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Snuff-induced lesions of the oral mucosa – an experimental model in the rat

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An experimental model in the white rat has been developed in order to study the influence of snuff on oral mucosa. A test canal in the lower lip, with one orifice buccally to the incisors and one on the lip side, was created by surgical means. The connection between the canal and the oral cavity was made to ensure the presence of saliva in the canal so that physiological conditions resembling those of the oral cavity were obtained. The canal was filled with snuff morning and night 5 days a week. The mean value for the maximal retention time of the snuff was 6 h. The animals tolerated the dose and time of exposure without signs of severe toxic symptoms. Histological examination of the canals after 9 months of exposure to snuff showed a mildly to moderately hyperplastic epithelium with hyperorthokeratosis. Locally deep proliferations of epithelium with acanthotic rete pegs could be seen. In the stratum basale hyperplasia with disturbed polarity and hyperchromatic nuclei and single mitosis were noted.

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The topical effect of unburned tobacco has mainly been studied in the hamster (Peacock et al. 1960, Dunham et al. 1966, Kandarkar & Sirsat 1977). Different mixtures of tobacco have been placed in the cheek pouches of the hamster as single depositions (Peacock et al. 1960) or as repeated long-term applications (Dunham et al. 1966, Kandarkar & Sirsat 1977). The retention has been prolonged by mixing the test substance with an inert material, such as beeswax, before administration (Moore et al. 1953, Dunham & Herrold 1962, Luthra et al. 1970). Epithelial lesions induced by pure tobacco have been detected in only one study (Kandarkar & Sirsat 1977). However, the cheek pouch of the hamster has been considered less suitable for inducing lesions in the mucosa due to the lack of significant amounts of saliva in the pouch (Kolas 1955, Levy 1963).

Few experimental investigations have been published in which the effects of unburned tobacco on the *oral* mucosa have been studied. Homburger (1971) has described an experimental model in the hamster in which the snuff was placed in a stainless steel webbing cartridge attached to the lower incisors. This allowed the test substance to come into contact with the gingivo-lingual mucosa. Alkaloid-free extracts of tobacco have been painted directly on the oral mucosa of mice (Mody & Ranadive 1959). The results of

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these investigations were negative. Yamamura et al. (1975) have described a model for studying the influence on the oral mucosa of chemical substances. The aim was to eliminate the protective effect of the saliva by mechanical means. This was achieved by the surgical creation of a cecal pouch covered by mucous membrane in the lower lip of the rat. The pouch, which had no connection with the oral cavity, opened onto the surface of the skin.

In this study this model has been modified to create an environment similar to the buccal cavity in man in order to study the influence of snuff on the oral mucosa.

Material and methods

Ten 3-month-old Sprague-Dawley rats (five males and five females)[1] were used. The mean weight of the males and females respectively was 390 g and 230 g at the start of the experiment. The rats were kept in Makrolon cages,[2] one or two rats per cage, with test and control animals as well as males and females separated. The bottoms of the cages were covered with soft wood bedding.[3] The animals received a standard pellet diet[4] and water *ad libitum*. The temperature was kept between 21 and 23°C and the relative humidity was kept at approximately 40%. The rats were subjected to at least 8 h of light per day. The general physical condition of the rats was noted at least 5 times a week and the weight of the rats was registered initially and thereafter once a month and when the animals were killed.

1. Anticimex AB, Stockholm, Sweden.
2. No. 3, Jacoby, Stockholm, Sweden.
3. Torrax, Anticimex, Södertälje, Sweden.
4. AB Astra-Ewos, Södertälje, Sweden.

Surgical method

The rats were anaesthesized by intraperitoneal injection of sodium pentobarbital (60 mg/ml).[5] The dose used was approximately 35 mg/kg body weight. 0.5 ml of a local anaesthetic (Xylocaine-Adrenaline® 20 mg/ml + 12.5 µg)[6] was infiltrated in the submucous tissue of the lower lip to minimize preoperative bleeding. The preparation thereafter began with excision of the mucous membrane in an area extending from lateral to the lower incisors to 3 mm dorsal to the midline of the lip on both sides. The width of the excision was about 2 mm (Fig. 1A). The edges of the wound of the oral mucosa were sutured tightly with catgut (3–0). The edges of the skin were sutured with nonresorbable material (3–0). This procedure resulted in a test canal covered with mucous membrane (Fig. 1B). Two teflon plates were applied with stainless steel wire (0.25 mm) pulled through the plate-lipplate (Fig. 1B, 1C) in order to join the surface and stabilize and protect the lip during the healing phase. The splints and sutures were removed after 14 days (Fig. 1D). In order to study whether or not the test canal was completely covered with epithelium, one rat was killed 14 days postoperatively by injecting an overdose of sodium pentobarbital (120 mg/kg body weight). One rat died due to complications of the anaesthesia. Two rats were excluded because of suture insufficiency. Of the 10 rats operated upon, 6 remained for the investigation.

Experimental design

The six animals were divided into two groups – a test (2 ♀, 2♂) and a control (1♀, 1♂) group. Fresh snuff (röda Lacket, pH 8.3)[7] was

5. ACO AB, Solna, Sweden.
6. Astra Läkemedel AB, Södertälje, Sweden.
7. Svenska Tobaks AB, Göteborg, Sweden.

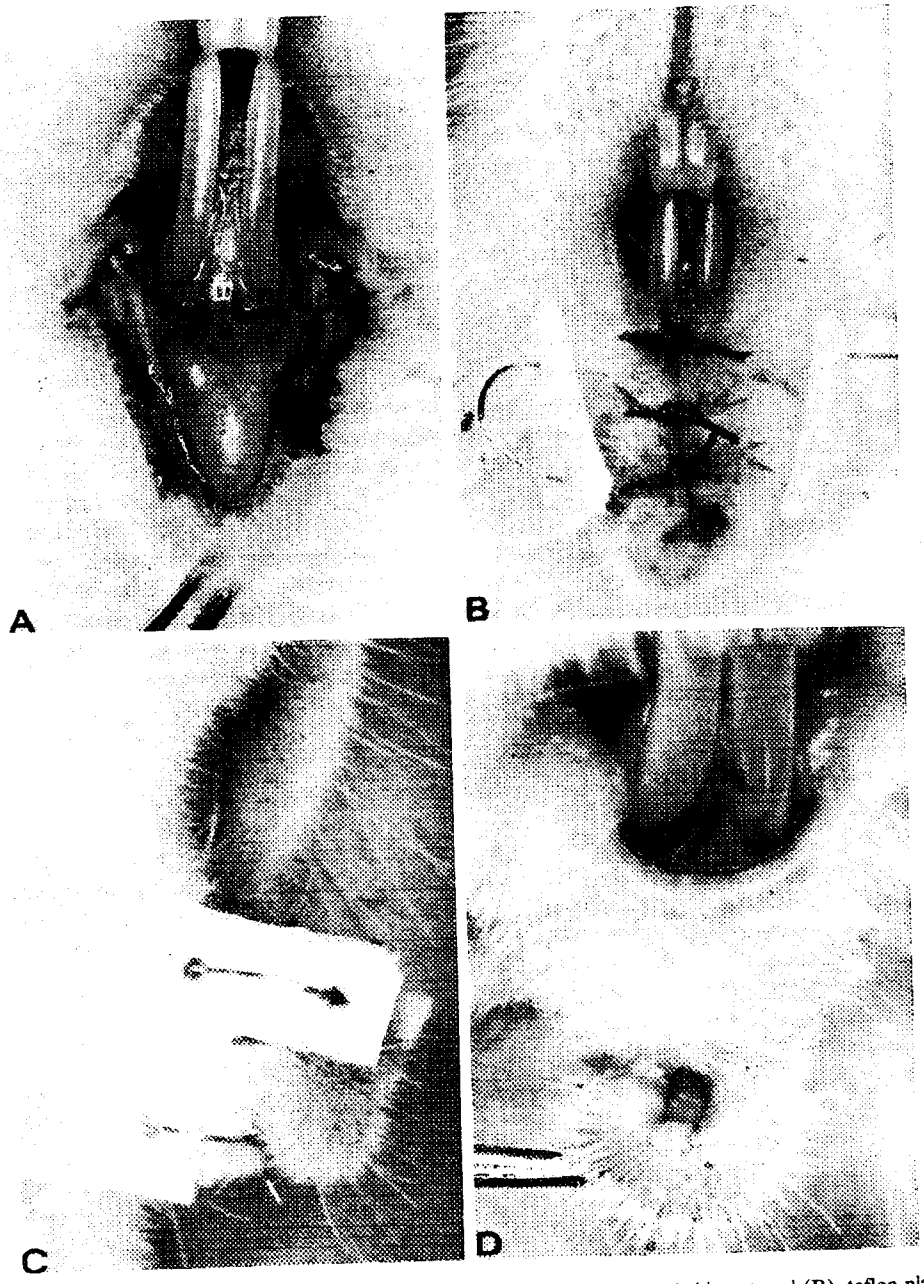
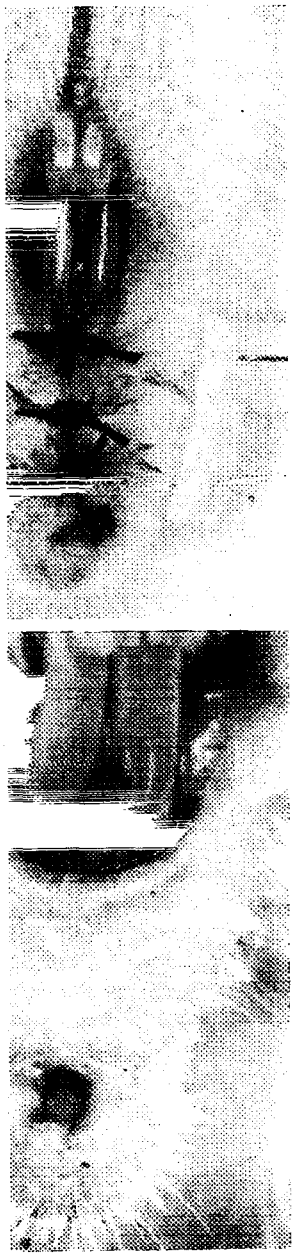


Fig. 1. Surgical method: mucous membrane excised (A), oral mucosa and skin sutured (B), teflon plates applied (B) & (C), sutures and splints removed (D).



and skin sutured (B), teflon plates

injected into the canals of the test animals in the morning (8 am) and at night (5 pm) five days per week for nine months. The snuff was applied by means of a plastic syringe.[8] It was injected from the lip side into the test canal until excess snuff was pressed out through the buccal opening, ensuring complete filling of the canal.

The test animals were killed after the termination of the experiment by injecting sodium pentobarbital 35 mg/kg body weight followed by intracardial aspiration of blood until cardiac arrest.

The control animals, not exposed to snuff, were killed in the same way after 12 months.

Model tests

Unless otherwise stated, all the measurements were performed 4.5 months after the start of the experiments.

Snuff exposure

The filling of the test canal with snuff was studied by mixing snuff and X-ray contrast (Endografin 70%)[9] before application in the canals. The rats were then anaesthesized with sodium pentobarbital (35 mg/kg body weight) and radiographs were taken.

The amount of snuff administered each time was calculated by means of the graduated syringe. The accuracy was 0.1 ml. By weighing corresponding amounts of injected snuff, it was possible to calculate the quantities applied in the test canal. The weight was calculated within an accuracy of 0.1 g. These measurements were performed twice for each of the test animals.

The retention time of the snuff was calculated for each of the rats in the test group by measuring the time the injected snuff remained in the test canal. The control was performed by visual inspection of the orifices

8. ANA-syringes, Scania Dental AB, Sweden.

9. Schering AB, Berlin. West-Germany.

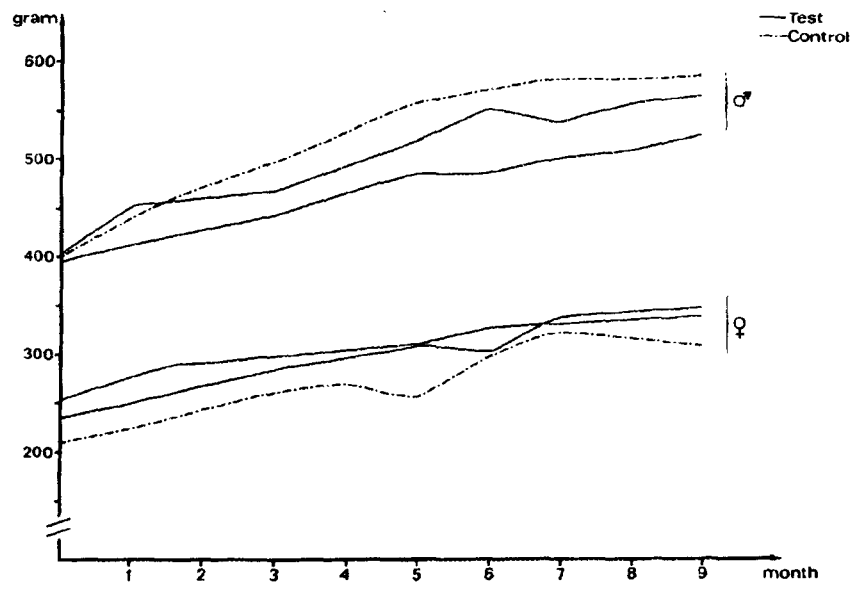


Fig. 2. Body weight curves for test and control animals during the experiment.

of the canal as well as by palpating the test canal every half hour. Two calculations per rat were performed on separate occasions without use of any anaesthetics.

Saliva measurements

Measurement of saliva produced in the canal or entering it from the oral cavity was performed by means of test bodies consisting of cotton-covered sticks. These were applied in the test canals in control and experimental animals for two min after flushing with water and careful drying of the canals. The increased weight of the test bodies was determined within in accuracy of 0.001 g.

The pH of the saliva of the oral cavity and the test canal was determined with litmus paper (pH 1–10)[10] both in test and in control animals. Produced saliva and pH were measured twice in each rat on separate occasions and always in the morning before fresh snuff was injected. No anaesthetic or exogenous stimulation was given.

Nicotine concentration

The nicotine concentration in the blood was determined in two of the test (1♀, 1♂) and one of the control (1 ♂) animals. Fresh snuff was applied in the canals of the test animals. The animals were then anaesthetized after 30 min and 10 ml blood was taken by means of intracardiac puncture. The samples were sent frozen for analysis[11] according to the method described by Falkman et al. (1975).

10. E. Merck AG, Darmstad, Germany.

11. AB Leo, Helsingborg, Sweden.

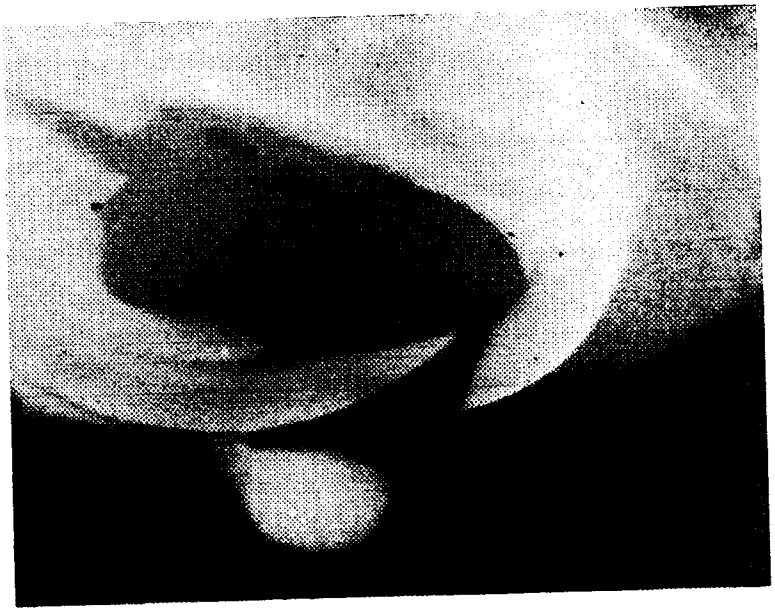


Fig. 3. Radiograph showing filling degree of the test canal with snuff.

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Fig. 4. The canal after 14 days' healing. A thin orthokeratotic layer covering a squamous epithelium with few shallow rete pegs (H & E Orig. mag. × 160).

Histological methods

Immediately after killing the animals, the lower lips were excised and the material put in chilled Histocon® and into cryostat chucks as soon as possible, frozen in isopentane and chilled to –140°C with liquid nitrogen. The sectioning was performed in a cryostat at –20°C. 8 µm sections were cut (Heyden et al. 1972). The test canals were sectioned perpendicularly to the longitudinal axis of the canals at three levels: anterior, central and posterior. The sections were stained with hematoxylin and eosin and according to Weigert-van Gieson. The lesions were classified according to the criteria given in "Definition of leukoplakia and related lesions: an aid to studies on oral precancer" (WHO collaborating Centre for Oral Precancerous Lesions, 1978).

Results

During the test period a hypersalivation was observed in the test group immediately after application of fresh snuff. This effect diminished during the experimental period. An increase in activity was observed among the test animals compared to the control rats after each injection of snuff and throughout the test period. No other differences in behaviour were observed between the two groups, and all experimental and control animals were in good condition at the end of the experiment.

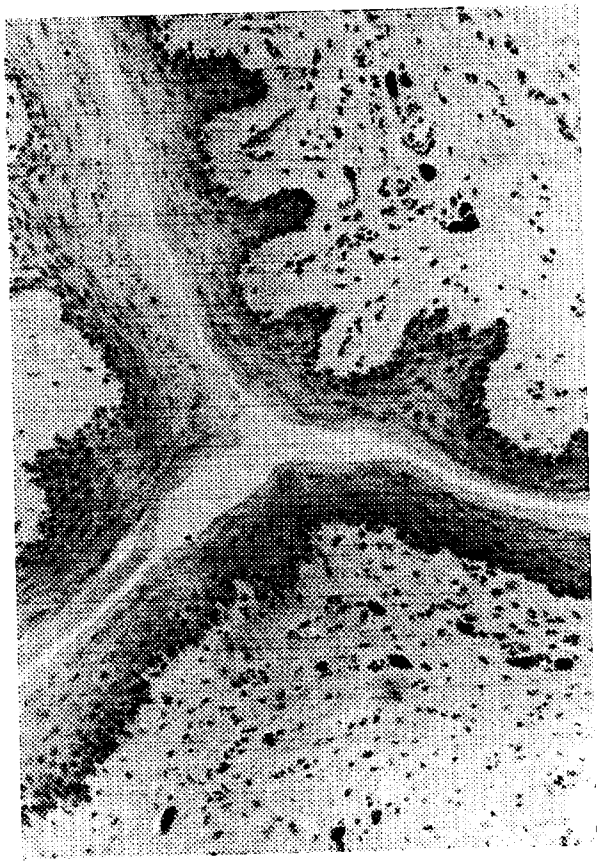

Fig. 5. The control animals after 12 months. Thickened orthokeratotic layer and slightly hyperplastic epithelium with a mild inflammatory reaction in the connective tissue (H & E Orig. mag. × 160).

The change in body weight during the test period is shown in Fig. 2.

The characteristic pattern for the degree of filling of the test canal after injection of snuff is shown in Fig. 3. The radiographs showed filling of the test canal out into contact with the mucous membrane (Fig. 3). No signs of exceptional thinning or thickening in the surrounding mucosa could be seen. Slight stricture was frequently seen in the posterior part of the test canal.

Snuff exposure

The mean volume of snuff administered was 0.2 ml (n = 8, range 0.2–0.3), which corresponds approximately to 0.2 g snuff.

The average volume of snuff injected each day was about 0.4 ml. If calculation of the quantity of snuff is based on the mean weight of the rats after 4.5 months (395 g, Fig. 2), the injected dose for each rat was approximately 1 g/kg/day. The mean value for the maximal retention time of the snuff in the test canal was 6 h (n = 8, range 5–8).

Fig. 5. The control animals after 12 months. Thickened orthokeratotic layer and slightly hyperplastic epithelium with a mild inflammatory reaction in the connective tissue (H & E Orig. mag. × 160).

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Saliva measurements

The average weight gain for the test bodies after 2 min of application in the test canals was 0.04 g (n = 12, range 0.01–0.06). The pH of the saliva for the test canals and oral cavity was within the range of 8–9 on both occasions.

Nicotine concentration

Determination of the concentration of nicotine in the blood showed 13 ng nicotine per ml blood in the control rat. For the test animals the values were 83 and 250 ng nicotine per ml blood.

Histological examination

After the healing phase the canal in the lower lip was covered with thin squamous epithelium, which comprised 5–10 cell layers. The surface was covered with a thin orthokeratotic layer. The border between the epithelium and connective tissue was even. A few shallow pegs of epithelium could be seen. There was no inflammatory reaction in the underlying connective tissue (Fig. 4). The control animals had a slightly hyperplastic epithelium with thickening of both the stratum granulosum and the stratum spinosum after 12 months. The earlier, very thin, orthokeratin layer was now somewhat thickened. In the lumen of the test canal focally desquamated keratin was found. Subepithelially in the connective tissue a mild inflammatory reaction was noted. The rete pegs extending down into the connective tissue were

Fig. 6. The test animals after 9 months. Hyperorthokeratosis with a mildly hyperplastic epithelium with rete pegs and mild inflammatory reaction in the connective tissue (H & E Orig. mag. × 160).

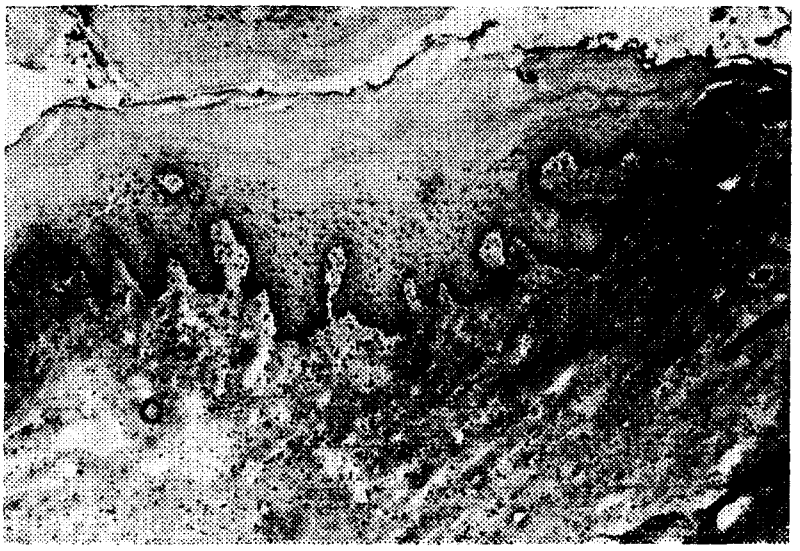

Fig. 7. The test animals after 9 months. Hyperorthokeratosis with vacuolated cells extending into the stratum granulosum. The epithelium moderately hyperplastic with deep rete pegs focally acanthotic. (Weigert-van Gieson Orig. mag. × 63).

slightly increased (Fig. 5). After 9 months of exposure to snuff the epithelium was mildly to moderately hyperplastic (Figs. 6, 7). Thus, in some areas the appearance differed only slightly from the control animals (Fig. 6), while in other areas the difference was marked. In certain parts marked hyperorthokeratosis was seen (Fig. 7), while in other areas a looser type of hyperorthokeratosis with focally vacuolated cells extending down into the stratum granulosum was found (Figs. 6, 7). The total width of the epithelium was often less in areas of compact keratinization than in areas with loose keratin and vacuolar degeneration. Locally the epithelium had proliferated, with the development of deep rete pegs, which were focally acanthotic (Fig. 7). Thickening of the epithelium was caused by the keratinization, but also by the widening of the stratum granulosum and stratum spinosum with the presence of acanthosis (Fig. 7). In the stratum granulosum large amounts of keratohyaline granules were present (Fig. 7). The border between the stratum basale and the connective tissue was always clearcut. Focal hyperplasia, with disturbed polarity and hyperchromatic nuclei as well as single mitoses, was seen in the stratum basale (Fig. 8). The intercellular spaces were widened with changed relation between cytoplasm and nucleus (Fig. 8). The connective tissue exhibited an inflammatory reaction varying in degree from mild to severe.

Discussion

Histological examination of the test canal after 14 days' healing showed that complete epithelialization had occurred in the mucosa of the test canal and on the skin side of the lip. The histological lesions among the test rats at the end of the experiment correlated well with the findings reported from human studies

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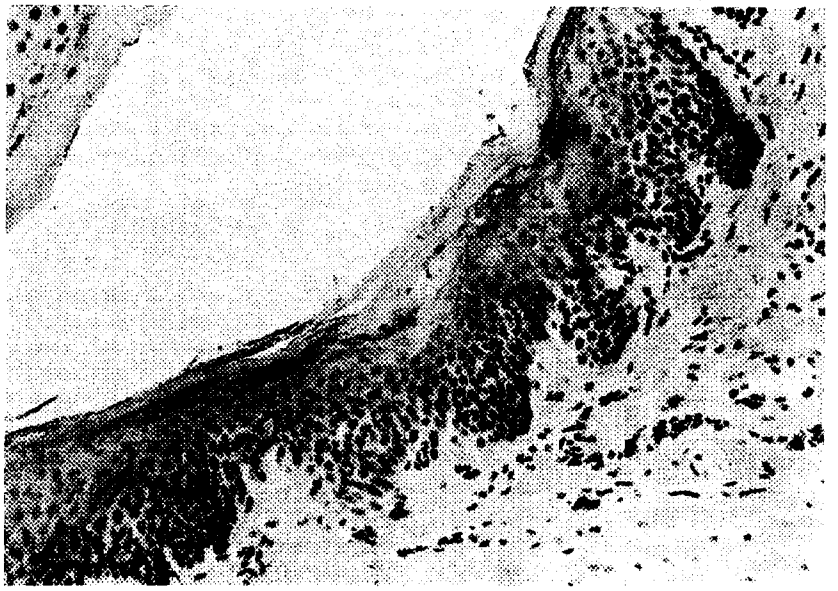

Fig. 8. The test animals after 9 months. Focal hyperplasia, disturbed polarity and hyperchromatic nuclei in the stratum basale (H & E Orig. mag. × 160).

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(Pindborg & Renstrup 1963, Roed-Petersen & Pindborg 1973, Axéll et al. 1976). The incidence of hyperkeratosis, hyperkeratotic lesions and slight dysplastic lesions among the test animals was higher than in human studies. These differences can be explained by the amount of snuff used, the time of retention and possibly by species differences. The histological findings in this study were in accordance with those of Kandarkar & Sirsat (1977). They inserted tobacco in the cheek pouches of hamster exclusively or after painting with vitamin A palmitate. However, it is not clear from their report which animals were merely exposed to tobacco. The slightly hyperplastic epithelium found in the mucosa of the control animals may be due to accumulation of desquamated epithelium, residues of food, hair etc., which may predispose to increased amounts of bacteria.

Measurement of the time of retention of the snuff in the canal was performed when the experiments had been in progress for 4.5 months, at which time the technique of application of snuff was well controlled. The exposure time varied between 10 and 16 h as snuff was administered morning and evening. This may be compared with the times found in clinical studies on human snuff users. These individuals were exposed to snuff for 15.7 ± 4.8 hours (Roed-Petersen & Pindborg 1973) and 6.8 hours (Axéll et al. 1976). The total amount of snuff given to the rats per day was calculated by measuring the amount of snuff used. The dose administered was 1 g/kg body weight. This is approximately five times larger than the corresponding dose in humans. In one study the snuff consumption was reported to be 14 g a day (Axéll et al. 1976), i.e., about 0.2 g/kg body weight. The animals seemed to tolerate the dose and time of exposure well, judging by the absence of severe toxic

symptoms during the experimental period. The analysis demonstrated a high absorption of nicotine, higher than has been found in human smokers (Armitage et al. 1975). Whether the absorption took place in the canal or in the gastrointestinal tract cannot be established from these results. The high pH of the saliva in the rats favours absorption of nicotine through the oral mucosa since the degree of absorption increases with increasing pH (Tibbling 1975). This supports the conclusion that the main part of the nicotine is absorbed in the test canal and not in the gastrointestinal tract.

The surgical design of the test canal aims at obtaining physiological conditions resembling those of the oral cavity. In experimental carcinogenesis in the rat it has been shown that the presence of saliva on the mucous membrane protects the oral mucosa (Wallenius 1966, Wallenius & Lekholm 1973). To ensure the presence of saliva in the canal, there was a connection between the canal and the oral cavity. The use of cotton-covered sticks to study the presence of saliva in the test canal is a crude method, but the weight gain clearly indicated the presence of saliva in the canal.

The surgical procedure was simple and fast and the animals tolerated the procedure well at this age.

Sprague-Dawley rats have previously been shown to be well suited for experiments with induced lesions in the oral mucosa (Wallenius 1966, Wallenius & Lekholm 1973, Lekholm & Wallenius 1976). Our surgical method was initially described in the rat (Yamamura et al. 1975).

The experimental model seems to fulfill the main requirements of an experimental model for studying the effects of snuff on the oral mucosa. Thus, there was good contact between the oral mucosa and the snuff, the retention time of the snuff was long, and the method of administering the snuff was simple. Furthermore, physiological conditions were similar in the test canal and the oral cavity. This experimental model can be used to induce lesions in the oral mucosa similar to those observed in man. The model is well suited for further studies of the factors which influence and modify the pathogenesis of snuff lesions.

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