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**Research Article**

# The Reversibility of Snuff-Induced Lesions: A Clinical and Histomorphological Study

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## Abstract

**Aims:** To evaluate the reversibility of clinical and histomorphological findings in snuff lesions 6 months after cessation of snuff use. **Design:** The study design was a prospective, open, non-randomized trial intervention of a smokeless tobacco cessation programme using nicotine gum as nicotine replacement therapy (NRT) for 3 months followed by a tapering period of 3 months. **Setting:** Twenty-six of 50 patients with a baseline biopsy agreed to have a second biopsy 6 months after cessation. **Measurements:** Before baseline biopsies were taken, the snuff lesions were classified on a 4-point scale of severity. After 6 months cessation the mucosa was re-examined, a second biopsy taken from the area of the lesion, and baseline and second biopsies compared. **Findings:** Eighteen subjects were analysed. Thirty-nine per cent of subjects showed a remaining clinical lesion 6 months after refraining from tobacco. The most predominant lesions were seen in the 73% of subjects still using NRT. The histomorphological picture was dominated by reductions in epithelial thickness, keratinisation, and inflammatory response after tobacco cessation, although 33% of subjects showed increased epithelial thickness and 39% had constant inflammatory reaction. **Conclusion:** In the significant group of patients, the mucosa was neither clinically nor histomorphologically completely normal 6 months after cessation of snuff use. Seventy-five per cent of the subjects with persistent lesions were using NRT on a daily basis. There are indications that nicotine alone may promote up-regulation of proto-oncogenes, which suggests causation. This limited study should be extended to focus on ex-snuff users with prolonged exposure to NRT.

**Keywords:** Chewing Tobacco;Snuff;Nicotine;Smoking Cessation;Tobacco Use Cessation;Smokeless Tobacco Cessation;Withholding Treatment.

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## Introduction

The clinical and histomorphological picture of snuff dippers' lesions among Scandinavian snuff users has previously been reported.[1-5] The clinical appearance has been classified on a 4 point scale.[5] A correlation between snuff exposure and clinical grade has been documented, and a correlation has also been shown between snuff use and certain cell changes, both superficial and deeply located.[2] It is generally accepted that snuff use has a negative impact on general health, and that cessation of snuff use is an important health care issue.[6-8] Habitual snuff exposure induces typical oral lesions, which have generally been considered reversible.[9] However, only a few studies have reported on the appearance of the snuff lesion before and after cessation.[10,11] Larsson et al.[11] found a clinically and histologically healthy mucosa in 16 subjects 3–6 months after cessation, while an animal study showed certain persistent histopathological changes 4 months after snuff administration had ceased.[12]

In this study of a group of long-term (> 10 years) regular snuff users who participated in a cessation programme including nicotine replacement therapy, we investigate the clinical and histopathological picture of snuff lesions 6 months after tobacco cessation.

## Materials and Methods

A smokeless tobacco cessation programme including NRT with 4 mg gum was previously developed for chronic snuff users.[13] Inclusion criteria were daily snuff use of more than 2 cans/week (>100 g) for 10 years or longer, agreed to an optional biopsy, and motivated to give up snuff use through a 12 month clinical survey. The exclusion criterion was ongoing habitual or occasional smoking during the snuff-using period. Of 280 responders screened over the telephone, the first 50 to fulfil the inclusion criteria were entered in to the study. All the subjects used loose snuff. A history of tobacco use was obtained at the start of the study and included number of years with the habit, daily hours of snuff

exposure, and daily consumption. (Table 1) At baseline all subjects were offered 4 mg nicotine gum (Nicorette McNeil AB) to use ad lib for 3 months followed by a 3 month tapering period. The study was conducted after informed consent in accordance with the declaration of Helsinki and approved by the Ethics Committee of Sahlgrenska University Hospital, Göteborg, Sweden (No. 5-91).

A clinical examination was performed and the snuff-induced lesion was photo-documented and graded[5], as follows: Degree 1: A superficial lesion with colour similar to the surrounding mucosa, with slight wrinkling and no obvious thickening. Degree 2: A superficial whitish or yellowish lesion with wrinkling and no obvious thickening. Degree 3: A whitish-yellowish lesion to a brown, wrinkled lesion with intervening furrows of normal mucosal colour and obvious thickening. Degree 4: A marked whitish-yellowish lesion to a brown, heavily wrinkled lesion with intervening deep and reddened furrows and/or heavy thickening. In addition to Axéll's classification the authors added Degree 0 for no visible lesion. Example of snuff induced lesion at baseline and at the 6-month follow-up (Figure 1).

A 10 × 5 mm biopsy was taken at baseline from the snuff-induced lesion using local anaesthesia (1.0 cc lidocaine with adrenalin 12.5 μg/ml; Dentsply Ltd, Sweden). At the 6-month visit, a second biopsy from the same mucosal area as the first was taken from subjects who had been abstinent since baseline. At baseline and at the 6-month control the mucosa was clinically examined and photo-documented. These photographs were used by the first and last authors to classify the snuff lesions, first independently and then jointly in cases where the grades differed.

For biochemical verification of the patients' self-reported tobacco use, saliva cotinine and expired carbon monoxide (CO) were analysed. Saliva samples of 1ml were taken and frozen in -20 C degrees and transported to McNeil AB, Helsingborg, Sweden, in order to determine cotinine levels by gas chromatography analysis. Cotinine is the major metabolite of nicotine and is frequently used to verify cessation compliance. Values > 15 mg/ml were considered to indicate on-going tobacco use.[14] To verify non-smoking status, CO analysis (Bedfont Smokerlyzer, Technical Instruments Ltd, Kent, UK) was performed at all visits. CO levels < 10 ppm were considered to indicate non-smoking status.

The biopsied tissue was immediately placed in chilled Histocon (Histo-Lab, Göteborg, Sweden). Within 24 hours the specimen was embedded with Tissue-Tek (Tissue-Tek OCT compound; Miles, Elkhart, IN, USA) on a piece of cork and frozen using isopentane chilled to -140$^{o}$C with liquid nitrogen.[15]. The biopsy was stored at -80$^{o}$C until sectioned (5 μm), and stained with haematoxylin and eosin. All lesions were classified according to the criteria given by Kramer et al.[16] The epithelial, mucosal and submucosal changes were assessed with regard to the width and type of the keratin layer, epithelial thickness, and degree and localization of stromal inflammation. The degree of inflammation was registered as follows: **1+:** Slight infiltration of inflammatory cells in small groups in the connective or interstitial tissue. **2+:** Moderate infiltration of the connective perivascular tissue. **3+:** Severe infiltration of the connective perivascular tissue.

The thickness of the epithelium and the keratinised surface layer was measured at the site of the rete pegs at 5 to 10 points evenly distributed in each section (Fig 1). The calculation was performed using Bioquant II Digital Morphometry (R&M Biometrics, Inc., Nashville, TN, USA) connected to an Olympus microscope. The measurements were performed blinded to all other subject data.

Student's *t*-test was used to compare the two biopsies, taken at baseline and after 6 months of cessation. Wilcoxon's signed-rank test was used to compare intra-individual inflammation. Kruskal Wallis non-parametric test was used to asses differences between multiple groups**.**

**Results**

Twenty-six patients agreed to have a second biopsy at 6 months taken from the same site. Eighteen subjects could be analysed regarding the clinical and histological outcome. Four subjects were excluded due continuous snuff use or smoking and biopsy specimen from 4 subjects were not possible to analyse. There was no significant difference in baseline data with regard to age and snuff exposure between those 18 patients and the 50 originally entered in the study (Table 1).

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| N | Age (years) | Snuff use Years | Snuff use Hours / day | Nr Cans Weekly | Mg of Nicotine / day |
|---|---|---|---|---|---|
| All=50 | 42.2 ± 10.7 | 20.8 ± 8.4 | 15.2 ± 2.1 | 5.0 ± 2.6 | 280 ± 144 |
| 2nd px=18 | 40.6 ± 9.8 | 22.3 ± 7.1 | 14.7 ± 2.7 | 4.7 ± 3.0 | 262 ± 169 |

**Table 1:** Age and tobacco exposure data of the n=50 subjects entering the smokeless cessation study compared with n=18 subjects who were abstinent at 6 months and having a second biopsy. All data are baseline data.

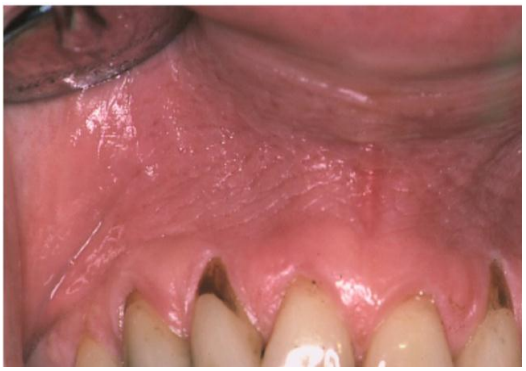

Figure 1a: Clinical picture at baseline; grade 3 lesion.

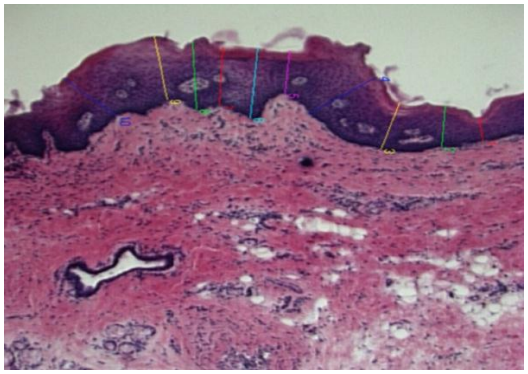

Figure 1b: Baseline biopsy. The epithelial and keratin thickness was measured along the colored lines depicted in the histomorphological picture.

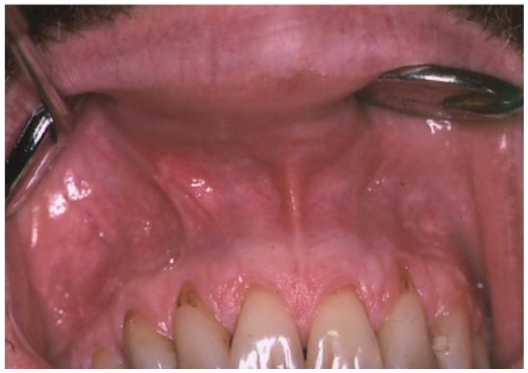

Figure 1c: Clinical picture at 6 months follow up shows a grade 1 lesion.

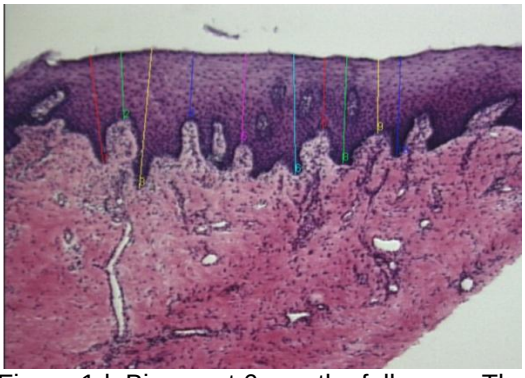

Figure 1d: Biopsy at 6 months follow up. The epithelial and keratin thickness was measured along the colored lines depicted in the histomorphological picture.

**Clinical evaluation**

There was no significant correlation between the severity of the lesion and the total exposure to snuff in terms of years with the habit, daily hours of snuff consumption, and amount consumed on a daily basis ($p > 0.05$; Table 2).

Of the 18 subjects, 39% [n=7] showed remaining lesions after 6 months (66% [n=2] with grade 4 lesions at baseline, 38% [n=3] with grade 3, and 40% [n=2] with grade 2. Five of these 7 subjects were still using NRT on a daily basis, 3 chewing the gum and 2 placing it under the lip, while two were nicotine-free (Table 2). Two subjects still on NRT exhibited no lesions. The remaining visible clinical lesions at the 6-month follow-up were classified as grade 1 or 2 lesions; according to Axéll.[5] All lesions were superficial with a whitish colour or a colour similar to the surrounding mucosa, some of them with slight wrinkling. The lesions were markedly reduced in size.

**Histomorphology**

In general, the snuff-induced oral lesions were characterized by evenly distributed, slight to moderate keratinisation, an increased epithelial thickness with rete pegs, and varying degrees of stromal inflammation. More specifically we noted that at baseline all specimens except one were hyperkeratinised. Nine of the lesions were parakeratinised, eight were orthokeratinised, and only one was not keratinised. Vacuolated epithelial cells with pycnotic nuclei were noted in few samples. Only one of the specimens showed a mild dysplasia in the epithelial basal cell layer.

A mild chronic inflammation in the lamina propria was visible in 45% of cases. The majority of the cells were lymphocytes and

macrophages with occasional plasma cells. One biopsy showed severe inflammation with a dense sub-epithelial infiltrate. This lesion was diagnosed as a lichenoid reaction pattern as well as a snuff lesion. At baseline, 8 biopsies showed a moderate inflammatory infiltration (2+) and 10 showed a mild inflammatory reaction (1+). At the second biopsy, one showed severe (3+), 7 showed moderate (2+), and 10 showed mild inflammation (1+).

After 6 months the epithelial thickness increased in 33% and decreased in 67%; mean thickness was 253±96 µm at baseline and 254±101 µm at 6 months. The mean total thickness of the keratin surface layer decreased significantly from baseline (52.5±31.5 µm) to 23.3±15.5 µm after cessation ($p = 0.001$). There was no significant difference between the epithelial thickness of the 32 subjects at baseline compared to the 18 subjects at baseline. Eight specimens exhibited orthokeratinisation, nine parakeratinisation, and one no keratinisation at baseline. At 6 months 15 were parakeratinised, only one remained orthokeratinised, and 2 were non-keratinised. There was no correlation between clinical scoring and degree of inflammation ($p>0.05$) or epithelial thickness ($p>0.05$) in histomorphology.

**Discussion**

The overall clinical and histomorphological picture after 6 months of abstinence was improved. However, at the site of snuff application 39% of the participants still exhibited clinical changes, although less severe, and the area of the affected mucosa had diminished in size. Only one lesion had the same classification as at baseline (grade 2), and the remaining lesions shifted from a higher to a lower grade, ranging from 2 to 0 (Table 2) on the Axéll et al. classification system.[5]

By contrast, in a study by Larsson et al.[11], tissue changes after snuff abstinence were found to be completely clinically reversible, although only 16 out of 29 subjects met the criteria of abstinence between 3 and 6 months. In our study, all subjects were confirmed tobacco abstinent prior to the second biopsy. A comparison of the data of the two studies shows that the age of the subjects and the clinical severity of the lesion at baseline were approximately the same. However, the mean duration of exposure to snuff was 10 years longer ($p = 000.1$) in the present study. We suggest that the differences between the two studies with regard to the reversibility of clinical findings are partly related to duration of snuff exposure.

Another contributing factor may be the fact that 71% of the subjects with remaining clinical lesions used NRT. These observations indicate that NRT may have a negative effect on the oral mucosa, as previously indicated by Andersson and Warfvinge[17], who demonstrated that nicotine is one of the ingredients in snuff with a local biological effect on the oral mucosa. This finding was substantiated by Wallström et al. exposing the oral mucosa to nicotine sublingual tablets.[18] Since tobacco is one of the main aetiological factors for oral squamous cell carcinoma and nicotine is one of the major alkaloids in tobacco, this finding should indicate caution regarding extensive use of oral nicotine in connection with tobacco cessation. Nicotine activates FOXM1, a proto-oncogene which has been shown to play an important role in oncogenesis. FOXM1 is up-regulated as an early event in human squamous cell carcinoma and it is enhanced by nicotine during malignant cell transformation.[19] We have previously, in a safety study, exposed the sublingual mucosa to 2–4 mg nicotine hourly for 3 months. This resulted in reversible superficial transient lesions at the site of application, verified by biopsy after cessation of the nicotine administration.[18] These findings also suggests that administration of NRT should be done with caution with regard to dose and duration, also taking into consideration the status of the oral condition, especially in patients with premalignant and malignant oral lesions.

The histomorphological picture at baseline was in agreement with previous reports: increased epithelial thickness, as well as increased thickness of ortho- or parakeratinisation and inflammatory reaction of varying severity and with only occasionally mild dysplasia.[1,2,20,21] However this study could not show a correlation between clinical picture and degree of inflammation nor epithelial thickness. This maybe explained by relative small number of patients.

At the 6-month visit we observed significant reduction of the keratin layer ($p = 0.001$). A decreased total epithelial thickness and a persistent chronic sub-epithelial

inflammation were also noted. However the findings were not statistically significant. This is in accord with the study from Hirsch et al.12, who exposed rats to snuff in a surgically created canal in the lower lip for 13 months, and sacrificed after a snuff-free interval of 1 or 4 months. At that time, the oral mucosa exhibited a slightly hyperplastic epithelium with little or no keratinisation. In 40% of cases, the rats showed a remaining inflammatory reaction. By contrast, Larsson et al.10 analysed biopsies from 16 abstinent subjects. Using a slight modification of the histological parameters suggested by Kramer16, they reported no histological changes and all samples were classified as normal.

In conclusion, we found that after long and extensive snuff use, snuff-induced lesions do not resolve completely, either clinically or histologically. We suggest that these patients be reviewed closely, especially as it has been reported that the risk for oral cancer among former snuff users were increased with borderline statistical significance.22-23 Because of the possible involvement of nicotine in malignant cell transformation, special attention should be given to patients using NRT as a cessation aid.

| Lesion | Patient No | Age (years) | a. Snuff duration (years) | b. Cans/ Week | c. Snuff Duration (hours/day) | Total Exposure (a x b x c) | Grade at 6 months | Nicotine Gum (mg/day) | Nicotine Gum use form |
|---|---|---|---|---|---|---|---|---|---|
| **Grade 4** | | | | | | | | | |
| | 01 | 47 | 30 | 7 | 15 | 3150 | 2 | 8 | Chewing |
| | 26 | 43 | 28 | 13 | 22 | 8008 | 1 | 40 | Chewing |
| | 48 | 35 | 20 | 3.5 | 15 | 1050 | 0 | 0 | |
| Mean | | 42 | 26 | 8 | 17 | 4069 | | | |
| SD± | | 6.1 | 5.3 | 4.8 | 4.0 | 3568.9 | | | |
| **Grade 3** | | | | | | | | | |
| | 02 | 32 | 15 | 2.5 | 12 | 450 | 1 | 4 | Chewing |
| | 05 | 42 | 20 | 2 | 9 | 360 | 0 | 0 | |
| | 20 | 45 | 25 | 7 | 11 | 1925 | 1 | 0 | |
| | 23 | 27 | 15 | 5 | 15 | 1125 | 0 | 0 | |
| | 28* | 28 | 14 | 2 | 15 | 420 | 0 | 0 | |
| | 31 | 37 | 8 | 2 | 15 | 240 | 0 | 0 | |
| | 36** | 34 | 20 | 7 | 15 | 2100 | 1 | 16 | Sublabial |
| | 43*** | 34 | 19 | 8 | 16 | 2432 | 0 | 32 | Chewing |
| Mean | | 35 | 17 | 4.4 | 14 | 1132 | | | |
| SD± | | 6.2 | 5.1 | 2.6 | 2.5 | 896 | | | |
| **Grade 2** | | | | | | | | | |
| | 14 | 47 | 33 | 1,5 | 16 | 792 | 0 | 20 | Sublabial |
| | 15 | 47 | 30 | 4,5 | 17 | 2295 | 1 | 12 | Sublabial |
| | 17 | 35 | 20 | 3 | 15 | 900 | 0 | 0 | |
| | 24 | 49 | 35 | 3 | 13 | 1365 | 0 | 0 | |
| | 34 | 69 | 20 | 2 | 15 | 600 | 2 | 0 | |
| Mean | | 49 | 28 | 2.8 | 15 | 1190 | | | |
| SD± | | 12.3 | 7.2 | 1.2 | 1.5 | 678.6 | | | |
| **Grade 1** | | | | | | | | | |
| | 40 | 38 | 24 | 4 | 15 | 1440 | 0 | 0 | |
| **Grade 0** | | | | | | | | | |
| | 10¨ | 42 | 25 | 7 | 14 | 2450 | 0 | 0 | |

**Table 2**. Snuff exposure and clinical grading of the lesions at baseline and at 6 months of 18 subjects.

^ Patient no 10 quit between telephone inclusion and baseline visit but was included in the study.

* Patient no 28 occasional party smoking.

** Patient no 36 snuff use during 1 week, 2 months prior second biopsy.

*** Patient no 43 daily smoking during 1 month, 2 months prior second biopsy.

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