Histologic changes associated with the use of loose and portion-bag packed Swedish moist snuff: a comparative study

Gunilla Andersson[1], Tony Axéll[1] and Åke Larsson[2]

Departments of [1]Oral Surgery and Oral Medicine, [2]Oral Pathology, Faculty of Odontology, Lund University, Malmö, Sweden.

Andersson G, Axéll T, Larsson, Å: Histologic changes associated with the use of loose and portion-bag packed Swedish moist snuff: a comparative study. J Oral Pathol Med 1989; 18: 491–497.

This study was to identify histologic tissue changes in the oral mucosa and to compare them in specimens from users of loose can-packed and portion-bag-packed moist snuff. The material consisted of biopsies from 252 regular snuff users, 184 using exclusively loose and 68 portion-bag snuff. An array of structural changes appearing in different combinations were identified among the 252 specimens. Two major patterns were recognized based on changes in the surface layer. Type 1 was characterized by an increased epithelial thickness with vacoulated cells and frequent chevron type changes. Type 2 showed a variably thickened surface layer with evidence of keratinization. Based on these findings, 14 carefully matched pairs of loose and portion-bag users were analyzed and compared. Loose snuff users showed predominately histologic Type 1 changes while portion-bag users showed more histologic Type 2 or only very discrete changes.

Key words: mouth, disease; oral mucosa, pathology; snuff; tobacco, smokeless.

Gunilla Andersson, Department of Oral Surgery and Oral Medicine, Faculty of Odontology, Lund University, S-214 21 Malmö, Sweden.

Accepted for publication August 15, 1989.

Use of smokeless tobacco is a common habit in many countries. However, the products are of many different types (1). In Scandinavia the overwhelming majority of snuff dippers use non-fermented moist snuff. During the last decade a new smokeless tobacco product, portion-bag packed snuff, has been introduced. Clinical changes associated with this type have recently been described (1), but no report of their histomorphology has been published.

Histologic changes seen in "snuff dipper's lesion" have been described in several previous studies (2–12). Common are hyperplasia of the epithelium with large vacoulated cells, some of which have been described as koilocytosis (12) and a chevron type of keratinization (10). In the connective tissue variable degrees of inflammation, amorphous changes, sialoadenitis and other degenerative changes in lip salivary glands have been observed (5, 7, 8, 11, 12). Slight epithelial dysplasia has been reported but the true frequency is a matter of dispute (4, 5, 7, 8, 12).

This study was to identify snuff-associated histologic tissue changes in the oral mucosa and to compare them in specimens from users of loose can-packed and portion-bag-packed moist snuff.

Material and methods

Material and examination procedure – The material for this study consisted of biopsies from 252 volunteers, all of whom were healthy men, regular snuff users for at least the last three months and with no other tobacco habit. For a detailed description of the recruitment procedure see ANDERSSON & AXÉLL (1). Loose snuff was used by 184 subjects of mean age 36.0 ± 11.6 yr, range 19–80 yr and portion-bag snuff by 68 individuals of mean age 36.9 ± 9.9 years, range 17–66 yr. Users of loose snuff consumed 23.6 ± 12.2 g/day during 10.8 ± 3.8 h and had done so for 13.1 ± 8.2 yr. The corresponding figures for portion-bag users were 11.3 ± 4.9 g/day during 10.3 ± 3.2 h and for 3.1 ± 2.5 yr.

At a first visit, all subjects were examined according to a standardized program, including questions on snuff habits, e.g. package form, brand, duration of habit, daily consumption and specified placement of the quid (one or more sites). A thorough clinical examination was carried out. Changes at the site(s) where the snuff was regularly placed were registered according to a four-grade clinical scale (7).

Among users of loose snuff, 10 (5.4%) Degree 1, 33 (17.9%) Degree

Table 1. Age and snuff exposure data of 28 matched subjects.

	Product	
	Loose snuff $n = 14$	Portion-bag snuff $n = 14$
Age, yr	34.7±12.1	36.9±10.0
Hours of daily snuff use	11.5± 3.4	11.2± 3.2
Grams of snuff used daily	17.0± 5.5	15.8± 4.9
Years with regular snuff habit	10.3± 8.0	4.4± 2.8

2, 130 (70.7%) Degree 3 and 11 (6%) Degree 4 lesions were registered (1, 7). Among users of portion-bags 13 (19.1%) subjects showed Degree 1, 31 (45.6%) Degree 2 and 24 (35.3%) Degree 3 lesions. No Degree 4 lesion was registered in this group. The higher proportion of clinically less severe changes among users of portion-bags compared with loose snuff users was statistically significant ($P<0.001$).

Biopsy procedure – From the central part of each changed area a biopsy was taken with a 6 mm punch using local anesthesia as infiltration well separated from the area of biopsy. The specimen was fixed in 10% neutral buffered formalin and embedded in paraffin. Five μ thick sections were stained with hematoxylin-eosin and PAS. Sections were also stained with rhodamine B and examined by fluorescent light (13) to evaluate the degree of keratinization. Sections of all 252 biopsies were examined light microscopically and compared with normal mucosa.

HPV-immunocytochemistry – Biopsies showing koilocytosis-like changes (14) were examined immunocytochemically, for the presence of HPV-antigen. Deparaffinised sections were incubated at room temperature with 0.15% H_2O_2 in methanol for 30 min, for blocking of endogenous peroxidase. Following a short rinsing in phosphate-buffered saline (PBS, 0.1 M pH 7.2), the sections were incubated with normal goat serum for 30 min followed by overnight incubation at 4°C with goat anti-human HPV (Dakopatts) diluted at 1:200 or 1:400. Negative controls included sections in which the primary antibody was omitted. Sections of a viral wart, run in parallel with the other sections, served as positive controls. For immunostaining, the ABC-technique was used. Sections were incubated with biotinylated antigoat IgG for 30 min, rinsed with PBS and incubated with the peroxidase-conjugated biotin-avidin complex for 30 min (Vectastain). Following further PBS rinsing, peroxidase activity was localized by incubating with 3'3 diaminobenzidine × 4 HCl (DAB 0.6%, Sigma) in 100 ml 0.05 M tris (pH 7.6) with 0.01% H_2O_2 for 1 h. Counterstaining was done with Mayers hematoxylin.

Matched pairs – In the clinical evaluation of the material the influence of package form and exposure data on the development of clinically more pronounced changes was assessed by means of stepwise logistic regression. Based on this evaluation 14 carefully matched pairs of loose and portion-bag users were selected (Table 1). The matched paired subjects used the same brand of tobacco either loose or wrapped in paper-bags, placed in the same site, and they exhibited closely similar patterns of snuff exposure in terms of hours of daily use and grams. Despite efforts to match for years of regular snuff habit there is a considerable difference between the groups due to the fact that portion-bags have only been available for a comparatively short time. The distribution of clinical degrees of changes among the matched subjects is shown in Table 2.

Based on findings in all 252 biopsies a selected number of histopathologic criteria (see below) were applied when studying the biopsies of the 14 matched pairs in an effort to identify traits specifically related to the package form of snuff.

Results

Histomorphology of the normal mucosa

In the present study, all the biopsies were taken from the midportion of the changed area, corresponding to the inside of the upper lip, close to the vestibular fornix. According to SCHROEDER (15), the epithelium of this part of the oral mucosa differs structurally from that of the buccal and of the alveolar mucosa. The epithelium is composed of small, ovoid, basophilic basal cells, which rather abruptly transform into a voluminous and pale "hydrosum" layer, with large polyhedral cells with a very low dye affinity. These cells seem to have a low density of cytoplasmic filaments and a high density of cytoplasmic ground substance. In routinely prepared sections (formalin fixation and paraffin embedding), they therefore often exhibit a vacuolated appearance (Fig. 1). In the upper-most "stratum distendum", they tend to form a more or less sharply flattened but still nucleated surface layer and these cells are variably condensed, forming a thin surface layer with pyknotic nuclei (Fig. 1). The final step of differentiation does not normally lead to a homogeneous surface layer in this part of the oral mucosa.

Histomorphology of the snuff-associated changes

In comparison with corresponding mucosa of a non-snuff user (cf. Fig. 1), an array of structural changes appearing in different combinations were identified among the 252 snuff users. Variable degrees of non-specific chronic inflammation were observed in all cases. The other changes have been defined as described below and these criteria have subsequently been applied when analysing specific subgroups of cases.

Changes of the surface layer – Two major patterns were recognized. In Type 1, the surface layer had an increased thickness and was composed of vacuolated cells with or without visible remnants of nuclei (Fig. 2). This feature was a commonly encountered finding and was always rhodamine B negative. It was not infrequently combined with a "chevron type pattern" (10) of piled-up, more stained cells with more well-preserved nuclei. These chevron type changes ("Christmas trees") tend to occur in those areas of the epithelium which cover the top of the connective tissue papillae (Fig. 2).

Type 2 showed a variably thickened surface layer with an eosinophilic stain and with a variable number of pyknotic

Table 2. Distribution of 28 matched subjects according to clinical grading.

Package form	Clinical degree*			
	1 $n=2$	2 $n=9$	3 $n=17$	Total $n=28$
Portion-bag snuff	2	7	5	14
Loose snuff	–	2	12	14
Total	2	9	17	28

* No clinical Degree 4 was encountered within the matched material.

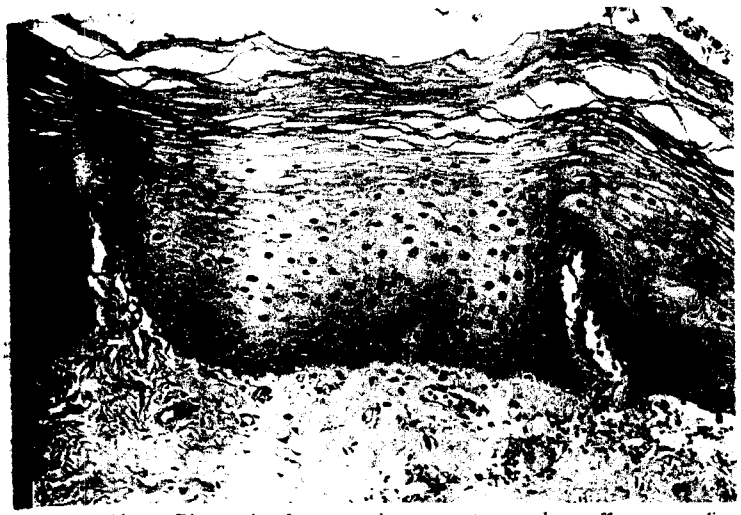

Fig. 1. 45-yr old man. Biopsy taken from normal mucosa not exposed to snuff, corresponding to area inside upper lip, in which snuff-users place their quids. A non-keratinized surface layer, with some artefactual splits, exhibits flattened cells with pyknotic nuclei. "Hydrosum" layer is composed of pale swollen cells. Distinct, basal cells form well-stained layer bordering underlying connective tissue. ×180.

nuclei (Fig. 3). A more or less continuous rhodamine B stain was always observed in this layer and/or in spots corresponding to the level of the granular layer.

Combinations of Type 1 and 2 were also identified, either appearing separate from each other or *mixed together* within the same surface area. Type 1 changes combined with a thin homogeneous structure-less surface zone, staining eosinophilic were also registered (Fig. 2). This zone always stained negative with rhodamine B (Fig. 8).

Atrophy and hyperplasia – This merely refers to the thickness of surface epithelium. In the photographically documented sections (24 × 36 mm negative size), we have arbitrarily defined the epithelium to be hyperplastic when a given area could not be completely reproduced within the horizontally orientated film rectangle at an original ×50 magnification (cf. Figs. 1–2). Accordingly, and in order to picture completely a hyperplastic epithelium within the given film area, the epithelial surface had to be orientated parallel to the short side of the film. In comparison with the thickness of normal epithelium, "epithelial hyperplasia" would then correspond approximately to a 1.5–2 fold increase of thickness.

Similarly, the epithelium has arbitrarily been defined to be atrophic when a more or less extensive loss of rete pegs was observed in conjunction with an over-all reduction of the thickness (Fig. 4).

Increased mitotic rate – The rate was recorded as increased, when more than two mitotic figures could be identified within any localized epithelial area at high power (orig ×100), corresponding to a surface area of approximately 0.08 mm^2 (Fig. 5).

Koilocytosis – Vacuolated epithelial cells are common in changes associated with snuff dipping (Fig. 2) frequently resembling truly koilocytotic cells. Such vacuolated cells are also a characteristic finding in normal epithelium of this part of the oral cavity (Fig. 1). In snuff dipper's lesions, vacuolated cells are often found to be piled up within the epithelium, sometimes extending all the way from the surface down to the basal cell layer. Problems may arise interpreting these cell changes as normal, degenerative or truly koilocytotic.

In snuff changes, vacuolated cells were classified as degenerative, due to the chemical etching of snuff, when appearing in continuity with the surface layers and extending into the deeper epithelial layers (Fig. 6). Accordingly, cells were interpreted as koilocytosis-like (cf. 14) when appearing in small narrow clusters with no obvious connection with otherwise continuous layers of degenerative cells. These presumably koilocytotic cells showed a vacuolated cytoplasm appearing as a clear rim round a pyknotic nucleus (Fig. 7).

Increased cellular density and basilar hyperplasia – In some snuff-induced changes, the degree of vacuolization of epithelial cells may be decreased rather than increased, in contrast with what is commonly observed in such changes. They give the impression of an increased cellular density, partly due also to a better dye-affinity of the cytoplasm of these cells (Fig. 5). An increased density may also be due to a changed nuclear-cytoplasmic ratio (Fig. 5). No distinction has ben made between these two patterns but both have been recorded as "increased cellular density". Occasionally, increased density is mainly localized to the basal part of the epithelium, "basilar hyperplasia" (Fig. 8).

Other histologic observations – Other observations have been made, all previously known from other studies of changes associated with snuff dipping, but appearing in the present study with such a very low frequency, that meaningful interpretations were difficult or impossible to make. Among these, eosinophilic leukocytes (7) were observed within the epithelium in a few cases. Similarly, eosinophilic connective tissue amorphous or hyaline changes (2, 7) could be recorded in only about 10 cases. Salivary gland involvement (8) could not be recorded in a meaningful way, due to the highly variable inclusion of gland tissue in the biopsies.

Epithelial changes suggestive of dysplasia, as defined by the criteria of WHO (16), were observed in only a few isolated cases. These findings will be the subject of a separate publication.

HPV-immunocytochemistry

Koilocytosis-like changes were identified in 17 cases. We were however unable to demonstrate convincingly positive HPV-antigen immunoreactivity in any of these biopsies. Further, none of these showed any dysplasia.

Matched pairs

We were able to match 14 pairs of loose and portion-bag snuff users (Table 2). No case of unequivocal dysplasia was recorded. Histomorphologic changes were distributed as shown in Table 3.

All the 28 cases showed some degree of non-specific inflammation, but we

Fig. 2. 35-yr old man with clinical Degree 4 lesion following dipping with loose snuff (14 h daily, 40 g a day, 16 y with regular habit). Type 1 surface change. Surface layer is heavily thickened due to piled-up vacuolated cells, with remnants of nuclei. Outermost surface zone is homogeneous and eosinophilic but it is rhodamine B negative. Cases exhibiting this type of surface change frequently fulfilled criteria of "hyperplasia". Spikes of cells within vacuolated layer form "chevron pattern". These spikes are clearly related to underlying connective tissue papillae (arrow), in this case slightly inflamed. No dysplasia. ×185.

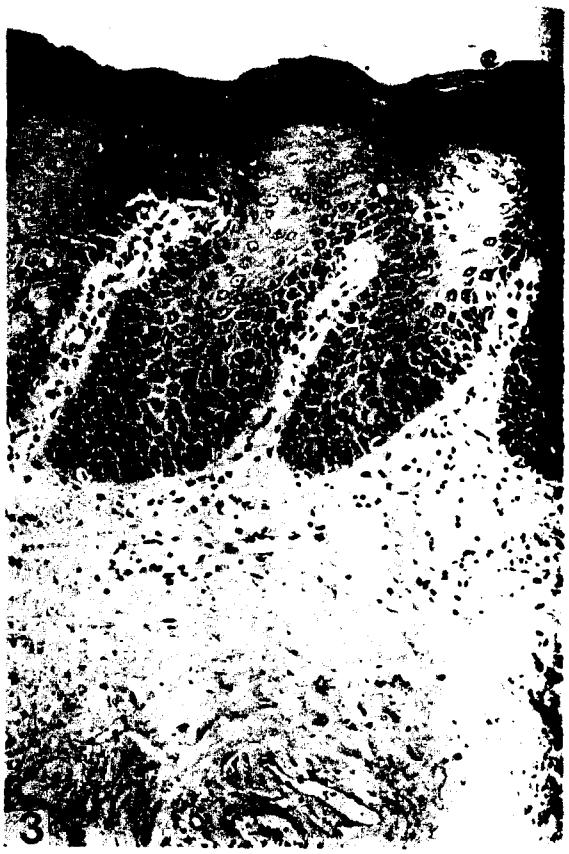

Fig. 3. 28-yr old man with clinical Degree 3 lesion following dipping with loose snuff (10 h daily, 17 g a day, 9 yr with regular habit). Type 2 surface change. Surface layer is keratinized (rhodamine B-positive, not illustrated). This is accompanied by slight inflammation and increased density of epithelial cells, exhibiting increased stainability, slightly enlarged nuclei and clearly visible nucleoli (cf Fig. 1) but no dysplasia. ×185.

were unable to detect any clear-cut differences between the two groups of snuff users. Cases with hyperplasia and increased mitotic rate were evenly distributed between the two groups. No Degree 4 lesion came out among the matched cases. When examined pairwise, we found that within each of six matched pairs (1–6), the clinical changes were of an identical grade, five (Nos. 1–5) of these also showing histologically almost identical surface changes. In the remaining eight pairs (Nos. 7–14), the portion-bag users showed clinical changes of a lower degree than their matched loose snuff users. Three of these pairs showed almost identical surface Type 1 changes (Nos. 7–9), but in the other five (Nos. 10–14), histologic surface changes differed within each of the pairs. Here, four of the loose snuff users showed clinical Degree 3 lesions (Nos. 10–13), three of these having Type 1 surface changes (Nos. 10–12). In contrast, all of the five portion-bag users had Degree 1–2 lesions (Nos. 10–14) and they all exhibited a histologic Type 2 change or a normal-looking epithelium.

These findings indicate that based on comparable snuff habits, loose snuff may cause clinically more pronounced changes (Degree 3) accompanied by histologic Type 1 changes. Portion-bag snuff, associated with less pronounced changes (Degrees 1–2) show more of histologically Type 2 or only very discrete changes.

Discussion

Snuff, as used here, causes clinical and histomorphologic changes of the mucosa. Among these, epithelial changes have attracted much attention, but detailed correlative clinical-histopathologic studies are still inconclusive.

By comparing two different habits of snuff use, we have been able to identify three major histologic patterns of surface change:

1. A thin eosinophilic surface zone, with opacified cells. We interpret this

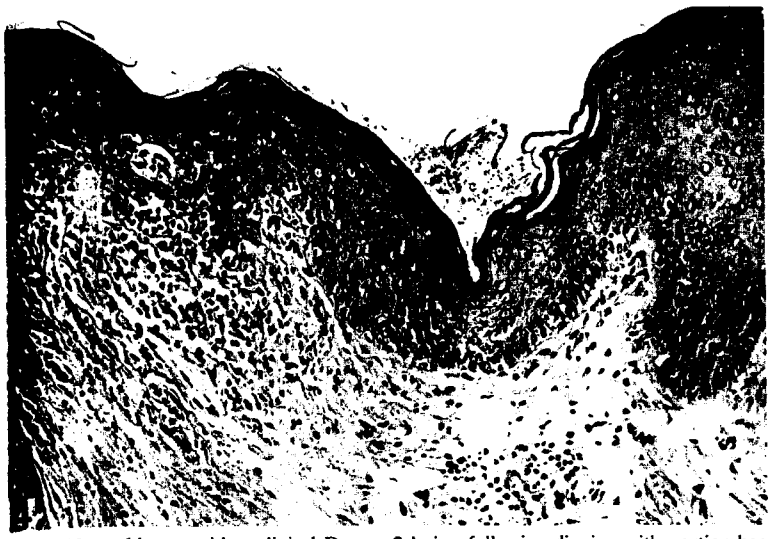

Fig. 4. 23-yr old man with a clinical Degree 2 lesion following dipping with portion-bag packed snuff (13 h daily, 24 g a day, 3 yr with regular habit). This corresponds to atrophy of the epithelium combined with keratinization (Type 2 surface change) and a moderate degree of inflammation. No dysplasia. ×180.

as a coagulative necrosis due to chemical etching by the snuff. It was variably observed in both habit groups. It may be misinterpreted as a keratinized layer but it stains negative with rhodamine B. We think that it may readily desquamate shortly following removal of the snuff quid. Hence, this layer is a variable finding in biopsies and not related to any specific habit of snuff use.

2. Vacuolization or swelling of the surface cells combined with aberrant desquamation causing a "pseudohyperplasia" of the epithelium. This is our Type 1 change which we interpret as indicative of an osmotic imbalance presumably caused by cell membrane alteration resulting in an osmotic absorption of water into the injured cells. This was a common finding in both groups of snuff users. The depth of vacuolated change varied, but was rarely found extending through the whole epithelial thickness. Thus, chemical cell membrane alteration by snuff is mostly restricted to the surface layers. The vacuolated cell surface change was occasionally accompanied by the characteristic "chevron pattern". Histomorphologically, this phenomenon seems to be the result of a persistence of more well-preserved cells, topographically associated with the underlying connective tissue papillae (10). Hence, these cells may be in a more favorable metabolic relationship to the underlying vascularized tissue.

3. The development of a keratinized surface layer, as evidenced by positive rhodamine B staining (13). This Type 2 change indicates an activation of the keratinization process in this area, which is normally non-keratinized. Such changes were observed in both groups of snuff users.

The crucial question is to what extent any of these three surface changes may be accompanied by serious damage to the underlying epithelial cell layers. Loose snuff tends to be more chemically etching than portion-bag snuff, i.e. causing more deeply-reaching effects, resulting in piling-up of significant numbers of vacuolated cells. All our cases of "hyperplasia" showed this characteristic change. Impeded desquamation may contribute to this thickening which may perhaps be associated with alterations of keratinocyte membrane lipids. This may be related to added rigidity and decreased fluidity of the cell membranes induced by tobacco components (17).

Occasionally, keratinization was observed. No studies seem to have been published on the cytokeratin pattern in snuff-induced changes and no conclusions can therefore be drawn about the way in which snuff may cause such a change. However, based on the present finding, keratinization per se is not accompanied by epithelial changes other than those seen in cases lacking keratinization.

Greer & Poulson (12) found koilocytotic changes in 26 of 45 snuff users and 6 of these 26 were HPV-positive. In contrast to this, we were unable to demonstrate HPV-antigen in any of the biopsies exhibiting koilocytosis-like changes. Perhaps with other techniques such as DNA hybridization, more information can be gained about any role played by HPV or other viruses in "snuff dipper's lesion".

The present study focused on the comparison between package forms and the 14 pairs were selected accordingly. No case suggestive of dysplasia was found within the matched material.

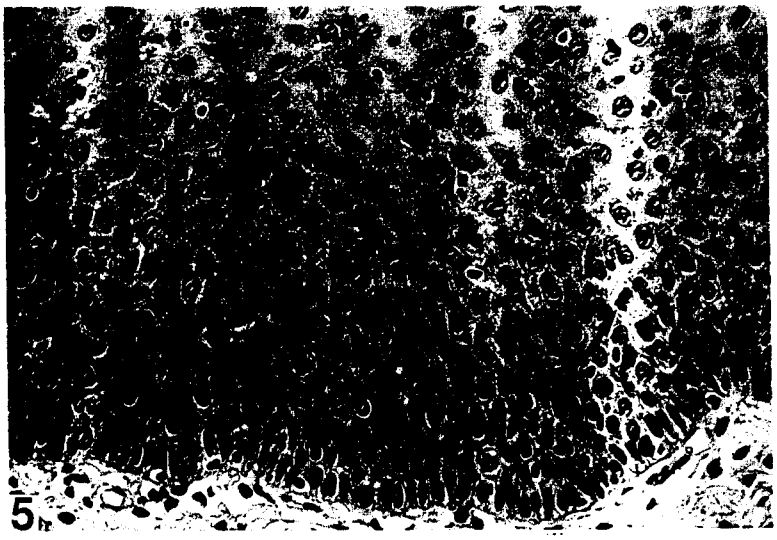

Fig. 5. 18-yr old man with a clinical Degree 2 lesion following dipping with portion-bag packed snuff (10 h daily, 16 g a day, 4.5 yr with regular habit). High magnification of basal-deep spinous cell layer, showing several mitoses (arrows). Spinous layer shows increased density, with enlarged cell nuclei and well stained cytoplasm. No dysplasia. ×360.

Fig. 6. 22-yr old man with clinical Degree 3 lesion following dipping with loose snuff (16 h daily, 17 g a day, 8 yr with regular snuff habit). Thick vacuolated surface layer is in direct continuity with vacuolated cells also in deeper spinous layers, the latter having koilocytosis-like appearance, with pyknotic nuclei. ×185.

Fig. 7. 32-yr old man with clinical Degree 4 lesion following dipping with loose snuff (13 h daily, 40 g a day, 11 yr with regular habit). Type 1 surface change, cf. Fig. 2. Localized area of deep spinous/parabasal layer shows koilocytosis-like cells (arrow) not clearly forming a continuum with swollen surface cells, cf. Fig. 6. ×185.

This could possibly be explained by the fact that regular long-term snuff users were not included due to the matching procedure (5). An increased mitotic rate was observed in several cases of both groups and in a few cases accompanied by an increased cellularity. In addition to inflammation these were the only changes found in the deeper tissue layers. Our interpretation is that they represent a reactive response to the surface change caused by the snuff. The damaging potential of snuff to the mucosa is however a controversial subject, especially concerning the incidence rate of precancerous/dysplastic development in snuff lesions (18). In the present study we have not yet completed our analysis of possible irreversible changes, which will require repeated biopsies.

In a previous study, based on a clinical grading system (1), loose snuff was

Fig. 8. 41-yr old man with clinical Degree 3 lesion following dipping with portion-bag packed snuff (7 h daily, 12 g a day, 10 yr with regular habit). Slightly swollen, opacified, rhodamine B-negative surface layer. Increased density is observed in basal parts of epithelium, "basilar hyperplasia". ×180.

Table 3. Matched pairs of snuff users. Histologic changes defined according to text.

Pair No.	Accession No.[1]	Surface layer[2,3]	Hyperplasia (H) atrophy (A)	Mitoses	Cell density[4]	Clinical degree
1	018	1	–	+	+	3
	243	1	–	+	–	3
2	062	1	H	–	–	3
	278	1	–	–	–	3
3	061	1	H	–	–	3
	364	1(2)	H	+	–	3
4	041	1	H	+	–	3
	353	1(2)	–	–	–	3
5	017	1	–	+	–	3
	244	1	A	–	–	3
6	037	2	–	+	+	2
	252	1	H	–	–	2
7	014	1(2)	–	–	–	2
	378	1	H	–	–	3
8	038	1	H	–	–	2
	292	1	–	+	–	3
9	056	1	–	+	+	2
	272	1	–	–	–	3
10	023	2	–	+	–	2
	318	1	H	+	–	3
11	074	2	A	–	–	2
	314	1	–	–	–	3
12	054	2	–	–	–	2
	279	1	–	–	–	3
13	031	N	–	–	–	1
	298	2	H	–	–	3
14	060	N	–	–	–	1
	334	2	–	+	+	2

[1]first case in each pair uses portion-bag snuff, second case loose snuff.
[2](2) Type 2 present in addition to predominant presence of Type 1.
[3]N=normally-looking mucosa.
[4]+ includes basilar hyperplasia and/or generally increased density.

found to be associated with a higher frequency of clinical Degree 3–4 lesions than portion-bag packed snuff. In the present study, having matched the 14 pairs with respect to amount and time of exposure per day of snuff, we found that clinical Degree 3 lesions were predominantly found among the loose snuff users. These were predominantly associated with histologic Type 1 changes with osmotically swollen cells. Among the portion-bag users, we found a tendency to exhibit Type 2 changes, with evidence of keratinization. Interestingly, all of these type 2 changes were associated with clinical Grade 2 lesions. Taken together, these findings indicate that portion-bag packed snuff results in less pronounced changes to the oral mucosa than loose snuff, but in the present study we have been unable to selectively study the effects of differences in habits. Further studies are in progress to elucidate this problem.

Acknowledgments – This study was supported by grants from Swedish Tobacco Research Council (Project No. 8716).

References

1. Andersson G, Axéll T. Clinical appearance of lesions associated with the use of loose can packed and portion-bag packed Swedish moist snuff – a comparative study. *J Oral Pathol Med* 1989; **18**: 2–7.
2. Pindborg JJ, Poulsen HE. Studies in oral leukoplakias. 1. The influence of snuff upon the connective tissue of the oral mucosa. Preliminary report. *Acta Pathol Microbiol Scand* 1962; **55**: 412–4.
3. Pindborg JJ, Renstrup G. Studies in oral leukoplakias. II. Effect of snuff on oral epithelium. *Acta Derm-Venereol* 1963; **43**: 271–6.
4. van Wyk CW. The oral lesion caused by snuff. A clinical – pathological study. *Med Proc* 1965; **11**: 531–7.
5. Roed-Petersen B, Pindborg JJ. A study of Danish snuff-induced oral leukoplakias. *J Oral Pathol* 1973; **2**: 301–13.
6. Frithiof L, Anneroth G, Lasson U, Sederholm C. The snuff induced lesion: a clinical and morphological study of a Swedish material. *Acta Odontol Scand* 1983; **41**: 53–64.
7. Axéll T, Mörnstad H, Sundström B. The relation of the clinical picture to the histopathology of snuff dipper's lesions in a Swedish population. *J Oral Pathol* 1976; **5**: 229–36.
8. Hirsch J-M, Heyden G, Thilander H. A clinical histomorphological and histochemical study on snuff-induced lesions of varying severity. *J Oral Pathol* 1982; **11**: 387–98.
9. Jungell P, Malmström M. Snuff-induced lesions in Finnish recruits. *Scand J Dent Res* 1985; **93**: 442–7.
10. Pindborg JJ, Reibel J, Roed-Petersen B, Mehta FS. Tobacco-induced changes in oral leukoplakia epithelium. *Cancer* 1980; **45**: 2330–6.
11. Archard HO, Tarpley TM. Clinicopathologic and histochemical characterization of submucosal deposits in snuff differ's keratosis. *J Oral Pathol* 1972; **1**: 3–11.
12. Greer RO Jr, Poulson TC. Oral tissue alterations associated with the use of smokeless tobacco by teen-agers. *Oral Surg* 1983; **56**: 275–84.
13. Clausen FP, Dabelsteen E. Increase in sensitivity of the Rhodamin B method for keratinization by the use of fluorescent light. *Acta Pathol Microbiol Scand* 1979; **77**: 169–71.
14. McLeod K. Prediction of human papilloma virus antigen in cervical squamous epithelium by koilycyte nuclear morphology and "warf scores"; confirmation by immunoperoxidase. *J Clin Pathol* 1987; **40**: 323–8.
15. Schroeder HE, *Differentiation of human oral stratified epithelia*. Basel: S Karger, 1981.
16. Smith C, Pindborg JJ. *Histological grading of oral epithelial atypia by the use of photographic standards*. World Health Organization's International Reference Center for Oral Precancerous Conditions. Department of Oral Pathology, Royal Dental College, Copenhagen 1969.
17. Sauk JJ, Norris K. Tobacco components induce alterations in keratinocyte lipid membrane fluidity and inhibit cell proliferation in culture. *J Oral Pathol* 1988; **17**: 30–3.
18. Main JHP, Lecavalier DR. Smokeless tobacco and oral disease. A review. *J Can Dent Assoc* 1988; **54**: 586–91.