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The influence of pH and nicotine concentration in oral moist snuff on mucosal changes and salivary pH in Swedish snuff users

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Abstract

The use of Swedish oral moist snuff is a widespread habit in Sweden. In 1999, 25% of the adult male population and 3.1% of the female population were users of snuff. The aim of the present study was to evaluate how variations in pH and nicotine concentrations of snuff affect the oral mucosa, clinically and histologically, salivary pH and daily nicotine intake in 20 habitual users of loose snuff.

The subjects were studied during use of their usual brand, after 12 weeks use of a snuff with lower pH and after another 12 weeks use of a snuff with both lower pH and lower nicotine concentration. Consumption data, oral soft tissue changes, salivary pH and nicotine intake were measured. Further, biopsies were taken from the central part of the clinically observed lesions and histological changes were analysed.

The subjects maintained their snuff consumption during the observation periods. The average salivary pH was higher during snuff use than in the morning. Further, it was higher shortly after the snuff was removed than during snuff use. After having switched to the snuff with both lower pH and lower nicotine concentrations, they showed a significant reduction in daily nicotine intake and developed significantly less pronounced clinical and histological changes. These results indicate that nicotine is one of the substances in snuff that has a biological effect on the oral mucosa. However, there also seems to be a synergistic effect between the pH and nicotine concentration in the snuff.

Key word: nicotine; oral mucosa; pH; saliva; snuff

Svensk sammanfattning

Betydelsen av snusets pH och nikotinkoncentration för förändringar i munslemhinnan samt pH i saliven hos svenska snusare

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I Sverige är bruket av vått snus mycket vanligt. År 1999 snusade 25% av den vuxna manliga befolkningen och 3.1% av kvinnorna var snusare. Syftet med denna studie var att utvärdera hur variationer i snusets pH och nikotinkoncentration påverkar munslemhinnan, kliniskt och histologiskt, samt salivens pH och det dagliga upptaget av nikotin.

Tjugo personer som regelbundet använde löst snus studerades när de använde sitt vanliga snus, efter 12 veckors konsumtion av ett snus med lägre pH samt efter ytterligare 12 veckor då ett snus med både lägre pH och lägre nikotinhalt använts. Konsumtion, graden av klinisk snusläsion, saliv pH och nikotinintag registrerades. Vidare togs biopsier centralt i snusläsionen för att bedöma histologiska förändringar.

Konsumtionen av snus var oförändrad under observationsperioderna. pH i saliv var högre under snusning än på morgonen före snusning. pH var också högre när snuset spottats ut än när det fanns kvar i munnen. När ett snus med både lägre pH och lägre nikotinhalt användes uppvisade snusarna ett signifikant lägre nikotinupptag samt mindre uttalade kliniska och histologiska förändringar.

Resultaten tyder på att nikotin är en av de substanser i snus som har en biologisk effekt på munslemhinnan. Det förefaller dock som om det finns en synergistisk effekt mellan pH och nikotinkoncentrationen.

Introduction

The use of Swedish oral moist snuff is a widespread habit in Sweden. In 1999, 13.6% of the population between 15 and 75 years of age were users of snuff. As mainly men use snuff, 25% of the male population and 3.1% of the female population are snuff users. Two types of snuff, loose and portion-bag packed snuff are produced in Sweden. The average daily consumption is 19 g of loose snuff and 10 g of portion-bag packed snuff (15). The most common way to use snuff in Sweden is to deposit 1-2 g loose snuff or a pouch of portion-bag snuff (1 g) in the vestibular area inside the upper lip. Both types of snuff are produced from ground tobacco and contains ~50% water and 0.8 to 0.9% nicotine. The pH of both loose and portion-bag packed snuff is alkaline (pH 7.9-8.6), but on average 0.5 pH units lower in portion-bag than in loose snuff.

Previous studies have shown that soft tissue changes of the oral mucosa and gingival margin are less pronounced, both clinically and histologically, among users of portion-bag packed snuff than in users of loose snuff (2). It has been assumed that the difference in tissue response between users of portion-bag and loose snuff is partly due to the pH differences of the two products, i.e. the higher the pH of the snuff the more severe are the changes in the mucosa. It has also been shown that nicotine has an impact on oral mucosal reactions, since snuff with lower nicotine content causes less severe changes (4).

Histological changes associated with the use of snuff have been thoroughly reviewed in previous studies (2, 13). In biopsies from this group of 252 snuff users, two major types of patterns were recognized based on changes in the surface layer. Type 1 was characterized by an increased epithelial thickness with vacuolated cells and frequent chevron type changes. Type 2 showed a variably thickened surface layer with evidence of keratinisation. Among this group of users, about 10% of the loose snuff users, showed epithelial changes suggestive of dysplasia as defined by the WHO (12). These tissue changes were reversible after cessation of habit (13).

The aim of the present study is to evaluate the effects of switching to a snuff with reduced pH and reduced nicotine concentration by observing changes in the oral mucosa and in biopsy specimens from snuff induced lesions in Swedish habitual users. Further, to evaluate how the salivary pH is affected by snuff use.

Material and methods

Snuff brands

The three types of snuff used in this study were loose snuff and manufactured in the same way, i.e. using the same heat treatment process. Snuff Brand A with pH 8.6 contained 0.8% nicotine, snuff Brand B with pH 8.0 contained 0.8% nicotine and snuff Brand C with pH 8.0 contained 0.4-0.5% nicotine. The pH of the snuff was calculated throughout the study periods to monitor any changes (see below).

Subjects

Twenty healthy volunteers were randomly selected among a population of 104 habitual users of loose snuff (Brand A) who participated in a previous study (2) and had a clinical thickening of the mucosa, classified as degree 3 or 4 lesions according to *Axéll* (5) (Fig 1A). They were given written information on the purpose of the study before giving a written consent to participate. The study was reviewed and approved by the institutional ethics committee at Lund University.

The mean age of the participants was 34 (s = 9) yrs. They reported a mean consumption of 36 (s = 17) g/day during 16 (s = 4) h/day. The participants had been regular snuff users for 18 (s = 6) yrs and had no other tobacco habit.

Study procedures and clinical examination

Before the start of the study the participants visited a dental clinic where age, medical history and number of years with regular snuff habit and daily consumption (h/day and g/day) were recorded and a clinical examination of the oral mucosa was carried out.

The subjects had used Brand A *ad libitum* for at least 12 weeks. During the following 12 weeks they used a snuff with a lower pH (Brand B) *ad libitum*. At the start of week 13, they switched to a snuff with the same pH as that of Brand B but with a lower nicotine concentration (Brand C) and used this *ad libitum*, for another 12 weeks.

Each participant visited the clinic at the end of weeks 4, 12, 16 and 24. Both the daily consumption during the weeks preceding the visit and total consumption between the visits was recorded. One hour before the visit to the clinic, a fresh pinch of snuff was placed in the mouth. At the visit, a clinical examination was carried out and consumption protocols, collected urine and morning saliva samples were delivered. Saliva samples were gathered both during and shortly after snuff use (see below).

Oral mucosal changes at the site(s) where the snuff quid was regularly placed were recorded and classified according to the degree of clinical severity on a four-point scale (5). This scale is based on clinical criteria including wrinkling, thickening and colour changes of the oral mucosa. The lesions were documented on colour slides and the photographs were coded. These were evaluated after collecting the total material. This evaluation was carried out blindly without information of the status of the subjects.

Histological analysis

Biopsies were taken at three occasions. Prior to the first biopsy, the participants had used Brand A snuff *ad libitum*. The second biopsy was obtained 12 weeks later after consumption of Brand B snuff and the third biopsy after an additional 12-week period with consumption of Brand C snuff. A complete set of three biopsies was obtained from 17 of the subjects. From the remaining three subjects, only biopsies from Brand B and Brand C lesions were obtained.

From the central part of the clinically observed lesion a biopsy was taken with a 6 mm punch using local infiltration anaesthesia well separated from the area of the biopsy. The snuff quid was removed immediately before anaesthesia i.e. approximately 5 min prior to the biopsy excision.

The biopsies were fixed in 10% buffered formalin solution and embedded in paraffin. Three-μm thick sections were cut from the central portion of each biopsy, and stained with haematoxylin and eosin for light microscopic analysis.

Affected mucosa was defined as areas with vacuolated cells and altered stainability (Fig. 2). The thickness of the altered part of the epithelial surface was evaluated with a computerized image analysis program (Image Access). One area at each side of the middle of the biopsy was measured and results are given as the mean value of these two measurements. The size of the areas was governed by the size of the computer screen and comprised approximately 1.5 mm's length of mucosa. The average thickness of the changes was computed by dividing the area of the affected mucosa with its length.

Further, a comparison with biopsies taken when the subjects took part in a previous study (2) using the same snuff (Brand A), was done. In the previous study the subjects did not have snuff in their mouth when they came to the clinic having removed it approximately 1 hour previously.

Sample collection

Urine. On day 6 of weeks 12 and 24, urine samples were collected for 24h. The volume and pH of the urine voids were measured and then 10 ml aliquots were transferred into glass tubes.

Saliva. On day 7 of the same weeks saliva samples were gathered in the following ways: at home in the morning before tooth brushing, eating and the use of snuff a 2 ml sample of resting whole saliva was collected. At the clinic, one hour after the intake of one pinch of snuff, a 2 ml sample of resting whole saliva was collected directly into a glass tube with the snuff still in the mouth. The snuff was removed, the mouth was rinsed with water and another 2 ml sample of resting whole saliva was collected in the same way.

Tobacco. At each visit to the dental clinic one sample of fresh snuff was collected from the snuff that was handed out to the participants for use during the next 4 or 8 study weeks and one sample was collected from the can that was currently used. Further, the pinch of snuff that had been in the mouth during the saliva sampling was collected.

Directly after collection, the urine and saliva samples and samples of snuff were immediately frozen (-18°C) and kept in the freezer until analysed.

Measurements of pH. Salivary pH was measured in all types of samples with a pH meter (EDT Instruments, UK). pHs of the snuff samples were measured as follows: 1 g of snuff was dissolved in 100 ml of water. The slurry was shaken for 10 min and the pH was measured in the continuously stirred solution using a pH meter. The pHs of all solutions were measured at the same occasion.

Analysis of nicotine and metabolites

Urine samples from the snuff users were divided into five portions a) analysed for nicotine and cotinine, b) for glucuronic acid conjugates of nicotine and cotinine, c) for *trans*-3'-hydroxycotinine, d) for glucuronic acid conjugate of *trans*-3'-hydroxycotinine and e) for nicotine-1'-N-oxide and cotinine-1-N-oxide.

Saliva samples were analysed for concentrations of cotinine (7). The concentrations of nicotine and its different metabolites in the urine were determined using gas chromatographic methods described previously (3, 4, 7). From these data the total amount of nicotine equivalents excreted in the urine during 24 h were calculated.

Statistical calculations

Means and population standard deviations were calculated for all parameters. Paired student's t-test was used to evaluate the effects of snuff pH and nicotine content on histological epithelial alteration. The degree of correlation between epithelial change and snuff consumption, between histological changes and nicotine metabolites in urine or saliva and between change in epithelial vacuolisation and change in saliva cotinine titre, was evaluated with Pearson's method (product moment correlation coefficient) and Spearman's non-parametric regression analysis.

Results

Consumption

The subjects had an average daily consumption of 49 (s = 19) g/day when they used Brand A. During the use of Brand B the average daily consumption was 43 (s = 21) g/day and during the use of Brand C it was 47 (s = 20) g/day. The consumption was the same throughout the 12 weeks study periods.

Exposure and uptake data

There was no change in pH of the snuff during consumption. Before use the average pH of snuff was 8.79 (s = 0.16) for Brand A, 8.03 (s = 0.24) for Brand B and 8.06 (s = 0.11) for Brand C. In the pinch of snuff the pH was 8.63 (s = 0.15), 7.86 (s = 0.18) and 7.90 (s = 0.18). The pH in the snuff from the box that was currently used was 8.62 (s = 0.52), 7.87 (s = 0.23) and 7.93 (s = 0.13).

As shown in Table 1 the average salivary pH was significantly higher ($P < 0.001$) during the day when the subjects used snuff than in the morning 6-8 hours after snuff use. Further, it was significantly lower with a pinch of snuff in the mouth than without snuff.

Figure 1A. 50-year old man with a clinically degree 3 lesion following use of Brand A for 32 yrs, 25 g/day during 19 hours.

Figure 1B. The same man showing a clinically degree 1 lesion after use of Brand B for 3 months followed by use of Brand C for another 3 months. The consumption data was the same as previously.

Figure 2. Section of upper lip mucosa from a 30 year-old male who has been using Brand A snuff for 12 weeks. Black line outlines the lower border of the area that has been measured.

Table 1. Average pH (standard deviation) in the saliva collected from 20 subjects at 3 different occasions; in the morning before any food or tobacco intake and in the middle of the day both with and without a pinch of snuff in the mouth.

Study period	pH in saliva		
Brand pH = 8.0	Morning	Afternoon	
	Without snuff	With snuff	Without snuff
Week 4, Brand B	7.03 (0.79)	8.22 (0.38)***[a]	8.49 (0.29)***[b]
Week 12, Brand B	7.04 (0.91)	8.15 (0.30)***	8.37 (0.43)*
Week 16, Brand C	7.05 (0.68)	7.87 (0.69)***	8.28 (0.47)**
Week 24, Brand C	6.96 (0.78)	8.05 (0.40)***	8.48 (0.25)***

a refers to the student's t test comparison between values in the morning without snuff and in the afternoon with snuff

b refers to the student's t test comparison between values in the afternoon, with and without snuff

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

The average salivary cotinine concentration was 368 (s = 174) ng/ml during consumption of Brand A and 439 (s = 176) ng/ml during the use of Brand B. After having switched to Brand C, the average salivary cotinine concentration fell significantly ($P < 0.001$) to 178 (s = 86) ng/ml.

Nicotine intake was estimated as the total amount of nicotine and metabolites excreted during 24 h expressed as nicotine equivalents. The average daily nicotine intake during use of Brand A was 29.9 (s = 9.4) mg and during the use of Brand B 30.7 (s = 14.2) mg. After having switched to Brand C the average daily nicotine decreased significantly ($P < 0.001$) to 14.3 (s = 7.3) mg.

To some extent the degree of clinical oral mucosal changes seemed to covariate with the concentration of salivary cotinine levels ($r = 0.37$; $P < 0.01$) and the nicotine dose ($r = 0.39$; $P < 0.01$)

Clinical observations

The pattern of snuff-induced oral mucosal lesions related to type of snuff used is shown in Table 2. At their first visit 16 subjects showed a degree 3 lesion and 4 (pats no 3,4,12,13) had a degree 4 lesion. After 4 weeks use of Brand B with a pH = 8.0 and a nicotine content of 0.8-0.9%, 6 subjects showed less pronounced clinical changes. After 12 weeks' use of Brand B, 11 subjects showed lesions with a lower clinical degree.

After switching to Brand C with the same pH (8.0) but with a lower nicotine content (0.4-0.5%), less pronounced changes was recorded in 4 subjects after 4 weeks use. At the end of the study after 12 weeks' use of Brand C, less pronounced changes were recorded in 8 subjects. In 2 of these subjects degree 3 lesions were recorded at recruitment and degree 1 lesions at the end of the study. At the end of the study, after having switched to a snuff with reduced pH and nicotine content, all but 3 subjects showed less pronounced changes.

At the second evaluation when intra-oral photographs were used, the same grading of the lesions as at the clinical examination was recorded.

Histological analysis

The mucosal samples displayed structural changes typical of lesions induced by Swedish snuff. The histological appearance of such lesions has been described in a previous publication (2). In most biopsies, the epithelium displayed changes denoted "type I" by *Andersson et al* (2). Briefly, the thickness of the surface layer of the epithelium was increased. The keratinocytes were heavily vacuolated and distended and appeared empty with the exception of variable remnants of visible nuclei. Many of the biopsies showed a so called "chevron pattern" with spikes of non-vacuolated, somewhat eosinophilic, cells above connective tissue papillae. There was no dysplasia according to the WHO criteria (12), although a slightly increased mitotic activity could be observed in some of the biopsies. A variable number of mononuclear cells was found in the adjacent connective tissue.

In a few biopsies "type II" alterations (2) were recorded. In these, the surface layer lacked distended vacuolated cells and was covered by a thin homogenous layer, sometimes resembling a stratum corneum. In those areas where only this homogenous surface zone was found, the thickness value of the surface alteration was set to 0.

The thickness of the surface alterations varied between 11 and 531 mm (Fig 3). There was no significant difference between the values for Brand A lesions (mean = 165 mm; s = 141) and Brand B (mean = 154 mm; s = 75), i.e. a decrease in pH did not affect the degree of surface alteration. However, when the nicotine intake or exposure was decreased, i.e. when the users changed from Brand B to Brand C the thickness of the surface alterations was significantly reduced (mean = 105 mm; s = 61, $P < 0.05$).

The biopsies from Brand A were also compared with biopsies from Brand A lesions in a previous study, where the time span between removal of the

Table 2. Frequencies of clinical grading versus type of snuff

Study periodand brand	Degree 1[a]	Degree 2	Degree 3	Degree 4
Recruitment, Brand A			16	4
Week 4, Brand B		3	16	1
Week 12, Brand B		7	13	
Week 16, Brand C	1	9	10	
Week 24, Brand C	2	11	7	

a Clinical grading

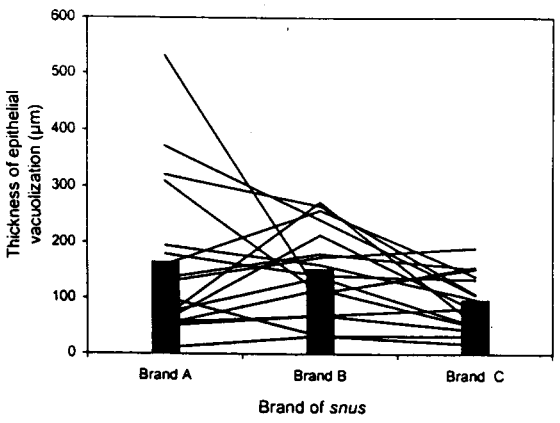

Figure 3. Graph shows the thickness of epithelial alteration (see Fig 2) in 17 snuff users who switched from Brand A to Brand B to Brand C snuff. Lines indicate individual values (mm) and bars denote the mean values.

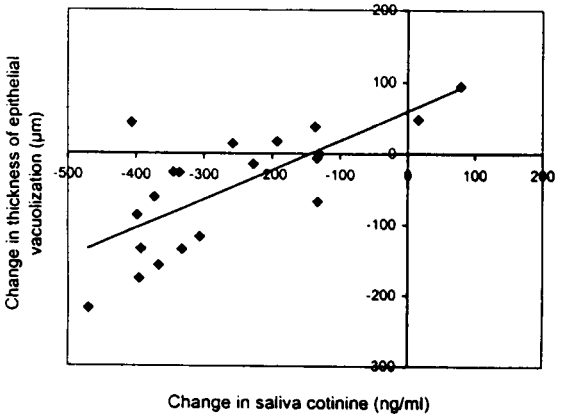

Figure 4. Graph shows the correlation ($r = 0.72$; $P < 0.001$) between change in saliva cotinine titre and change in epithelial alteration (see Fig 2) in 20 snuff users who had switched from Brand B to Brand C snuff.

snuff quid and the biopsy excision was about 1 hour. The lesions from the previous study had a smaller mean thickness of the epithelial vacuolisation zone (mean = 93 mm; s = 59) than the Brand A lesions of the present study and also smaller than what was found in the Brand B and C lesions. The values varied from 0 to 222 mm.

The concentration of saliva cotinine was almost unaffected when users switched from Brand A to Brand B but decreased ($P < 0.001$) when users changed from Brand B to Brand C. The decrease in the thickness of surface alteration was also greatest in those users who displayed the largest decrease in cotinine value (Fig 4; $r = 0.72$; $P < 0.001$).

Discussion

The subjects in this study consumed more than twice as much snuff daily (43-49 g *vs* 19 g) as the calculated average amount consumed by Swedish snuff users (15). These consumption data show that the subjects in this study were heavy snuff users. There was almost no change in consumption when switching from Brand A to Brand B and C. A previous study performed on users of portion-bag snuff (4) has shown that snuff users do not or do only to a small extent compensate for lower nicotine, on switching to a product with half the amount of nicotine delivery, by increasing their consumption or changing their behaviour. These results were confirmed in this study. Further, the saliva cotinine concentrations and the nicotine intake decreased significantly when a snuff with lower pH and lower nicotine content was used.

Clinically less pronounced lesions were recorded in the oral mucosa of 11 subjects after a pH reduction of the snuff. These results are in accordance with a previous study (2) in which users of portion-bags showed less pronounced lesions than users of loose snuff, which have a pH value about 0.5 units higher than that in the portion-bags. After switching to the snuff with lower nicotine content as well, another 6 subjects showed milder lesions. This is also in accordance with a previous study showing less pronounced lesions in users of portion-bags after switching to a snuff with half the amount of nicotine (4). The reduction was evident after 4 weeks but the lesions were further reduced after an additional 8 weeks indicating that three months may be an appropriate study period for snuff-induced lesions.

The clinical oral mucosal changes seemed to covariate with the concentrations of salivary cotinine levels and the nicotine dose to some extent, which is in accordance with a previous study (4). Further, in this study we found a significant correlation between the change in epithelial vacuolisation and saliva cotinine values whereas no correlation was observed between epithelial alteration and nicotine dose. However, cotinine is a rather poor indicator of nicotine uptake compared to nicotine titres in urine since cotinine is related to the metabolic activity of the individual, so the relevance of this correlation may be questioned.

We have previously reported that the four-point clinical scale sub-grouping was related to the histological appearance in tissue biopsies (2). In the

present study, the clinical and histological changes were reduced in parallel but were not actually correlated. Thus, there was no significant histological effect on the epithelium when only pH was reduced. This implies that the clinically observed lesions comprise more than just vacuolisation of the epithelial surface layer, e.g. oedema of the lamina propria. The histological lesion also seems to be a rather acute effect as the thickness of the vacuolated zone was smaller when the time span between snuff removal and biopsy increased from five minutes to one hour. Thus, standardized biopsy procedures are imperative in the study of snuff-induced lesions.

The snuff users in this study had an elevated salivary pH (7.9-8.5) both during and directly after snuff consumption compared to the average pH (6.5-6.9) in resting saliva in a general population (8). However they returned to almost normal values (~ 7) after one night of abstinence. Directly after the snuff had been removed, the pH of the saliva was somewhat higher than during consumption, which may be due to the fact that the ionic strength of the saliva is lower after than during snuff use. Despite the fact that the snuff users had an alkaline salivary pH both during and shortly after snuff use, mucosal changes were recorded only at the sites where the pinch of snuff was placed.

The amount of epithelial vacuolisation was unchanged when only pH was lowered but decreased significantly when also nicotine content was lowered. *Hoffman et al* (11) have shown that the pH of a snuff brand determines the fraction of unprotonated nicotine, which is most readily absorbed through the oral mucosa. *Brunnemann et al* (6) showed that 59% of nicotine is present in unprotonated form at pH 7.99 while only 1% is present at pH 5.84. It is tempting to draw the conclusion that the histological effect is primarily nicotine dependent. A possible mechanism would be a receptor mediated depolarisation of the keratinocytic cell membrane leading to an influx of cat ions and chloride and subsequently water. In vitro, it has been shown that cultured keratinocytes (16) and fibroblasts (1) become vacuolated when subjected to clinically relevant (10) concentrations of nicotine and that they normalize when nicotine is withdrawn from the culture medium. The effect may thus be physiologically regulated via keratinocytic nicotine cholinergic receptors (9) although a toxic effect cannot be ruled out. Future studies, using in-vitro models, are planned to address this question and to find out if other substances in tobacco have biological effects on the keratinocytes.

The histomorphology of the snuff-induced lesions in the present study are in accordance with studies by *Wedenberg et al* (17) and *Merne et al* (14). In those studies, immunohistochemistry was used for detailed analysis of cell proliferation and cell cycle regulation, with somewhat contradictory results. Analysis of the present material using additional techniques are planned to further elucidate the biological effects of snuff at a molecular level.

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