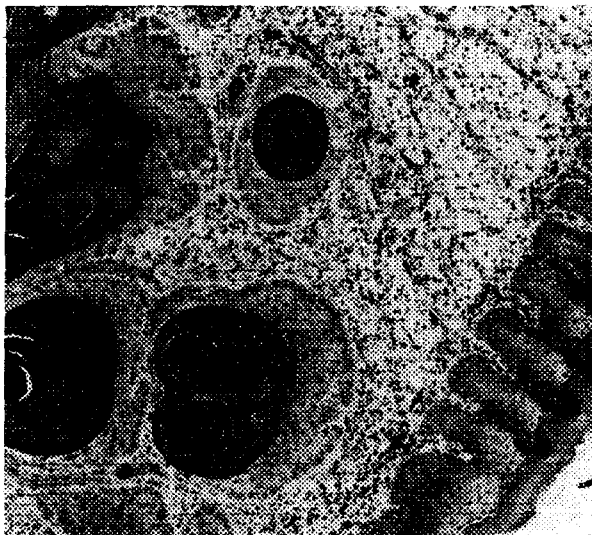


Fig. 6. Well-differentiated invasive squamous cell carcinoma of the tongue in a Group IV rat. H & E, $\times 96$.

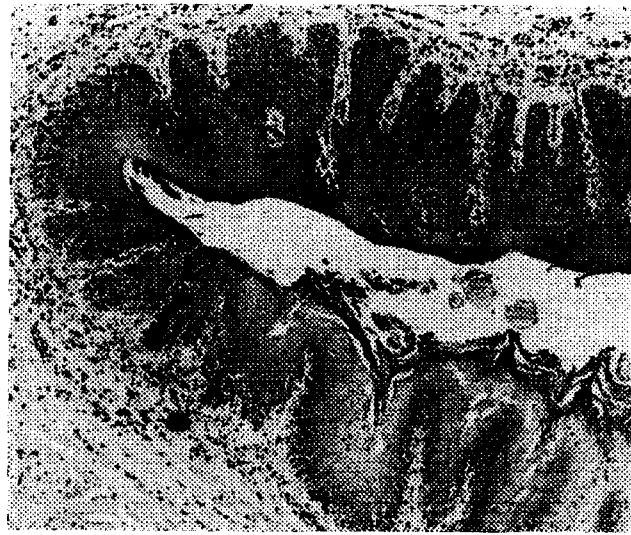


Fig. 8. Section of the lip canal in a rat exposed to snuff only. Note the marked diffuse hyperplasia and focal dysplasia of squamous epithelium. H & E, $\times 66$.



Fig. 7. Squamous cell papilloma of the lip in a snuff-exposed rat. H & E, $\times 33$.

followed by snuff (Group IV, ten tumors). Of these tumors, six in Group I, seven in Group III, and eight in Group IV were carcinomas. In contrast, no such tumors were encountered in the control groups (II and V). The difference in tumor incidence between Groups I and IV and Groups II and III is statistically significant ($P \leq 0.01$ and 0.05). Thus, the present study demonstrates that long-term snuff administration has the potential to induce malignant tumors. Wallenius and Lekholm (16) have shown that 100% of rats treated with 4-NQO for 6 to 8 mo developed oral squamous cell carcinomas. In this study, 14% of the rats exposed to snuff only developed intraoral carcinomas. Thus, snuff has a significantly lower level of carcinogenic activity than does 4-NQO. However, 10 weekly applications of snuff during a 2-yr period result in approximately the same incidence of intraoral cancers as 12 applications of 4-NQO administered over a 4-wk period followed by up to 104 wk of observation.

With regard to oral cancer, our figures are similar to the results obtained by Hecht *et al.* (9), although the number of oral tumors is somewhat higher in our study. This may be related to the fact that snuff was administered 10 times per wk



Fig. 9. Gross appearance of sarcoma of the lower lip in a rat exposed to snuff only.

in our study in comparison to 5 times per wk in their study and that we used another brand snuff.⁴

Also, TSNA were present in relatively high concentrations in our commercial snuff. The mean value of 28 samples of snuff was: NNN, 5.14 mg/kg; NNK, 0.89 mg/kg; and NAT, 5.09 mg/kg. NPYR was the only volatile *N*-nitrosamine present at detectable levels in all snuff samples (Table 1).

Our results show that snuff did not exert any promoting capability in the oral cavity or elsewhere when the rats were initiated with 4-NQO in the hard palate for 4 wk, since the number of tumors in Groups I and IV was virtually identical.

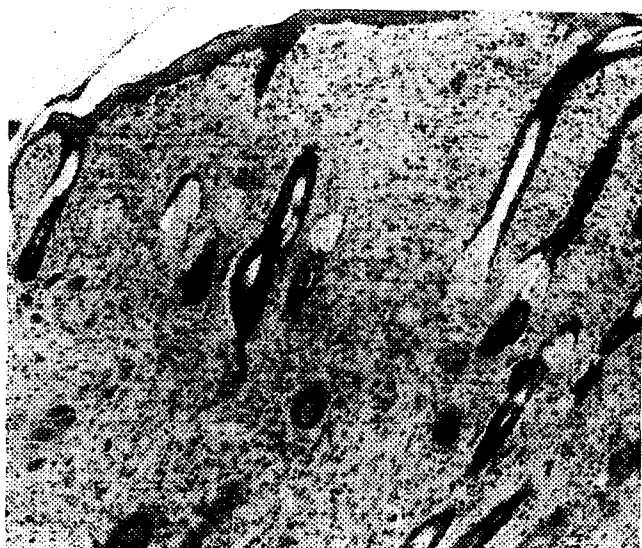


Fig. 10. Micrograph of the tumor in Fig. 9. H & E, $\times 66$.



Fig. 11. Micrograph of the tumor in Fig. 9, showing bone invasion. H & E, $\times 132$.

In a recently finished study, initiation was performed in the lip canal with 4-NQO.⁵ No promoting effects of snuff in the lip or oral cavity were found, similar to the results of the present study.

NNK administration is strongly associated with the development of liver tumors. This compound, which is present in snuff, is likely to be responsible for the development of the three hepatomas in Groups I and IV. Snuff by itself can be carcinogenic for the lip, oral cavity, nasal cavity, and forestomach. This is probably due to its high concentration of TSNA, which have been shown to be organ-specific carcinogens to these organs (13). Other chemicals in snuff may also contribute to its inflammatory and neoplastic effects.

Five lip sarcomas were seen in Groups I and IV (Figs. 10 to 12). One of the sarcomas in Group IV metastasized to the lungs, which has not been reported previously in 4-NQO-induced sarcomas. Since no lip sarcomas were seen in Group III, it seems most likely that these tumors were related to snuff

⁵ P.-A. Larsson *et al.*, unpublished observation.



Fig. 12. Section of the lip canal in a snuff-exposed rat. Note the inflammatory response with extensive foreign body giant cell reaction. H & E, $\times 96$.

exposure rather than 4-NQO. United States snuff used in the present study was quite coarse and irritating and resulted in a significant inflammatory reaction (Fig. 12) which may have contributed to the development of these sarcomas.

Whether the difference in tumor incidences between Groups III and IV reflects a tumor-promoting effect of snuff is difficult to assess, but it seems unlikely since snuff by itself (Group I) induced as many tumors as 4-NQO followed by snuff. The results of this study indicate that snuff may exert tumorigenic effects in different organs of the rat (oral cavity, nasal cavity, esophagus, forestomach, and liver). This is in accordance with the hypothesis proposed by Hecht *et al.* (9). It is supposed that the high content of TSNA in snuff initiates organ-specific tumors, not only local tumors at the site of application. The epidemiological studies performed so far have focused on the relationship between snuff and oral cancer. The present study suggests that future epidemiological studies should evaluate the general tumorigenic effects of the snuff habit.

ACKNOWLEDGMENTS

The authors thank Samuel M. Cohen, M.D., Ph.D., for his advice and comments. The authors also thank Claudia Borgeson, Lori Davis, Patrik L. Johansson, John Parr, Takao Sakata, Margaret St. John, and Scott Tibbels for excellent technical assistance as well as Shirley C. Murray for excellent secretarial help.

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