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Effect of long-term application of snuff on the oral mucosa: an experimental study in the rat

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The long-term effect of snuff exposure was studied in Sprague-Dawley rats using a surgically-created test canal in the lower lip to retain snuff. The rats received standard snuff (n = 42) and highly alkaline snuff (n = 10) for 9–22 months, whereupon they were killed. Untreated rats with identical test canals (n = 15) served as controls. A complete post-mortem examination was performed. One rat exposed to standard snuff for 9 months developed a squamous cell carcinoma of the oral cavity. However, exposure to standard snuff usually resulted in a mild to moderate hyperplasia of the epithelium, hyperorthokeratosis and acanthosis. Rats exposed to snuff for 18–22 months showed vacuolated cells penetrating deeper into the epithelium with hyperplastic and atrophic lesions. In a few rats, severe dysplastic changes developed in the crevicular epithelium. Rats exposed to alkaline snuff differed little from the first group except that there was focally atrophic and ulcerated epithelium and less fibrosis. Pathological findings outside the oral cavity were rare. Squamous cell hyperplasia of the forestomach was found in rats exposed to snuff for 18–22 months, possibly caused by ingested snuff. In conclusion, this study has shown that exposure of rats to snuff for 10–16 hours per day 5 days a week for most of their life-span resulted in lesions mainly restricted to the epithelium and the underlying connective tissue of the surgically created test canal.

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Dysplastic lesions of the oral mucosa associated with snuff exposure have been reported in a few prospective studies from Scandinavia (Roed-Petersen & Pindborg 1973, Hirsch et al. 1982). However, Axéll and co-workers (1978) demonstrated a clear association between snuff exposure and the development of oral cancer in a retrospective study. It has been stated that snuff-associated carcinomas are low-grade lesions with a long induction time (Roed-Petersen & Pindborg 1973). This is supported by the findings of Axéll and co-workers (1978), who found that the incidence of tumours was highest in a group of male snuff-dippers aged 71–80 years. However, Hirsch et al. (1982) found dysplastic lesions among snuff-dippers with a relatively short history of snuff-dipping. It has not been possible to establish whether those lesions were reactive or pre-neoplastic. Thus, inconsistency exists in the available data on the risks of snuff-dipping. An experimental rat model has therefore been developed which allows the study of the effects of snuff on the

Fig. 1. The test canal in the lower lip with one orifice buccally to the incisors and one on the external skin.

oral mucosa (Hirsch & Thilander 1981). This model permits investigation of the long-term effect of snuff and has been used in this investigation to determine the occurrence of histopathological lesions in the oral mucosa and other organs in the rat after exposure to snuff for 9, 12 and 18–22 months.

Material and methods

Three-month-old, male and female Sprague-Dawley rats (n = 78) supplied by Anticimex AB, Stockholm, Sweden, were used. The mean weights of the rats at the beginning of the experiment were 354 g (males) and 237 g (females). The rats were housed in plastic cages (Makrolon cages No. 3, Jacoby, Stockholm, Sweden), 2–4 in each cage. The test and control animals, female and male, were separated. The cages were lined with soft wood bedding (Torrax, Anticimex, Södertälje, Sweden). The animals received standard pelleted food (Astra Ewos, Södertälje, Sweden) and tap-water *ad libitum*. The temperature in the animal house was maintained at 21°–23°C and the relative humidity was kept at approximately 40%. The rats were subjected to at least 8 h light per day. The rats were observed with respect to their general physical condition at least five times every week and were weighed monthly.

Surgical procedure. A test canal was created in the lower lip of each rat. After 10 days of healing, this canal was covered with mucous membrane and had one orifice buccally to the lower incisors and another on the external skin in the most anterior part of the lip (Fig. 1). The surgical procedure has been described in detail by Hirsch & Thilander (1981).

Experimental design. Owing to operative and post-operative complications (mostly suture insufficiency), 11 animals were not included in the study. Thus, a total of 67 rats remained for

the experiment. These animals were divided into three groups – two test groups (26♀, 26♂) and one control group (7♀, 8♂). Approximately 0.2 g of fresh snuff (Röda Lacket, Svenska Tobaks AB, Göteborg, Sweden, pH 8.3) was injected by means of a plastic syringe (Hirsch & Thilander 1981) into the canals of the animals in the first test group (21♀, 21♂). The second test group of rats (5♀, 5♂) were given highly alkaline snuff by the same method (Röda Lacket, Svenska Tobaks AB, Göteborg, Sweden, pH 9.3). The only difference between the two brands of snuff was that in the second brand the pH was raised by adding 50% more $NaCO_3$ (1% of the total weight). The snuff was administered daily at 8 am and 5 pm five days a week. The estimated average time of daily exposure to snuff was 12 h (Hirsch & Thilander 1981). The 15 control animals underwent the same surgical procedure as the test rats, but did not receive snuff.

The rats in the first test group (n = 42), which received standard snuff, were killed after 9 months (6♀, 6♂), 12 months (7♀, 7♂) or when moribund after 18–22 months (8♀, 8♂). The animals in the second test group (5♀, 5♂) were killed when moribund after 18–22 months. The rats of the control group were killed after 9 months (2♀, 2♂), 12 months (3♀, 3♂) or 18 months (2♀, 3♂). All animals were killed by injection of sodium pentobarbitone, 35 mg/kg body weight, followed by intracardiac aspiration of blood until cardiac arrest occurred.

Morphological examination. A complete postmortem examination was performed on all rats, with histological examination of the heart, kidneys, adrenal glands, urinary bladder, spleen, stomach, small intestine, large intestine, liver, thyroid gland and any other grossly abnormal organ or tissue. The test canal in the lip including the crevicular epithelium of the lower incisors was sectioned transversally by multiple step sections.

Histological methods. The test canal in the lip was placed in ice-cold Histocon® (Histolab, Göteborg, Sweden). The specimens were frozen in isopentane chilled to about –140°C with liquid nitrogen (Heyden et al. 1972). The other organs were fixed in 4% buffered formalin and embedded in paraffin. Tissue sections of the lips (8 microns) were cut in a cryostat at –20°C. Five micron sections of the other material were cut and all specimens were stained with hematoxylin-eosin and according to the Weigert-van Gieson method.

The histopathological lesions in the test canal were classified in accordance with the criteria given in "Definitions of leukoplakia and related lesions: an aid to studies on oral precancer" (WHO Collaborating Centre for Oral Precancerous Lesions, 1978).

Results

There was no statistically significant difference in weight gain between the experimental groups and the control rats, although the male rats in the control group exhibited more rapid weight gain between 2 and 12 months. The animals in the test groups remained in good condition until approximately the ninth month, when a general decrease in physical activity was noted. A similar behavioral change occurred among the control rats after approximately 14 months. The condition of the rats was further markedly impaired by 18 to 22 months and they were, therefore, killed. The body weights of the test and control rats are shown in Fig. 2.

Morphological findings in the oral cavity and mucosa. Only one macroscopic tumour was observed. This rat belonged to the first test group and the tumour was detected after 8.5

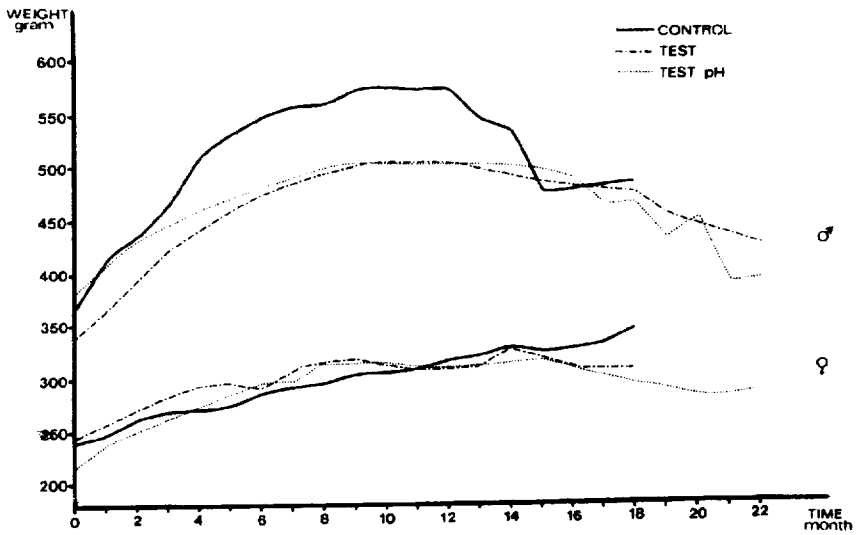

Fig. 2. Body-weight curves for test and control animals during the experiment. 'Test' = rats given standard snuff. 'Test ph' = rats given alkaline snuff.

months. It was ulcerated and situated on the left side of the oral cavity, extending from the incisors and involving both the upper and lower jaws. The tumour was a moderately well-differentiated squamous cell carcinoma invading the bone (Fig. 3).

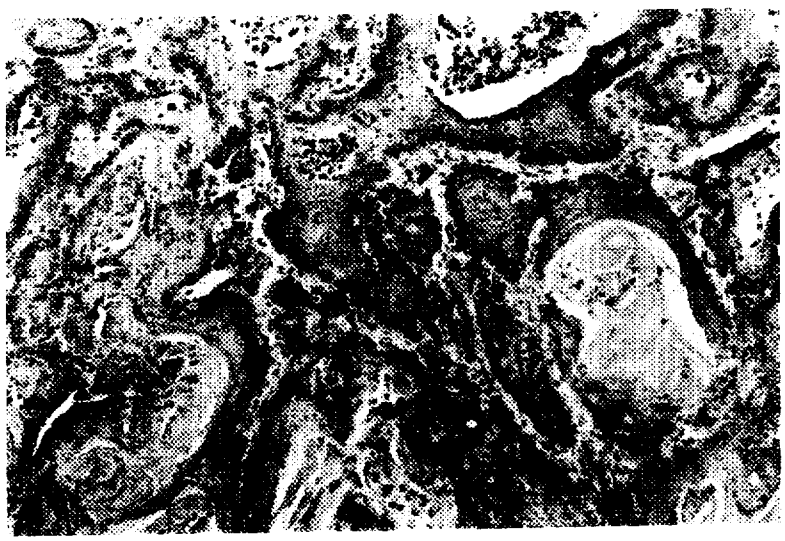

Figure 3. Moderately well-differentiated keratinizing squamous cell carcinoma after 8.5 months of snuff exposure. H&E, ×160.

Fig. 4. Transverse section of the test canal after 12 months of snuff exposure. Moderate epithelial hyperplasia with hyperorthokeratosis and acanthosis is seen. H&E, ×60.

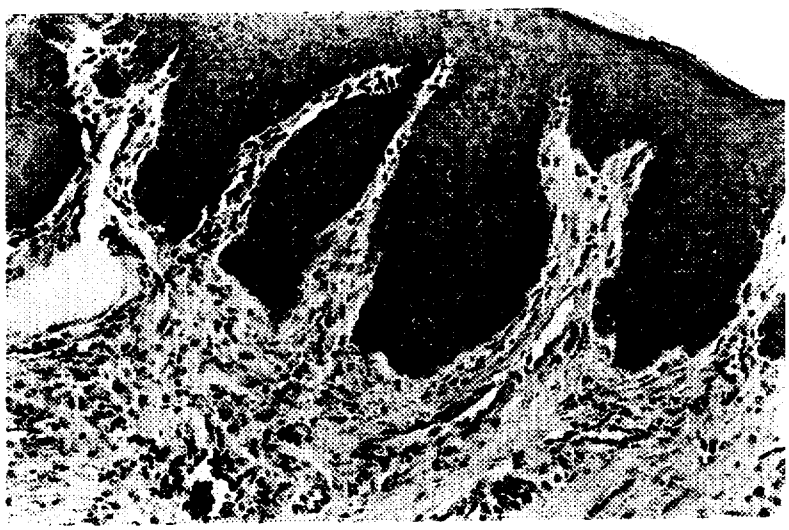

Fig. 5. Epithelial hyperplasia and disturbed polarity and underlying mild inflammatory reaction after 9 months of snuff exposure. H&E, ×160.

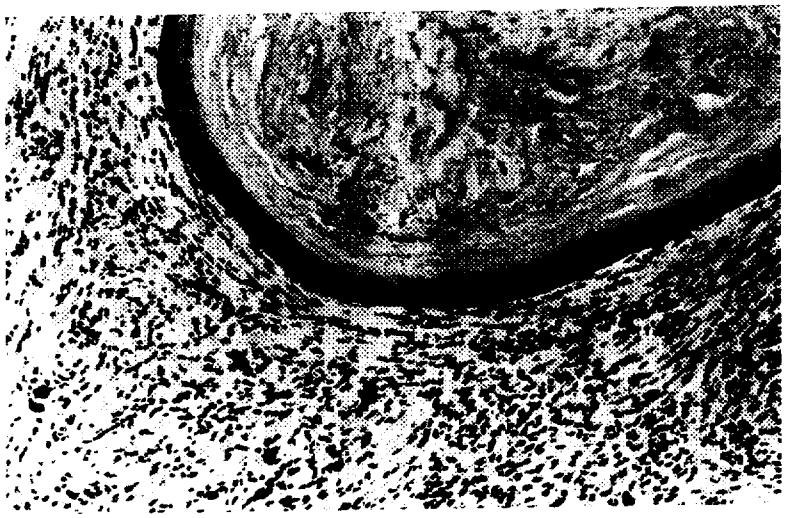

Fig. 6. Section of the test canal after 22 months of snuff exposure showing marked atrophy of the squamous epithelium and hyperorthokeratosis. H&E, ×160.

Fig. 7. Severe dysplasia in the crevicular epithelium associated with an extensive inflammatory reaction in a test rat after 22 months. H&E, ×160.

Fig. 8. Focal ulceration in the test canal after 22 months of exposure to alkaline snuff. H&E, ×160.

The squamous epithelium exhibited a generalized, mild to moderate hyperplasia after 9–12 months of snuff exposure and focally, severe hyperplasia was often seen. Hyperorthokeratosis was observed, with vacuolated cells extending down into the granular layer. Focally, there were acanthotic proliferations of the epithelium with development

Fig. 9. Marked squamous cell hyperplasia of the forestomach after 22 months of snuff exposure. H&E, ×74.

of deep rete pegs (Fig. 4). Increased amounts of keratohylaine granules were present.

The border between the basal cell layer and the *lamina propria* was always sharply delineated (Fig. 4). Areas of focal hyperplasia, disturbed polarity, hyperchromatic nuclei and occasional mitotic figures were seen basally (Fig. 5). The underlying connective tissue exhibited a mild to moderate inflammatory reaction composed of lymphocytes, histiocytes and mast cells.

The oral mucosa of rats exposed to standard snuff for 18 to 22 months differed only slightly from that of rats exposed for 9 to 12 months. Thus, the vacuolated cells extended somewhat deeper into the covering epithelium. There was also a tendency towards widened intercellular spaces with an altered cytoplasmic and nuclear ratio in the *stratum basale*. The inflammatory reaction of the underlying connective tissue varied between mild and severe, but a more prominent fibrosis was noted. The rats exposed for 22 months exhibited hyperplastic lesions together with atrophic lesions (Fig. 6). Severe dysplastic changes were also found in the crevicular epithelium of the lower incisors in two rats in this group (Fig. 7).

The second test group differed very little from the first (18–22 months), but the fibrosis was less prominent and the epithelial lining focally was atrophic and ulcerated (Fig. 8). Vacuolated cells were less frequent in this group. The inflammatory reaction was similar to that of the rats given standard snuff for 18 to 22 months.

The lips of the control animals mostly showed mildly hyperplastic epithelium which was covered with orthokeratin. The rete ridges extending into the connective tissue were slightly increased and the inflammatory reaction was mild or absent. In general, there was a clear difference in appearance of the lesions of the test rats compared to the controls. However, even the test rats had focal areas of normal mucosa. The incidence of the lesions in the various groups of rats is given in Table 1.

Table 1.
The incidence and distribution of histopathological lesions in the lip mucosa of snuff-exposed Sprague-Dawley rats. I = slight, II = moderate, III = severe.

		Snuff exposure, months				Control rats, months		
Type of lesion		9 (n=12)	12 (n=14)	18–22 (n=16)	Alkaline snuff 18–22 (n=10)	9 (n=4)	12 (n=6)	18 (n=5)
Atrophy			3	5	3			
Ulceration			1		3			
Hyperorthokeratosis	I	5	4	5	4	4	2	3
	II	3	1	3	1		2	
	III	4	9	8	5			1
Vacuolation	I	4	7	4	1			
	II						1	
	III	1		2				
Hyperplasia	I		4	4	2	4	4	4
	II	10	7	10	2			
	III	2	3	2	3			
Acanthosis	I	4	9	9	7			
	II	3	3	2	1			
	III	5		1	3			
Dysplasia	I	3	2	4	2			
Inflammation	I	4	10	9	7	1	1	2
	II	8	4	5	1			
	III			2	2			
Fibrosis	I	4	3	3	8	2	2	
	II	5	3		2	1	1	
	III	3	8	13				

Table 2.
The type, incidence and distribution of histopathological lesions outside the oral cavity of snuff-exposed Sprague-Dawley rats.

Type of lesion and location	Snuff exposure, months				Control rats, months		
	9 (n=12)	12 (n=14)	18–22 (n=16)	Alkaline snuff 18–22 (n=10)	9 (n=4)	12 (n=6)	18–22 (n=5)
Follicular tumour (thyroid gland)	8	9	15	8	3	4	4
Fibroadenoma (mammary gland)	2	2	3	2	1	1	2
Fibrous histiocytoma (skin)	1	1					
Squamous cell papilloma (skin)			1				
Neurofibroma (skin)			1				
Papillary squamous epithelial hyperplasia (forestomach)			4	2			
Adenomatous polyp (colon)			1	1			
Rat nephrosis		1	6	3		1	2

Morphological findings outside the oral cavity. Pathological findings outside the oral mucosa were rather rare. The type, incidence and distribution of various lesions are given in Table 2. The most frequent lesion was follicular tumour of the thyroid gland, found in 64–93% of the rats, the incidence being higher with increasing age of the rats. Spontaneous tumours of the mammary gland were also quite common, with an incidence of approximately 20% after 9 months. Histologically, the breast tumours were fibroadenomas. A few skin tumours were seen among the test rats, for example fibrous histiocytoma, squamous cell papilloma and neurofibroma. The rats which had been exposed to snuff for 18–22 months had an increased incidence of marked squamous papillary hyperplasia of the forestomach (Fig. 9) but no overt forestomach tumours were detected. Nephroses, kidney lesions seen mainly in aging male Sprague-Dawley rats, were observed as the rats grew older.

Discussion

In general, the test canal exposed to snuff exhibited a markedly higher frequency of hyperorthokeratinized, atrophic and ulcerated, mildly dysplastic and fibrotic lesions compared to the control rats (Table 1). In human snuff-dippers fewer atrophic and ulcerated lesions were found (Hirsch et al. 1982).

The increased thickness of the squamous epithelium and degree of stromal inflammation found in this study are in accordance with the results of earlier clinical investigations (Pindborg & Renstrup 1963, Roed-Petersen & Pindborg 1973, Axéll et al. 1976, Hirsch et al. 1982).

The occurrence of vacuolated cells was lower than in human studies (Axéll et al. 1976, Hirsch et al. 1982). A particularly low incidence was seen in the second test group, exposed to alkaline snuff (10%). The differences between experimental and clinical results are difficult to explain. They may be

species-related, but may also be due to the rats having had greater snuff-exposure than the humans (Hirsch & Thilander 1981). The results of this investigation do not allow any conclusions concerning the relationship between the time of exposure to snuff and the severity of the lesions. This is in agreement with the clinical data reported by Hirsch et al. (1982). However, it may be noted that there was a rather high incidence of atrophic lesions in the test groups after 18–22 months, as well as the presence of dysplasia in both the test canals and the crevicular epithelium of the lower incisors. If snuff is stored under poor conditions, the pH may rise. The average pH found under poor storage conditions is 9.3 (personal communication, Svenska Tobaks AB, Göteborg, Sweden, 1981), i.e. the same pH as in the second brand of snuff tested in this experiment. It has been suggested (Roed-Petersen & Pindborg 1973, Axéll et al. 1976) that tissue damage such as development of a thick layer of vacuolated cells may be related to the pH of the snuff. Experiments performed by Sunanda et al. (1975) support this assumption. They treated rats kept on a vitamin B-deficient diet with an alkaloid-free U.S. tobacco extract, alone or in combination with a 20% solution of slaked lime, for up to 22 months. They found more marked histological changes in the group painted with both tobacco and lime.

In U.S. tobacco products such as fermented snuff, high levels (29.1 p.p.m.) of the first organic carcinogen isolated in unburned tobacco (N-nitrosonornicotine) were found by Hecht et al. (1975). These findings were confirmed by Hoffmann & Adams (1981a). They analysed snuff obtained from the United States, Germany, Sweden and Denmark in order to determine the levels of tobacco-specific N-nitrosamines (TSNA) in these products. N-Nitrosonornicotine (NNN), a moderately active carcinogen, 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a relatively strong carcinogen, and N-nitrosoanatabine (NAT), so far not bioassayed.

According to Hecht et al. (1975), additional NNN is formed by the reaction of salivary and tobacco nitrite with nornicotine and nicotine. Analyses of the saliva of snuff-dippers revealed that the TSNA are extracted from the snuff during dipping (Hoffmann & Adams 1981a). It is, therefore, considered of importance that the test-tobacco tested contains alkaloids, and that the test canal be moistened with saliva, prerequisites fulfilled in this experiment. The specific brand tested in our investigation was found to contain from 15 to 106 p.p.m. of TSNA; the concentration of NNN ranged from 8.2 to 77 p.p.m., NNK from 1.6 to 3.8 and NAT from 5.0 to 25 p.p.m. in this brand (Hoffmann & Adams 1981b). There is no correlation between pH and NNN levels (Hecht et al. 1975) but a high pH could contribute significantly to more rapid absorption of NNN and nicotine (Hilfrich et al. 1977).

This study has shown that exposure of rats to snuff for 10 to 16 hours a day, 5 days a week, for most of their lifespan results in lesions mainly restricted to the epithelium and connective tissue of the test canal. One rat developed a squamous cell carcinoma of the buccal mucosa. This tumour may be spontaneous or induced. It appears that spontaneous tumours of the oral mucosa are extremely rare, as in 5 studies, comprising approximately 1,900 Sprague-Dawley rats, only 1 spontaneous tumour was reported (Johansson 1981) to occur in the oral cavity (Thompson et al. 1961, Wallenius 1966, Prejean et al. 1973, MacKenzie & Garner 1973). The possibility that the present tumour was induced by snuff therefore cannot be completely ruled out. Axéll et al. (1978) calculated the association between oral cancer and snuff-dipping in Sweden and found it to be 0.5 per 100,000 male snuff-dippers. The tumour incidence in this experiment may accordingly be expected

to be very low in view of the limited number of animals used.

One lesion which may be of interest is papillary hyperplasia of the squamous epithelium of the forestomach (Fig. 9), which was found only in snuff-treated rats and then after long exposure (18–22 months). It is reasonable to assume that this lesion was caused by snuff which had been swallowed by the rats. Whether the lesion is reactive and reversible or pre-neoplastic and has the potential to develop into a true forestomach tumour cannot be determined from this study.

Our rat model has a low incidence of spontaneous tumours. This is, of course, a great advantage when one is studying the effect of weak carcinogens or promoters. Barnes has stated (1975) that we are profoundly ignorant of the consequences of exposure to low concentrations of weak carcinogens, particularly over a long period of time. Dayan declared (1977) that suitable animals models for studying the effects on the oral cavity of weak carcinogens or promoters, are not available. However, we believe our model to be suitable for testing long-term effects on the oral mucous membrane of snuff alone or in combination with various substances.

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