

Harm and harm reduction in smokeless tobacco users

An in vitro and clinical study

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Abstract

Background: This thesis describes the effects of smokeless tobacco (ST) and its derivatives on the oral mucosa; the development of, and results of applying, a cessation program for snuff users with long and extensive exposure; and, the persistent clinical and histomorphological changes after cessation.

Methods: Three of the studies were open prospective non-randomized clinical intervention trials using nicotine replacement therapy (NRT) of either 4 mg nicotine chewing gum or 2 mg nicotine lozenges. Clinical examinations, biopsies, and histomorphological analyses were performed. In the fourth study *in vitro* assays were performed to investigate the effect of snuff extract, alkaloids, and selected tobacco-specific nitrosamines (TSNA) on the accessory function of rat oral epithelium cells and T cells.

Results: Of 280 participants motivated to discontinue snuff use, 50 were selected to treatment. After 3, 6, and 12 months, 58%, 46%, and 30% respectively were tobacco abstinent. Compliance was confirmed by measuring cotinine and carbon monoxide (CO) levels. Four subjects were still on NRT after 12 months, but tobacco-free since baseline. Twenty subjects abstinent after 6 months had a second biopsy from the site of snuff application. Of these, 40% showed remaining clinical lesions, the most significant of which were seen in the 75% of subjects still using NRT. The histomorphological picture was dominated by reductions in epithelial thickness, keratinization, and inflammatory response after tobacco cessation, although 30% of subjects showed increased epithelial thickness and 35% had increased or constant inflammatory reaction. A shift from ortho- to parakeratinization was noted in 80% of lesions. Of 30 individuals who used lozenges for 6 months, 8 presenting lesions had a significantly higher nicotine exposure ($p < 0.05$) than those without lesions. All lesions appeared between 1 and 6 weeks after treatment began. After 3 months of NRT, all lesions had resolved but one, which was healed at the 6-month control. In the mitogen (concanavallin A) driven *in vitro* model using rat oral epithelium cells with accessory Langerhans cells (LC), T cells incubated with various concentrations of extract of Swedish moist snuff (SS) showed a significant inhibition of cell proliferation at 12.5% ($p < 0.05$), and a concentration of 4% reduced T cell proliferation by 50%. Alkaloids and TSNA in concentrations similar to those in SS had no significant effect on cell proliferation. No mitogenic capacity was detected in the SS extract, alkaloids, or TSNA, although *N*'-nitrososornicotine (NNN) showed a tendency to be stimulatory in an *in vitro* assay with T-cells and rat oral epithelial cells.

Conclusion: Snuff cessation with NRT is a promising way to achieve a tobacco-free state. Compliance to treatment was high regardless of outcome, although almost all subjects gained weight, which correlated with a significant increase both in diastolic blood pressure in the success group and in total cholesterol values. Tissue samples from those with extensive exposure to snuff who were still using NRT on a daily basis 6 months after cessation were neither clinically nor histomorphologically completely normal. SS extract can evoke an immunosuppressive effect on T-cell proliferation using cells from oral epithelium as accessory cells. This effect was more pronounced when the complete SS extract was employed compared to when single components were used. These findings may indicate a local immunosuppressive effect of ST on the oral mucosa. Daily repeated sublingual exposure to nicotine for 3 months appears to be a safe form of administration with mild and transient effects in individuals devoid of clinical lesions.

Keywords: Cessation, nicotine replacement, oral mucosa, smokeless tobacco, snuff, reversibility, immunotoxicity, Langerhans cells, rat oral epithelium, nicotine.

List of original papers

This thesis is based on the following original publications, which will be referred to by their Roman numerals.

- I. Bengt Hasséus, Mats Wallström, Bengt-G. Österdahl, Jan-M. Hirsch, Mats Jontell.**
Immunotoxic effects of smokeless tobacco on the accessory cell function of rat oral epithelium.
Eur J Oral Sci. 1997 Feb;105(1):45-51.
- II. Mats Wallström, Lars Sand, Fredrik Nilsson, Jan-M Hirsch.**
The long-term effect of nicotine on the oral mucosa.
Addiction. 1999 Mar;94(3):417-23.
- III Mats Wallström, Gunilla Bolinder, Bengt Hasséus, Jan-M Hirsch**
A cessation program for snuff dippers with long and extensive exposure to Swedish moist snuff. A 1-year follow-up study.
Acta Odontol Scand. 2010 Sep10. [Epub ahead of print]
- IV. Mats Wallström, Magnus Kjelsberg, Anne Christine Johannessen, Jan-M Hirsch.**
The reversibility of the snuff-induced lesion – a clinical and histomorphological study.
(Submitted for publication September 2010.)

Other publications not included in this study

Mats Jontell, S Watts, Mats Wallström, L Levin, Kent Sloberg

Human papilloma virus in erosive oral lichen planus.

J Oral Pathol Med. 1990 Jul;19(6):273-7.

Ali M Idris, Salah O Ibrahim, Endre N Vasstrand, Anne Christine Johannessen, Johan R Lillehaug, Bengt Magnusson, Mats Wallström, Jan-M Hirsch, Rune Nilsen.

The Swedish snus and the Sudanese toombak: are they different?

Oral Oncol. 1998 Nov;34(6):558-66.

Lars Sand, Mats Wallström, Jamshid Jalouli, PA Larsson, Jan-M Hirsch.

Epstein-Barr virus and human papillomavirus in snuff-induced lesions of the oral mucosa.

Acta Otolaryngol. 2000 Oct;120(7):880-4.

Mats Wallström, Fredrik Nilsson, Jan-M Hirsch.

A randomized, double-blind, placebo-controlled clinical evaluation of a nicotine sublingual tablet in smoking cessation.

Addiction. 2000 Aug;95(8):1161-71.

Lars Sand, M Hilliges, PA Larsson, Mats Wallström, Jan-M Hirsch.

Effects of long-term administration of cancer-promoting substances on oral subepithelial mast cells in the rat.

Anticancer Res. 2002 Sep-Oct;22(5):2623-7.

Jenny Gustafson, Christina Eklund , Mats Wallström, Göran Zellin, Bengt Magnusson, Bengt Hasséus.

Langerin-expressing and CD83-expressing cells in oral lichen planus lesions.

Acta Odontol Scand. 2007 Jun;65(3):156-61.

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Abbreviations

ANA	anabasine
CO	carbon monoxide
Con A	concanavallin A
EC	epithelial cells
FTND	Fagerströms Tolerance Test for Nicotine Dependence
FTQ	Fagerströms Tolerance Questionnaire
Hrs	hours
IARC	International Agency for Research on Cancer
IC ₅₀	inhibitory concentration
LC	Langerhans cells
NAB	N'-nitrosoanabasine
NDMA	N-nitrosodimethylamine
NHANES	National Health and Nutrition Examination Survey
NIC	nicotine
NK cells	natural killer cells
NNK	4-(N-nitrosomethylamino-1-[3-pyridyl]-1-butanone
NNN	N'-nitrosonornicotine
NRT	nicotine replacement therapy
SS	Swedish moist snuff
ST	smokeless tobacco
SD	standard deviation
TSNA	tobacco-specific nitrosamines
Yrs	years

1 Introduction

These investigations are part of a research program initiated by the late Dr Per-Åke Henrikson at the Department of Oral & Maxillofacial Surgery, University of Gothenburg, Sweden, during the early 1970s and constitute a continuation of a number of studies investigating issues related to snuff consumption.

Unburned, or smokeless, tobacco (ST) is produced and sold worldwide and consumed by hundreds of millions of people (1). Moist snuff, an ST product sold mainly in the USA and the Scandinavian countries (2), is a mix of finely ground tobacco, water, and flavoring, which is sold either “loose” or in pouches similar to small teabags. A “pinch” of loose snuff or a pouch is usually placed between the upper or lower lip and gum. The amount of snuff consumed and daily exposure time varies (3-6). Continuous exposure to high levels of nicotine is addictive (7) and contributes to a wide range of pharmacological effects on visceral and circulatory functions. Besides nicotine, the snuff dipper is exposed to more than 3000 chemicals in snuff, including carcinogenic tobacco specific nitrosamines (TSNAs) (8, 9). The use of snuff results in local and generalized toxicological reactions as well as increased risk for oral or pancreatic tumor development, cardiovascular disease, and diabetes(4, 10-17).

Various toxicological reactions in the oral mucosa have been related to the use of ST (1) and toxicological reactions equivalent to those seen in humans have also been observed following studies in laboratory animals (18-22). *In vitro* investigations have shown that ST extracts have an immune-stimulating potential (23), but that, conversely, they also have a number of general negative effects on the immune system (24, 25). It is reasonable to assume that the immune system of the oral mucosa at the site of ST exposure could be affected, so it is also of interest to elucidate some of the most potentially harmful chemicals found in ST.

Nicotine replacement therapy (NRT) is widely used to aid tobacco cessation. Various forms of administration are available, all of which involve absorption of nicotine through either the mucosal membrane or the skin (26). There is little published information regarding the direct effect of this extensive exposure to nicotine on the oral mucosa (27, 28). It would therefore be valuable to evaluate the long-term effects of locally applied nicotine on the oral mucous membrane.

The negative health consequences of moist snuff use and its relatively high prevalence, especially among adolescents and young adults (29-31), make it necessary to promote snuff cessation. There are few specially designed cessation programs available to ST users (32, 33), and NRT is especially uncommon for this purpose (34-37).

The assistance of NRT has increased the success rates for educational and behavioral methods of smoking cessation significantly (38, 39). ST cessation without NRT or with NRT in low doses has been reported, but with significantly lower rates of success (35, 40). It is therefore important to explore further opportunities for achieving good results in snuff cessation in a highly nicotine-dependent group of users.

The clinical and histomorphological picture of snuff dippers' lesions among Scandinavian snuff dippers has previously been reported (4, 11, 41-43). Habitual snuff exposure induces these typical oral lesions, which have generally been considered reversible (44). However, only a few clinical studies and one animal study have reported on the appearance of the snuff lesion before and after cessation (20, 45, 46).

It is still an open question to what extent snuff-induced lesions in the oral mucosa are reversible. In the light of a possible malignant cell transformation later in life, apparent in available reports (17, 47), this is an important issue that needs attention.

1.1. Smokeless tobacco and snuff habits

Americans Indians were probably the first people to use snuff and to chew or smoke tobacco (48). Tobacco was used in those cultures for several reasons including medical treatment, prevention of fatigue and hunger on long distance treks, and various ritual and ceremonial uses.

During the 16th century the use of tobacco spread all over Europe. The French ambassador Jean Nicot introduced snuff in 1560 to the French Royal Court to cure Queen Catherine de Medici's severe migraine, recommending her to inhale particulate tobacco nasally. The botanical name derived from his surname was established when Carl von Linné named the plant *Nicotiana Tabacum* in his system of plant classification in 1753 (48). The word "tobacco", from the Spanish "tobaco," derives from an Arawak language word for a roll of tobacco leaves or the tube or pipe in which the plant was smoked, while the name in the Caribbean for the plant itself was "petun." Locally, however, in parts of Mexico, the plant was also referred to as "tabac." In 1828 the active ingredient of tobacco was isolated and called nicotine (48).

The use of snuff also spread throughout Africa, Japan, and China, where it was fashionable among the Ching Dynasty. The Chinese believed that snuff cured toothache, provoked sweating, and alleviated constipation. The use of snuff by European royalty during the 16th, 17th, and 18th centuries gave respectability to the habit and increased its popularity.

In many Swedish cities, snuff has been manufactured since the beginning of the 18th century, but in Gothenburg production started in the early 1650s (49). In 1915 the production of snuff in Sweden was taken over by the Swedish Tobacco Monopoly and was gradually redirected to Gothenburg. In the 1960s the tobacco monopoly was abolished and the company converted to Svenska Tobaks AB and later, in 1992, to Swedish Match AB.

ST is the collective name for tobacco that is consumed unburned, either orally or nasally. Although it is banned by governmental regulation in some countries, ST for oral use is manufactured and consumed on all continents (1, 50, 51) under various names including betel-quid, chimo, gudhaku, gutkha, gul, iq'milk, khiwam, kahaini, maras, maras powder, mishri, nass, naswar, plug, shamma, toombaak, moist snuff, snus,

or some other variant depending upon the locale (1). Though the names differ, however, the product is used similarly throughout the world, either tucked as a small portion or “quid” in the mouth or chewed or sucked for a certain period of time (52).

Moist snuff is the most popular form of orally used smokeless tobacco in North America and parts of Europe, particularly the Scandinavian countries (53). Earlier data on the prevalence of daily snuff use in Sweden varies from 7% of men over 45 years of age in the southern part of the country (54), to 24% of the male population and 5% of the female population in central Sweden, and 30% of men and 6% of women in the north (55). Gradually the number of users has increased in the southern and central regions of Sweden, regional variations have diminished, and the prevalence of daily snuff use throughout Sweden was recently reported as 19% of men and 4% of women (56).

Since 1971 an annual drug habit survey has been conducted among schoolchildren in Sweden, and it is clear that the use of snuff has varied over time. In lower secondary school children from 1983 to 2000 the prevalence of daily or occasional snuff use rose among 15-year-old boys from 16% to 25% and among 15-year-old girls from 2% to 8%; by 2009, however, it had declined to 15% and 4% respectively. Smoking has increased during the last years (30).

Data on snuff use in Norway has been collected by Statistics Norway since 1985. From 1988 to 2009 the prevalence of daily snuff use increased among men aged 16 to 74 years from 3% to 11%, and 2% of women were using snuff daily by 2009. The highest prevalence was registered in the age group 16 to 24, where 21% of men and 7% of women were daily users (31).

In the USA in 1970, smokeless tobacco use was most prevalent among adults over 65 and the dominant form was chewing tobacco. Among younger males 16 to 24 years of age, 2.2% used ST. By 1987 this had changed and 6.1% of men over 65 used ST compared with 8.9% of men aged 16 to 24. In 1995, 19.7% of males in higher education reported use of ST and 80% of those used moist snuff (57). Eaton et al. conducted a nationwide US survey and found that 8.9% of all students reported current ST use (29). The overall prevalence in males was 15%, while only 2.2% of females reported current use. The highest prevalence was documented among white males (20.1 %), followed by Hispanic (7.5 %) and black males (5.2 %) (29).

1.2 Manufacturing process, alkaloids and nitrosamines

ST is mainly produced from *Nicotiana Tabacum*, although *Nicotiana rustica* Linn is used in Turkey for the production of the ST specific to that region (58).

Moist snuff consists of 40% to 45% finely ground air- or fire-dried tobacco mixed with water (45–60%), sodium carbonate (1.5–3.5%), sodium chloride (1.5–3.5%), moisturizer (1.5–3.5%), and flavoring (<1%) (59).

The chemical composition of ST varies due to the type of tobacco used and undergoes substantial changes during, curing, processing, and storing (60).

Over the years chemical analyses performed on ST have shown it contains very large numbers of different chemicals (61, 62); Hoffmann et al. (2001) found 23 *N*-nitrosamines and 28 pesticides, which brought the number of known constituents in tobacco to a total of 3095 (9).

All ST products contain nicotine (NIC), which is highly addictive, and the speed of absorption is a major determinant of addiction (7, 63). The level of unprotonated nicotine affects the absorption rate and degree of trans-mucosal nicotine absorption, which is facilitated when the tobacco product is more alkaline (64, 65). The pH and the level of unprotonated nicotine vary among the tobacco products and snuff brands, and the ones with the highest content of unprotonated nicotine had the highest market shares.

TSNAs are widely considered to be among the most important carcinogens in ST and cigarette smoke (8, 66); about 30 carcinogens have been identified in smokeless tobacco. The high levels of TSNA observed in snuff are primarily due to their formation during curing, fermentation, and aging, but they are also produced endogenously during consumption (67) from the precursor alkaloids, nicotine, nor nicotine, anatabine, and anabasine where nicotine, nor nicotine, and anabasine are the major contributors. Hoffmann et al. provided the most comprehensive insight into the levels of major tobacco carcinogens in the leading brands of most snuff sold in the USA (68). Since the middle 1980s the concentrations of nitrosamines in some brands on the USA market and in Sweden have declined by up to 85% (69, 70), although Richter et al. (2008) found a 20-fold difference in the range of sums for total carcinogenic TSNAs (3).

1.3 General and oral toxicological effects

1.3.1 General effects

The evidence is robust for the negative health effects of smoking resulting in severe morbidity and mortality. In aggregate, one of every ten deaths worldwide is caused by tobacco (71). The evidence on ST is less extensive in terms of numbers of scientific publications. When searching the PubMed database using MESH terms combining adverse effect and smoking versus adverse effect and ST the number of hits are 27,597 and 870 respectively. Adverse effect and smoking/ST results in 294 hits. The number of review articles returned by applying the same search strategy for smoking tobacco and smokeless tobacco are 4207 and 132 respectively.

The biological effects and toxicity of ST extract have been studied using different human, as well as animal, cells. A low concentration of extract stimulates cell growth while an increase inhibits cell growth (19, 72-74). With increasing concentrations, a number of cytotoxic effects occur, such as inhibition of cell differentiation, apoptosis, DNA and protein synthesis, decreased cell adhesion, and cell membrane leakage (24, 72, 75-78). Studies *in vitro* have shown that extract of ST has an immunostimulatory potential (23), but also a depressing effect on lymphokine-activated killer-cell activity

(24). Furthermore, natural killer cells (NK-cells) in peripheral blood from rats exposed to oral snuff showed decreased activity *in vitro* (25). ST exposures have also been demonstrated to affect the immune system by reducing the number of Langerhans cells (LC) in the oral mucosa (79). These cells represent a peripheral outpost and provide the oral mucosa with an immunosurveillant competence (80). LC have the ability to internalize and process antigens, and after migration to the regional lymph nodes, the cells are able execute their function as accessory cells and present antigens to naive T cells (81). Upon stimulation, T cells expand clonally, leave the lymph nodes, and find the site of antigen entrance where these cells orchestrate the local immune response (82). It is reasonable to assume that the immune system of the oral mucosa at the site of SS exposure will be affected. Thus, investigating the *in vitro* effect of SS extract on immune cells may reveal effects that can be expected *in vivo*.

Diploid, non-tumorigenic Chinese hamster embryo fibroblasts have been transformed with aqueous tobacco extract in combination with herpes simplex virus type 1 and isolated. Transformants grew in soft agar and were tumorigenic in nude mice, and cytogenetic analysis revealed clonal chromosome abnormalities (83).

1.3.2 Oral effects

Pindborg and Renstrup (11) were among the first to describe the clinical and histomorphological picture of the snuff-induced lesion in Scandinavia. These lesions in connection with snuff use have been described both clinically and histomorphologically in a number of articles. The reactions are generally benign epithelial lesions resulting in surface cell-death keratinization without any sinister finding. The connective tissue findings are chronic inflammation and fibrosis including the minor salivary gland (4, 10, 11, 41, 42). A dose relationship between exposure and the severity of the lesions has been found (4, 84, 85).

The association between ST and gingival recession has been reported from the USA and from Sweden. Earlier data from younger individuals show the presence of gingival recession and inflammation in users of ST (86). This agrees with later descriptions of gingival recession in the area adjacent to snuff application (41, 84). However, data reported on the effect of ST on periodontal disease is conflicting. Epidemiological data from 12,932 adults in the US NHANES study showed ST users were twice as likely as non-users to have severe periodontal disease at any site (87). Three Swedish studies, however, were not able to confirm increased bone loss in Swedish snuff consumers (88-90).

ST users of chewing tobacco showed more decayed and filled tooth surfaces than non-users, probably due to the high sugar content (91). Among adolescents in Sweden tobacco users showed higher epidemiological caries data than non-tobacco users (92). From nine dental clinics within the Public Dental Service in the City of Gothenburg, 2145 patients aged 14 to 19 answered a questionnaire concerning their tobacco habits. Tobacco was used in some form by 27% of the adolescents and 9% were using snuff. The habit increased with age. All caries epidemiological data were significantly higher among patients with any tobacco habits compared to non-users. It can be concluded that there is a correlation between the tobacco habit and increased caries prevalence. However, dietary and oral habits were not examined and should be investigated before

any definite conclusions can be made regarding the effect of the tobacco habit per se on the development of caries.

1.3.3 Smokeless tobacco use and cancer

The International Agency for Research on Cancer (IARC) (93), in their monograph *Tobacco habits other than smoking; betel-quid and areca-nut chewing; and some related nitrosamines*, concluded “there is sufficient evidence that the use of smokeless tobacco can cause oral cancer in humans and that chewing tobacco may increase the risk for oral cancer development.” The issue was reviewed again in another IARC monograph (1), and the conclusion was that several studies in a number of countries have identified the use of smokeless tobacco as a cause of oral cancer. The working group stated that “there is sufficient evidence in humans to establish smokeless tobacco as carcinogenic, i.e. smokeless tobacco causes cancer of the oral cavity and pancreas.” For esophageal and pancreatic cancer, IARC also reported a positive association with ST.

In Sweden the amount of TSNA in snuff has been reduced compared with many of the commercial oral snuff brands found in other areas (70). This is due to an improvement in its production, including a shift to anaerobic fermentation among other things. Since a high proportion of the male population in Sweden (20%) (56) uses snuff regularly, studies regarding its cancer risks are urgently needed. Three Swedish-based case controlled studies (47, 94, 95) on oral snuff found no significant association between snuff use and the risk for head and neck cancers. In one of the articles, however (47), a nearly fivefold elevated risk for head and neck cancer was reported in the subgroup of men with snuff use and no history of smoking, and in both studies noted in the IARC analysis (1), a borderline statistically significant increase was found for the risk for oral cancer among former snuff users. A stronger statistically significant association between Swedish snuff use and oral cancer was found in a population-based survey of 9976 men, and the authors concluded that snuff-related risks for oral cancer should not be dismissed lightly (17).

Hirsch et al. reported eight clinically and histopathologically well-documented cases of oral cancer in Swedish males at the site of snuff application (96). Well-documented data have now been collected from 12 such patients with a mean age of 70 years, who had used snuff for a mean of 46 years at the time of cancer diagnosis (in manuscript 2010).

Studies and data on moist snuff and cancers other than oral and pharyngeal cancers have also been evaluated by IARC in a monograph that concludes that “there is sufficient evidence in humans for the carcinogenicity of smokeless tobacco” (1). Smokeless tobacco has been shown to cause cancers in the pancreas as well as in the oral cavity. The IARC conclusion regarding pancreatic cancers was in part based on a prospective cohort study from Norway where a significantly increased risk was found in the group of ever-users compared with non-users of snuff (16). This finding was confirmed two years later in a Swedish retrospective cohort study (97). The Norwegian study also reported modest but non-significant increases in risks for oral and pharyngeal, esophageal, and stomach cancers (16). In addition, they found sufficient evidence from studies conducted in laboratory animals to show the carcinogenic potential of moist snuff (1).

1.3.4 Smokeless tobacco and cardiovascular effects

The evidence of the risk for cardiovascular disease from smokeless tobacco is limited. Clinical epidemiological studies in humans have focused on cardiovascular disease including myocardial infarction (fatal and nonfatal), atherosclerosis, stroke, hypertension, and metabolic effects, and four cohort studies (13, 98, 99) have found a significantly increased risk for mortality from all cardiovascular disease and from myocardial infarction and stroke. A systemic review with meta-analysis confirmed an association between the use of ST and risk of fatal myocardial infarction and stroke (100).

While case-controlled studies of smokeless tobacco show no significantly increased risk of cardiovascular disease (101-103), there is an inconsistency in the evidence. Hypertension is a major risk factor for the development of cardiovascular disease and a large Swedish cross-sectional study reported a higher prevalence of hypertension among snuff users than among subjects who had never used tobacco (104). In a prospective study of long-term use of smokeless tobacco, snuff users showed a moderately increased risk to develop hypertension compared to non-users (105). However, a Swedish 10-year follow-up prospective study investigating the association between snuff use and components of metabolic syndrome found that high consumption of snuff (>4 cans/week) significantly increased the risk for obesity and triglycerides, but not for hypertension (106).

Due to the high background rates of cardiovascular disease, even a small increase in risk that is clearly possible could represent a large public health impact in countries that have a high prevalence of smokeless tobacco use (1).

1.3.5 Smokeless tobacco and diabetes

In a Swedish study the associations between cigarette smoking and use of oral moist snuff and impaired glucose tolerance and type 2 diabetes was investigated (107). The results indicated that users of moist snuff with a consumption of ≥ 3 cans per week have study for heavy smokers with a consumption of more than 25 cigarettes per day. Another study from northern Sweden, however, found no increased risk of type 2 diabetes and impaired glucose tolerance among current or former snuff users compared with never users (108).

Henley et al. concluded in two large studies among men in the US that there is no association between the use of spit tobacco (snuff or chewing tobacco) and mortality from diabetes, but there is a significantly increased risk for mortality from cardiovascular disease in current ST users (98).

1.3.6 Smokeless tobacco, pregnancy, and fertility

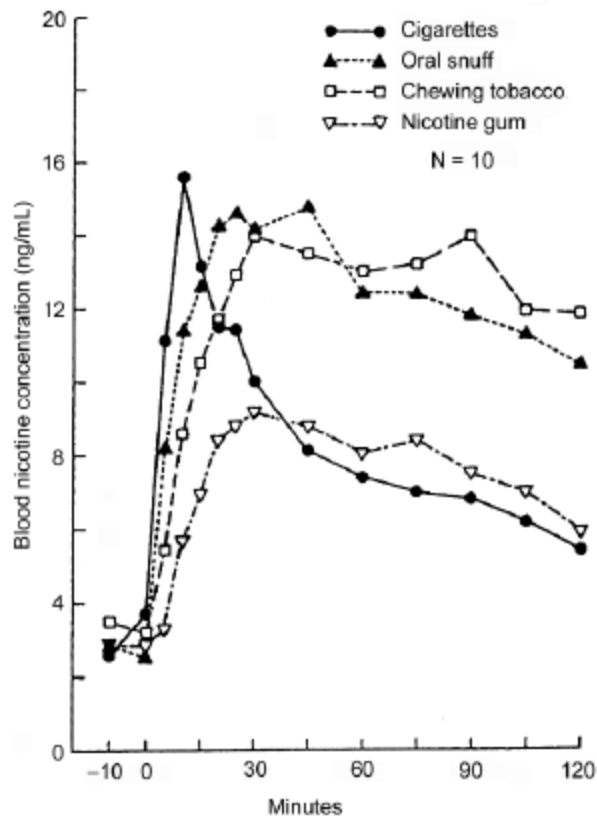
The Council of Scientific Affairs of the American Medical Association reviewed the health effects of smokeless tobacco and confirmed that ST adversely affects pregnancy outcome (109). The pregnancy outcomes of birth weight, pre-term delivery, and pre-eclampsia were evaluated in Swedish women using snuff during early pregnancy compared to non-tobacco users (110). Both snuff use and smoking were associated with preterm delivery, with a twofold risk for snuff use and a 60% increased risk for

smokers. Snuff users also showed a nearly 60% increased risk for preeclampsia. A large population-based cohort study investigated the association between use of Swedish snuff during pregnancy and risk of stillbirth in 7629 pregnant women. It was concluded that the use of snuff during pregnancy was associated with a high risk of stillbirth, but the mechanisms of the association differ from those understood for smoking during pregnancy. Light smoking was associated with the same risks as snuff use, while heavy smoking (i.e. more than 10 cigarettes daily) markedly increased the risk of stillbirth (111).

1.4 Nicotine addiction

Dependency on snuff is a result of the psychoactive effect of nicotine combined with learned or conditioned factors, genetics, and social and environmental factors (including tobacco product design and marketing). Stimulation of nicotinic cholinergic receptors releases a variety of neurotransmitters in the brain. One of them, dopamine, signals a pleasurable experience and is critical for the reinforcing effects (effects that promote self-administration) of nicotine and other drugs of abuse (for review see Benowitz (112)). A withdrawal syndrome associated with smokeless tobacco use has been identified that is qualitatively similar to that from cigarette smoking (113).

In a study of 10 volunteers, Benowitz et al. measured plasma nicotine levels after the use of a single dose of moist snuff, chewing tobacco, smoking, and nicotine chewing gum. The levels were approximately the same for all products except for the nicotine gum, which delivered less nicotine. The amount of nicotine absorbed for snuff and chewing tobacco was twice as much that absorbed by smoking due to the prolonged exposure to those oral products (Fig 1) (114). The slower uptake of nicotine from ST products had been interpreted to mean that non-smoked tobacco would not be as addictive as smoked tobacco because it delivers less immediate and frequent reinforcement of nicotine (63). In a review article Tomar et al. concluded that pH is a major determinant of the speed with which nicotine absorption occurs in oral use of smokeless tobacco (64). This was confirmed in a study on the pharmacokinetics and pharmacodynamics of different brands of moist snuff tested in men (115). That study also found that snuff products are capable of rapidly delivering high doses of nicotine and that nicotine plasma levels in snuff users are as high as concentrations observed in smokers.



Adapted from Benowitz *et al.* (1988)

Fig 1. Blood nicotine concentrations during and after smoking cigarettes (average, 1 and 1/3 cigarettes), use of oral snuff (2.5g), use of chewing tobacco (7.9g) and chewing nicotine gum (two 2-mg pieces). Benowitz *et al.* 1988.

Nicotine has a half-life of about two hours, and its main metabolite is cotinine. Cotinine, with a half life of 18 to 20 hours, is used as an indicator of nicotine uptake (116).

Many snuff users swallow the juice, and it is possible that the some of the nicotine absorption occurs in the intestine. This may result in higher serum cotinine levels (117, 118). The metabolism of nicotine occurs in a two-phase process described by Yildiz in a review article (119). Nicotine is oxidized to cotinine in the liver and most of the cotinine is further metabolized before being excreted. Some metabolism may take place in other organs such as the brain or lungs and may play a role in susceptibility to nicotine consumption (120)

1.5 Prevention

The onset of ST use usually occurs during adolescence and young adulthood, and the prevalence is much higher in males than in females (2, 121). Primary prevention has no

one magic key; different strategies must be combined to be effective. The school is an important arena for prevention because of its access to almost all children. Many school-based education campaigns aimed at preventing children from starting smoking have been studied, mostly in North America, but the studies have shown mixed results. No evidence exists that information campaigns alone are effective, but where educational campaigns also train young people to resist the social influences and peer pressure that encourage them to smoke, they can be effective (122). Comprehensive strategies that use several components have generally been found more effective than information-based interventions, which alone have shown limited or no effect (123, 124).

A recent review of both reviews and meta-analyses has suggested that school-based smoking prevention programs can have significant long-term effects if they are interactive programs targeting social influences or social skills, if they involve at least 15 sessions including students in the 9th grade, and if they show substantial short-term effects (125). A Swedish intervention study showed effects of such programs in smokers, but no significant effects in snuff users (126). These results could reflect the fact that the focus of the study was on preventing smoking and cancer, not on preventing the use of snuff. Tobacco education must be imparted through schools, existing government health programs, and hospital outreach programs (126). The WHO has implemented six prevention strategies in their MPOWER package including: Monitor tobacco use and prevention policies, protect people from tobacco smoke, offer help to quit tobacco use, warn about the dangers of tobacco, enforce bans on tobacco advertising, promotion and sponsorship, and raise taxes on tobacco (127).

1.6 Nicotine withdrawal symptoms, NRT, and intervention

Nicotine has a key role in explaining why people are addicted to snuff, because it can act as a primary reinforcer, a stimulant, a euphoriant, and an anxiety-reducing drug (128). Nicotine is metabolized with a half-life for elimination of two hours. If the blood nicotine level falls below an individual threshold level, the tobacco user may experience withdrawal symptoms. Through frequent ST intake the snuffer can regulate the intake of nicotine and maintain a sufficient blood level to cope with the withdrawal symptoms. ST users may continue their habit in order to achieve the positive effect of nicotine or to avoid the withdrawal symptoms (negative reinforcement), (129). The American Psychiatric Association has listed Nicotine Withdrawal Disorders (130) as dysphoric or depressed mood; insomnia; irritability; frustration or anger; anxiety; difficulties concentrating; restlessness; decreased heart rate; increased appetite, and weight gain. Craving or a strong urge to smoke or to use ST is considered a withdrawal symptom by some authors (131).

Different treatment interventions include hypnosis, acupuncture, behavior modification, antidepressant medication as a smoking cessation aid, and NRT. The latter was introduced in the early 1970s to help smokers succeed in quitting. Nicotine was bound to an ion exchange resin and made into a chewing gum (132) containing 1, 2, or 4 mg of

nicotine perpiece. To increase absorption of the nicotine, the gum was buffered with a small amount of alkali.

Successful smoking cessation therapy involves both behavioral and pharmacological treatments (133). A Cochrane systematic review concluded that all kinds of NRT can be helpful to people quitting regardless of setting (39).

Although numerous studies have focused on smoking, relatively few have considered intervention for the cessation of ST use. Earlier studies showed very disappointing results for ST cessation compared to smoking cessation (34, 134, 135). When chronic smokeless users attempt to quit, they are frequently unsuccessful, with between 75% and 100% of subjects in control groups relapsing within 3 to 12 months (36). More promising is a meta-analysis of 14 randomized controlled trials of ST cessation interventions, 8 on behavioral interventions and 6 on pharmacological interventions (136). The overall conclusion was that behavioral therapies, especially when involving an oral examination, are the most effective interventions, and NRT may also be effective.

1.7 Harm reduction

The concept of harm reduction was introduced by Henningsfield in the mid-1990s as a relatively new topic in strategic discussions on tobacco control and smoking (for review see Nordgren (137)). Harm reduction has since been the focus of much work in the scientific world, especially concerning the question of whether or not smokeless tobacco can be used as a gateway away from smoking tobacco. As defined by the Institute of Medicine report, *Clearing the Smoke, Assessing the Science Base for Tobacco Harm Reduction*, “a product is harm-reducing if it lowers total tobacco-related mortality and morbidity even though use of that product may involve continued exposure to tobacco related toxicants” (138).

Different authors have proposed snuff as a means of harm reduction (55, 139-142). Advocates of such strategies argue that the public health burden of smoking could be substantially reduced if a sufficient number of smokers made the change to less hazardous products. Several studies, however, neither support the concept nor show evidence of the health effects supposed to be gained from decreasing delivery levels of toxicity by switching from cigarette smoking to ST use (2, 54, 143-145). It can be concluded that all of these articles focus on the primary objective of reducing death and disease, but the gateways differ from study to study.

The health service focuses on problems related to our patients' health and to their quality of life i.e. primary prevention. The areas focused on in order to promote well-being are well known. The therapist often meets “healthy” tobacco users, and it has been established that repeated counseling in connection with visits to the health service has an effect on tobacco consumption. The initial information and discussion often results in reduced consumption of tobacco products, but rarely in total abstinence. The

result is associated usually with the time the therapist invests with the patient and whether there is a specific program for the patient to follow (146) .

A different problem arises when the patient is referred to the hospital for treatment of a disease and the therapist is faced with the sick tobacco user. The problem then is often that the tobacco use is a negative prognostic factor for treatment outcome that makes reconstructive surgery hazardous and radiotherapy associated with more negative reactions in both soft and hard tissues. The challenge here is to reduce tobacco exposure as quickly as possible, and solutions to this challenge have not been extensively investigated (147-149).

2. Aims of the study

The aims of the overall study comprising the four studies reported in this dissertation were to evaluate the effects of commercial Swedish moist snuff, nicotine, and its derivatives on the functional capacity of rat oral epithelial cells and T-cells *in vitro* (Study I) and on the oral mucosa (Study II); to develop and evaluate a snuff dipping cessation program (Study III); and to evaluate the reversibility of snuff-induced lesions after six months of avoidance of exposure to moist snuff (Study IV).

3. Study Design

In an *in vitro* model (150) we investigate the functional assay of the immunocompetent cells of the oral mucosa exposed to Swedish moist snuff extract, alkaloids, and nitrosamines (Study I).

Three of the studies (Studies II, III, and IV) were prospective open non-randomized intervention trials including baseline oral examination and tissue biopsy from the affected area, minor physical examination, short cessation advice, NRT recommendations, and five follow-up visits within 12 months.

4. Materials and methods

4.1 Samples

4.1.1 Animals (Study I)

In the experiments with epithelial cells and T cells, 15 to 30 inbred 8–10-week-old Lewis' rats were used. Experiments with spleen cells were included to obtain suitable concentration ranges for the different materials used in the study. These experiments required the spleens of 1 to 5 rats, sacrificed by exposure to saturated CO₂ and cervical dislocation. Cervical or mesenteric lymph nodes were dissected and pooled and spleens were removed by blunt dissection after incision through the ventral abdomen. Oral mucosal membranes were obtained by dissecting the buccal mucosa from the underlying musculature.

4.1.2 Human subjects (Studies II, III, and IV)

In the study of the effects of nicotine on the sublingual mucosa (Study II) 30 healthy subjects, aged 20 years or older, fulfilling the inclusion criterion of smoking at least 10 cigarettes per day for the last 3 years, were recruited from a waiting list at the Smoking Cessation Clinic, Sahlgrenska University Hospital, Gothenburg. The 30 subjects were 12 males with a mean age of 45.2 years (range 29.3–62.4) and 18 females with a mean age of 39.4 years (range 25.8–50.6).

Subjects for the studies on smokeless tobacco cessation (Study III) and reversibility of snuff-induced lesions (Study IV) were recruited from a health survey, tobacco cessation waiting list, and advertising in a local morning newspaper. Out of 280 responders, who were screened over the telephone, the first 50 subjects (48 male and 2 female) to fulfill the inclusion criteria of daily snuff use of more than 2 cans/week (>100 g snuff) for 10 years or longer, agreed to a biopsy, and motivation to give up snus use through a 12-month clinical survey were entered in to the study. Their mean age was 42.2 years (SD \pm 10.7). All the subjects used loose moist snuff.

4.2 Smokeless tobacco, alkaloids, and TSNA (Studies I, II, III, and IV)

In the *in vitro* experiment (Study I) commercially available Swedish moist snuff (SS) ("Röda Lacket" brand, Svenska Tobaksbolaget AB, Gothenburg, Sweden) was used and SS extract was prepared as described earlier (151). Briefly, SS was dissolved in a cell culture medium and incubated at 37°C for 1 hr. The solution was centrifuged to remove undissolved tobacco. Alkaloids and nitrosamines present in SS include ANA (100µg/g), NAB (0,20 mg/kg), nicotine (NIC; 10 mg/g), NNN (2.5 mg/kg), NNK (0.80 mg/kg), and NDMA (0.001 mg/kg) (152-154). The alkaloids and TSNA are commercially available. To obtain concentrations of alkaloids and TSNA which correspond to those

found in SS, NAB, NNN, NNK, ANA, NDMA, and NIC were diluted in cell culture medium.

A dissolvable tablet containing 2 mg of nicotine bound to β -cyclodextrin was used to study the effect on the sublingual mucosa (Study II).

Only loose moist snuff was used by the subjects in studies III and IV.

4.3 Functional assays (Study I).

Spleen cells: In toxicity experiments, spleen cells were transferred to culture plates containing a cell culture medium. SS-extract (0.2%, 0.8%, 3%, 12.5%, and 50% concentrations), alkaloids, or TSNA were added to each culture well. To each well a mitogen con A was added. Following 48 hr of incubation, titrated thymidine was added to each well. After an additional 24 hr, the cells were harvested onto fiberglass filters and radioactivity was counted in a liquid scintillator. All experiments were repeated at least three times.

In pre-incubation experiments, the spleen cells were pretreated for 4 hr at +37 °C with SS-extract, alkaloids, or TSNA dissolved in a cell culture medium at concentrations given above. **Epithelial cells and T cells:** In toxicity experiments, epithelial and T cells were transferred to culture plates. SS-extract, alkaloids, or TSNA (concentrations in culture wells corresponding to concentrations in SS) were added to each well. Con A was added and incubations and cell harvesting were performed according to the protocol described for spleen cells.

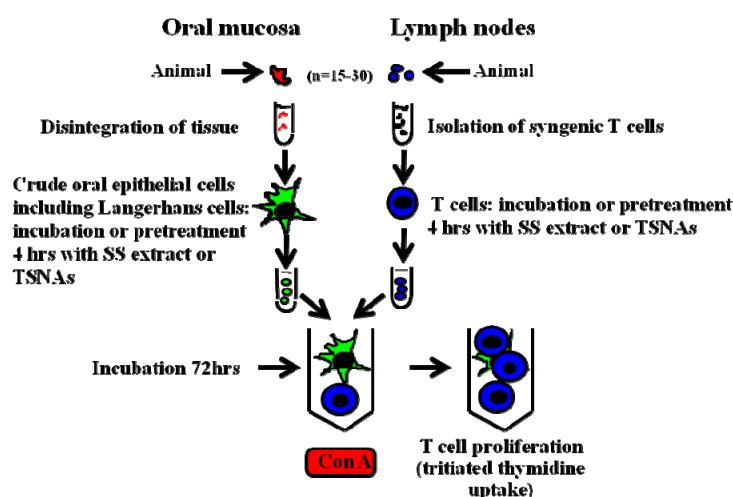


Fig. 2. Experimental design in study I assessing the effect of SS-extract and TSNA on the functional capacity of rat oral epithelial cells and T cells.

In preincubation experiments, the epithelial cells and the T cells were pretreated for 4 hr at +37°C with the various concentrations of SS-extract, alkaloids, or TSNA. After repeated washes, cells were transferred to culture plates and incubated in a cross-over fashion: pretreated epithelial cells (EC) and untreated T cells; untreated EC and pretreated T cells; untreated EC (2×10^4 cells/well) and untreated T cells (2.5×10^5 cells/well). Con A was added and subsequent incubations and cell harvests were performed as described above (Fig 2).

4.4. Histological analysis (Studies II and IV)

Both studies involved histomorphological analysis.

In the study of the long-term effect of nicotine on the sublingual mucosa (Study II) an optional biopsy was obtained at the 6-month visit from those subjects who agreed, regardless of outcome. The biopsy, 3 mm in diameter, was taken from the site of application of the nicotine tablet under local anesthesia (Xylocaine-adrenaline 20 mg/ml + 12.5 µg epinephrine, Astra, Sweden). The specimens were fixed in a 4% solution of formaldehyde and transferred for routine analysis.

To evaluate the reversibility of snuff-induced lesions in subjects who refrain from ST (Study IV), two biopsies were obtained on two different occasions, one at baseline and a second after 6 months of abstinence, from the same mucosal area. A 10 × 5 mm mucosal excision was performed under local anesthesia (1.0 cc lidocaine with adrenalin 12.5 µg/ml; Dentsply Ltd, Sweden). The biopsied tissue was immediately placed in chilled Histocon (Histo-Lab, Gothenburg, Sweden). Within 24 hours the specimen was embedded with Tissuetek OCT compound (Tissuetek; Elkhart, IN, USA) on a piece of cork and frozen using isopentane chilled to -140°C with liquid nitrogen (155). The tissue was stored at -80°C until sectioned (5 µm), and stained with hematoxylin and eosin. The thickness of the epithelium and the keratinized surface layer was measured at the site of the rete pegs and the calculation was performed using Bioquant II Digital Morphometry.

4.5 Cessation program (Study III)

A total of 6 visits were scheduled over one year. At first visit (baseline) medical and tobacco histories were obtained, height and weight were recorded, and blood pressure and heart rate were measured after 5 minutes rest with the subject in a supine position. Subjects were permitted to continue ongoing medications. A 20 × 10 mm biopsy (Fig 3) was obtained from the oral snuff lesion and sent for routine examination. A venous blood sample was taken for serum cholesterol analysis. The day of the first visit was also the quit day and all subjects were recommended 4 mg nicotine chewing gum regardless of ST dependence, for a maximum of 3 months.



Fig 3. Biopsy from snuff lesion

This was followed by an individualized tapering period for 3 months when recommended up to the 6-month follow-up visit. Subjects were instructed to chew the gum until a slight tingling sensation was felt in the throat and then to place the gum under the upper lip where the snuff quid normally was usually placed. Withdrawal symptoms were elicited through open questions at each visit and recorded (130).

Follow-up visits were made at 2 and 6 weeks, and at 3, 6, and 12 months; at all visits body weight was checked, oral mucosa was clinically examined, especially at the former site of application, and pathological changes were recorded and photo-documented. We used a single cessation technique consisting of primary face-to-face counseling according to Kottke et al. (156) supplemented with medical examinations at five recurrent visits and oral screening. Motivational information was combined with the subjects' own inspection of the residual or former mucosal lesion. In addition at all visits the participants were reminded about the health benefits of tobacco cessation.

4.6 NRT medication (Studies II and III)

The dosage recommendation of the 2 mg nicotine tablet in Study II was based upon the subjects' nicotine dependence score according to the Fagerströms Tolerance Questionnaire (FTQ) (157). Subjects who scored $FTQ < 7$ were considered to have low dependence were recommended to take 1 tablet per hour (max 20 tabl/day). Those with high dependence ($FTQ \geq 7$), were recommended to take 2 tablets per hour (max 40 tabl/day). Subjects were instructed to place the tablet under the tongue until it dissolved.

In the cessation study (Study III) all subjects were recommended 4 mg nicotine chewing gum, 10 pieces per day for a maximum of 3 months. This was followed by an individualized tapering period of 3 months, with a 2 mg nicotine chewing gum recommended for use up to the 6-month visit.

4.7 Medical data (Study III)

Height and weight were recorded. Blood pressure and heart rate were measured after 5 minutes rest with the subject in a supine position. A venous blood sample was taken for serum cholesterol analysis. Subjects were permitted to continue ongoing medication. The medical data was collected at all visits.

4.8 Assessments

Oral mucosal rating (Studies II, III and IV)

A combination of objective and subjective measures, used only in this study, was used to produce a rating score for the site of application of nicotine. The objective part of the rating was based on the clinical type of lesion: normal mucosa, hyperplastic, hyperkeratotic, atrophic, or erosive. Subjective severity was rated as mild (no or only slight symptoms), moderate (enough discomfort to interfere with oral habits), or severe (inability to perform normal oral habits) (Fig 4).

Oral Mucosa
Examination of floor of mouth

No reaction

	Lesion	Mild	Moderate	Severe
1.	Hyperplastic			
2.	Hyperkeratotic			
3.	Atrophic			
4.	Erosive			

Area of lesion (mm²)

Fig 4. Score sheet for objective and subjective sublingual findings

Photos of the lesions and clinical records were used to classify the snuff lesions at baseline and any remaining lesions at the 6-month follow-up.

In study IV the clinical appearance of the snuff-induced lesions was graded according to Axéll et al. (158), with a grade 0 (normal mucosa) added to evaluate the reversibility after cessation of snuff-use.

Biochemical verification (Studies II and III)

The subjects' self-reported tobacco use, saliva cotinine, and expired carbon monoxide were analyzed. Random samples of 1 ml saliva were frozen at -20°C and transported to McNeil Health AB, Helsingborg, Sweden to determine cotinine levels through gas chromatography analysis. Cotinine is the major metabolite of nicotine frequently used to verify cessation compliance. At the 12-month follow-up all subjects' cotinine levels were verified. Values exceeding 15ng/ml were considered to show ongoing tobacco use (116). To verify non-smoking status, a CO analyzer (Bedfont Smokerlyzer, Technical Instruments Ltd, Kent, UK) was used at all visits. A CO level < 10 ppm was considered to indicate a non-smoker. The oral mucosa was examined to trace obvious signs of ongoing snuff use using the Axéll classification (158).

4.9 Outcome measures (Studies II and III)

Outcomes were success (continuous, self-reported complete abstinence from any tobacco use from the second visit until the endpoint at 12-month follow-up) (159) and failure (self-reported tobacco use, cotinine > 15ng/ml, exhaled CO > 10ppm, continuing use of NRT at the 12-month follow-up, or lost to follow-up).

5. Statistics and Ethics

5.1 Statistical Methods

In studies II, III, and IV parameters were evaluated using descriptive statistics. Mann-Witney U-test was used to detect differences for non-paired data and Wilcoxon signed rank test was used for paired data. For correlation analyses linear regression was used. All analyses were performed with the statistical software SPSS 12.0. A *p*-value < 0.05 was considered significant. In Study I statistical significance was calculated by analysis of variance (ANOVA) and by Fisher's Least Significant Difference (LSD) test for pairwise comparisons between groups as a posthoc test.

5.2 Ethics

All subjects in studies II, III, and IV were informed in accordance with the Declaration of Helsinki and the studies were approved by the ethics committee at the Sahlgrenska University Hospital and the Swedish Medical Product Agency. The animal study (I) was approved by the ethics committee at the Sahlgrenska University Hospital.

6. Results

6.1 Demographic data (Study II, III and IV)

The Study II sample consisted of 12 males (mean age = 45.2 yrs, range 29.3–62.4) and 18 females (mean age 39.4, range 25.8–50.6).

The Study III sample comprised 50 snuff users (mean age = 42.2 ± 10.7 yrs; mean snuff duration = 20.8 ± 8.4 yrs; mean snuff duration = 15.2 ± 2.1 hrs/day; mean nicotine dose = 280 ± 144 mg/day).

In Study IV 26 participants were abstinent at the 6-month visit. Twenty-one agreed to have a second biopsy taken from the same site as previously, but only 20 specimens were possible to analyze. There was no significant difference between the 20 patients who contributed a second analyzed biopsy sample and the 50 patients originally entered in the study with regard to age and snuff exposure: mean age (yrs \pm SD) = 40.6 ± 9.3 , mean snuff duration (yrs \pm SD) = 22.1 ± 6.2 ; mean snuff duration (hrs/day \pm SD) = 15 ± 2.5 ; mean nicotine dose (mg/day \pm SD) = 275 ± 162 .

6.2 Cessation outcome (Study II and III)

In Study II, complete abstinence was not the primary objective and was therefore not stressed. At 6 months, 13% were abstinent and at 12 months only 7% remained abstinent. All were found in the $FTQ \geq 7$ group. No subject in the $FTQ < 7$ group was abstinent after 6 weeks. When allowed occasional slips after week 2, the abstinence rate was 40% after 6 months and 27% at 12 months, all mainly in the $FTQ \geq 7$ group.

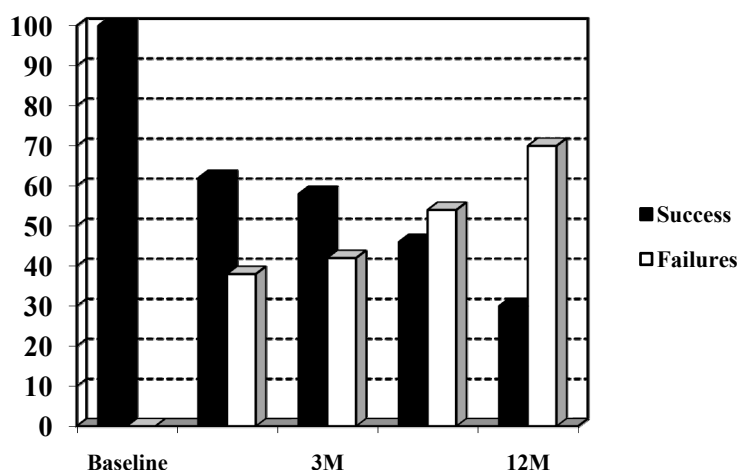


Table 1. Cessation outcome.

In Study III 15 subjects (30%) were self-reported and biochemically verified as snuff- and NRT-free at the 1-year follow-up. At the 3-, 6-, and 12-month follow-up visits 58%, 46%, and 30% respectively were tobacco abstinent (Table 1). Four subjects were still substituting NRT at the 12-month follow-up, but had been tobacco-free since baseline. According to outcome measures, these 4 subjects had a cotinine value exceeding 15ng/ml and were therefore regarded as failures. With these 4 subjects included in the success group, however, the outcome was a 38% success rate.

6.3 Reversibility of lesions in the exposed mucosa (Studies II and IV)

The floor of the mouth (Study II)

The mucosa was clinically healthy at baseline. During the study 10 lesions were registered in 8 subjects in the floor of the mouth, hyperplastic in 4 subjects and hyperkeratotic in the other 4. Atrophic mucosa was found in 2 subjects (diagnosed on 2 occasions). All lesions appeared between 1 and 6 weeks after the start of the study. At the 3-month visit all lesions had resolved but one, which had healed at the 6-month control (Table 2).

Subject no.	Weeks/ months (smoking status*)	No. of tablets mean/day	Clinical type of lesion	Severity	Adverse events**
1	2W (AB)	35	Hyperplastic	Mild	Burning/ smarting
	3W (AB)	17	Hyperplastic	Mild	—
	6W (SL)	25	Hyperplastic	Mild	—
	3M (AB)	30	Hyperplastic	Mild	—
4	6M (AB)	15	Normal	—	—
	6W (AB)	31	Hyperplastic	Mild	Burning/ smarting
5	3M (AB)	34	Normal	—	—
	2W (AB)	16	Atrophic	Moderate	Burning/ smarting
6	3W (AB)	19	Hyperkeratotic	Mild	—
	6W (AB)	19	Normal	—	—
	2W (AB)	22	Hyperkeratotic	Mild	Burning/ smarting
8	3W (AB)	38	Normal	—	Burning/ smarting
	3M (SL)	37	Hyperkeratotic	Mild	—
	6M (SM)	—	Normal	—	—
	2W (AB)	21	Hyperplastic	Mild	—
20	3W (AB)	32	Hyperplastic/ hyperkeratotic	Mild	—
	6W (AB)	37	Hyperplastic	Mild	—
	3M (AB)	34	Normal	—	—
23	1W (AB)	36	Hyperplastic	Mild	—
	2W (AB)	36	Normal	—	—
31	2W (AB)	24	Atrophic	Mild	—
	6W (AB)	19	Normal	Mild	—
31	6W (SL)	20	Hyperkeratotic	Mild	—
	3M (AB)	14	Normal	—	—

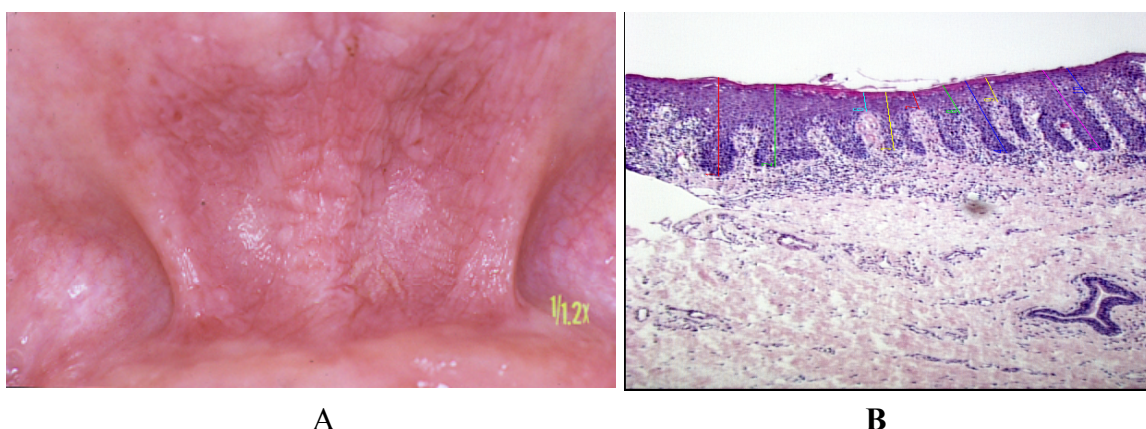
*Smoking status: AB-abstinence, SL-occasional slips, SM-smoking. ** Only AE from the area of application reported.

Table 2. Mucosal lesions registered in the floor of mouth.

The complaints were of a mild and transient character. No complaints or clinical alterations were noted at 6 months when tablet treatment stopped or at the follow-up at 12 months. There was an overall consistency between the clinical appearance of the lesions and their appearance in the photographic documentation, except for 3 occasions where photos indicated a lesion that was not clinically recorded. In these uncertain cases the primary diagnosis from the clinical control visit was considered to remain valid. At 6 months 11 subjects (36%) had an optional biopsy taken from site of application that showed normal or essentially normal mucosa.

Snuff mucosal area (Study IV)

There was a correlation between the severity of the snuff-lesion and total exposure to snuff in terms of years with the habit, daily hours of snuff consumption, and amount consumed on a daily basis when the subjects registered at baseline. The most prominent grade 4 lesions were in subjects with the most tobacco exposure (Fig 5A). After 6 months of cessation 50% of subjects with grade 4 lesions, 40% with grade 3, and 50% with grade 2 lesions at baseline showed remaining lesions at 6 months for a total of 8 out of 20 abstinent subjects (40%). Six of these 8 subjects were still using NRT on a daily basis, 3 chewing and 3 placing the gum under the lip, while 2 were nicotine-free. Two subjects still on NRT exhibited no lesions. The remaining visible clinical lesions were classified as grade 1 or 2 lesions, according to Axéll (158).



A **B**
Fig 5 Clinical picture of snuff induced lesion (A) and histomorphological picture (B)

In general, the snuff-induced oral lesions were characterized by evenly distributed, slight to moderate keratinization, an increased epithelial thickness with rete pegs, and varying degrees of stromal inflammation. At baseline, eleven biopsies showed a moderate inflammatory infiltration while nine showed a mild inflammatory reaction. At the second biopsy, one showed severe, six showed moderate, and 13 mild inflammation. After 6 months the epithelial thickness increased in 30% and decreased in 70%, with the mean thickness at baseline and at 6 months being $253.1 \pm 103.7 \mu\text{m}$ and $236 \pm 82.6 \mu\text{m}$, respectively. The total thickness of the keratin surface layer decreased significantly after cessation ($p=0.001$) (Fig 5B)

6.4 NRT use (Studies II and III)

In Study II compliance was high. The average daily dose in users varied for the subjects with a FTQ ≥ 7 during the first week from 7 to 38 tablets per day (mean 23 tablets) and for subjects with a FTQ < 7 from 3 to 17 tablets per day (mean 7 tablets). During the tapering period (3 to 6 months) medication ranged for FTQ ≥ 7 from 1 to 18 tablets per day (mean 7.9 tablets) and for FTQ < 7 from 2 to 10 tablets per day (mean 4 tablets). At 6 weeks one subject was using more than the recommended maximum dose of 40 tablets per day (44 tablets).

In Study III the compliance to pharmaceutical treatment was also high regardless of outcome. NRT was initially used as recommended by 30 subjects out of 41 attending the visit at 2 weeks (73%), as shown in Table 3. After 3 months 21 subjects out of 39 attending (54%) were using NRT. At 6 months, after the 3-month tapering period, 12 subjects out of 38 (32%) attending were still using NRT. At the end of the study 7 subjects out of 45 (16%) remaining in the study were using NRT. Of these 7 subjects 4 were tobacco-abstinent but were still regarded as failures due to their cotinine levels. Of those who were tobacco abstinent, 59% were using NRT at the 3-month visit, 35% at 6 months, and 21% at 12 months. No correlation was found between the amount of snuff use before cessation and the amount of NRT use during the study.

Visit	Attending the visit (n)	NRT use All group n (%)	Mean daily use mg/day	Range mg/day	NRT use No tobacco n (%)
Baseline	50				
2 weeks	41	30 (73)	19.0	6-60	24 (59)
6 weeks	38	25 (66)	17.9	4-60	23 (60)
3 months	39	21 (54)	11.6	2-48	17 (44)
6 months	38	12 (32)	7.3	4-40	8 (21)
12 months	45	7 (16)	16.9	2-40	4 (9)

Table 3. Pharmaceutical treatment during tapering period until endpoint.

6.5 Craving, withdrawal symptoms (Studies II and III)

In Study II the subjects reported desire/urge to smoke as the most dominant withdrawal symptom throughout the study. The mean value at baseline was 2.63 and decreased to 1.87 after 12 months. Intensity of withdrawal symptoms were reduced over time.

Withdrawal, registered as cravings for snuff, was the predominant symptom in Study III also. Cravings for snuff among the abstinent subjects decreased over time, but by the end of the study 20% in the success group still were subject to cravings.

6.6 Side effects (Studies II and III)

Twenty-nine subjects in Study II reported 121 events, 19 with very little intensity, 85 with mild, 15 with moderate, 2 with severe, and 1 with serious intensity. The serious adverse event was reported after 6 months. No subject was withdrawn from study drug due to adverse event.

In Study III the reported side effects of gum use were hiccup and gastro-intestinal symptoms.

6.7 Medical data (Study III)

Body weight and BMI

Almost all subjects gained weight during the study; the success group participants gained on average 5.1 kg (min -4 kg; max +18 kg) and the failure group 2.4 kg (min -1 kg; max +7 kg). The BMI values in the success group were significantly higher at follow-up than at baseline.

Blood pressure, heart rate, and cholesterol

The physical examination revealed normal blood pressure values (<140 mmHg systolic, < 85 mmHg diastolic) in 88% of the subjects, both at baseline and at follow-up. In 12% of the subjects borderline or light hypertensive blood pressures were observed. At follow-up there was a significant correlation ($p<0.05$) between an increase in diastolic blood pressure and weight gain in the success group, whereas no significant increase in systolic blood pressure was registered.

Heart rate decreased from a mean of 80 beats/minute at baseline to 69 beats/minute at follow-up in the success group, though the difference was not significant. This difference extrapolated to a 24-hour mean value corresponds to an average of 10,000 fewer heartbeats per day in each subject. In the failure group a decrease in heart rate was also found (3 beats/minute), but it was less pronounced and also not significant.

Total serum cholesterol was measured twice and there was a significant increase in mean cholesterol values in the success group ($p<0.05$), but not in the failure group. There was also a significant correlation between increase in body weight and cholesterol measurements in the success group ($p<0.05$) but not in the failure group.

6.8 Cytotoxic effect (Study I)

SS-extract

A significant inhibition of spleen cell proliferation ($p < 0.05$) was noted at a concentration of 0.8%. Already at a concentration of 2% proliferation was reduced by 50% (inhibitory concentration [IC_{50}] = 2%).

When epithelial cells, including LC, and T cells were incubated with the various concentrations of the SS-extract, a similar response was obtained to that noted for spleen cells. A significant inhibition of cell proliferation occurred at 12.5% ($p < 0.05$); and at a concentration of 4%, the T cell proliferation was reduced by 50% (IC_{50} = 4%).

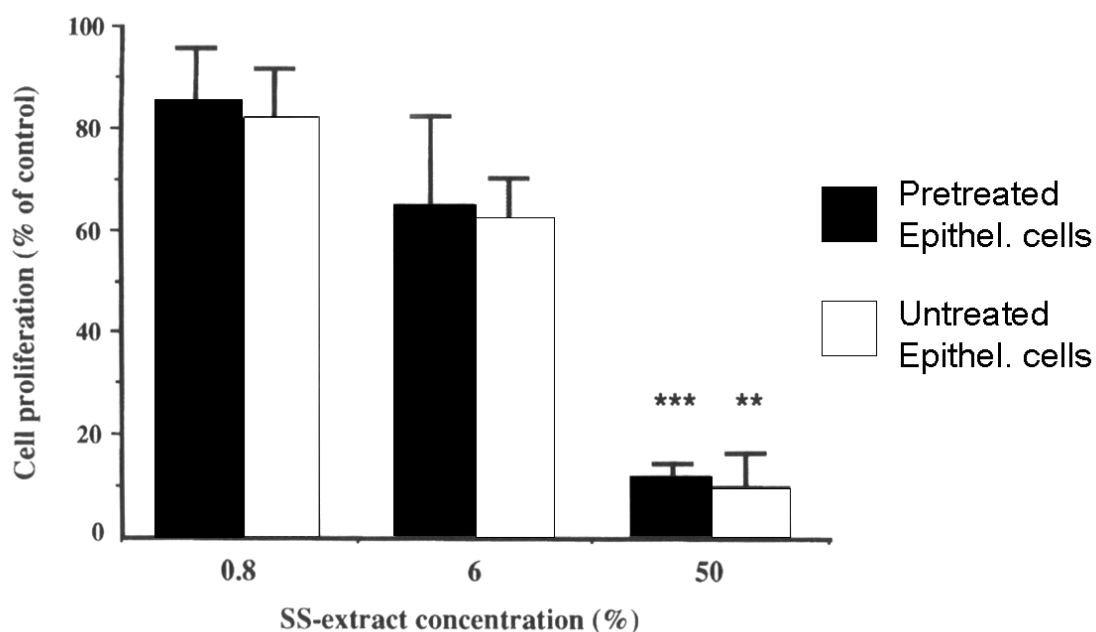


Fig 6. Pretreatment of oral and epithelial cells and T cells with SS-extract of Swedish moist snuff. Data expressed as T cell proliferation in relation to proliferation in assays with untreated cells.

Stimulation test

The ability of epithelial cells and T cells to functionally recover from SS-extract exposure was tested by pre-incubation with three different concentrations of SS-extract. The pre-incubated epithelial cells were then incubated with untreated T-cells and the pre-incubated T-cells with untreated epithelial cells. Both epithelial cells and T cells recovered from the cytotoxic effects at a concentration less than 6%. When epithelial cells and T cells were pre-incubated separately with an extract concentration of 50%, a significant permanent reduction of cell proliferation was observed compared with non-exposed cells (Fig 6).

When the various alkaloids and TSNA were present during the entire incubation of epithelial cells and T cells, no significant difference in cell proliferation was observed for any of the substances tested. A negative outcome was also revealed after pretreatment of the two cell populations (fig. 6). Although NNN showed a tendency to be stimulatory and NAB to be inhibitory, there were no significant differences between controls and pretreated cells (fig.7).

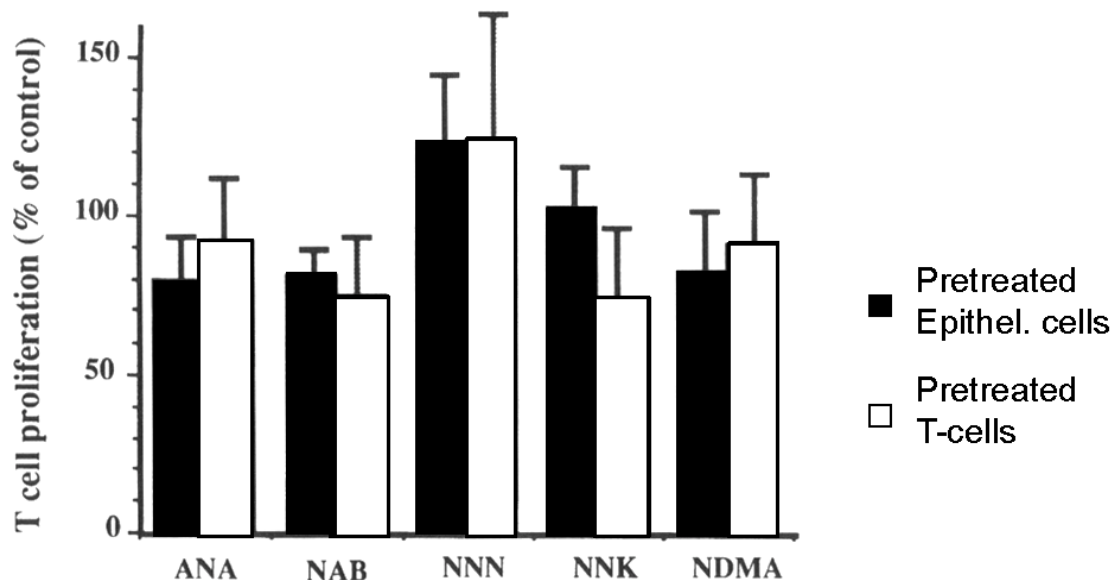


Fig 7. Pretreatment of oral epithelial cells and T cells with the TSNA's ANA, NAB, NNN, NNK and NDMA. Data expressed as T cell proliferation in relation to proliferation in assays with untreated cells.

7 General Discussion

Methodological considerations

In vitro experiments have been widely used to study the toxic effects of ST. These methods enable the study of effects in different cell types following exposure to ST.

In Study I we exposed different types of immune cells to ST extract and TSNA's. Inhibitory concentrations of the different substances in cell cultures were registered. As in all *in vitro* studies it is difficult to apply these data to *in vivo* situations, because testing the toxic effect of varying dilutions of ST extract in cell culture wells does not reflect the *in vivo* situation. However, an *in vitro* assay is a valuable way to elucidate cell response to selected tobacco chemicals or to the joint action of a complete SS, and it gives an indication of the possible effects *in vivo*.

In Study II the effect of nicotine lozenges on the sublingual mucosa was evaluated in a group of smokers. These subjects already presented an oral mucosa exposed to the effects of cigarette smoking for a long period at the site of application. Clinically the mucosa was considered healthy but we had no histological baseline data. After completion of the medication period there were no histopathological alterations in the mucosa. However, we could only obtain biopsies from 11 subjects at the 6-month follow-up because the biopsies were optional and not all subjects consented. The clinical lesions observed in some subjects at the site of application of the lozenges may be due to the absorption of varying amounts of nicotine in combination with mechanical irritation. The inter-individual variation in nicotine exposure was substantial but not all high users of nicotine showed clinical lesions. Because the subjects' oral mucosa normalized over time, the nicotine lozenges were shown to be safe for use in both low and high consumers of tobacco.

In Study III the outcome of a ST-cessation program was assessed. The main limitation of the ST-cessation study was that there was no placebo group, therefore it is not possible to evaluate the cause of success. There could be several reasons for successful outcome of the program, including the selection of highly motivated subjects, the use of NRT, the use of individual counseling, and the use of motivational feedback involving a soft tissue biopsy.

In Study IV we investigated the reversibility of snuff-induced lesions in the oral mucosa. The mucosa in snuff users was neither clinically nor histomorphologically completely normal 6 months after cessation of snuff use. It is important to emphasize that the study revealed not only that clinical lesions persisted, but also that changes at the cellular level were evident. Only one examiner (MW) registered the clinical conditions of the subjects, but two independent observers examined the clinical photos and the photos were used in the analysis.

Smokeless tobacco-cessation treatment outcome

An ST-cessation program was designed and evaluated. The program included both pharmacological (gum 4 mg) and intensive behavioral treatment, biopsy of the snuff-induced lesion, and follow-up visits for one year.

The end point outcome after one year showed a success rate of 30%. Data from previous ST-cessation studies based on both behavioral and pharmacological therapy are usually limited to three or six months' follow-up, not validated and point prevalence figures, and with results varying from 11-29% (136). In light of the long-term results in previous studies, our 30% sustained abstinence (from 2 weeks until end point), biochemically verified after one year, was considered promising. Our results are comparable to some ST-cessation long-term follow-ups at 12 months (160, 161) and smoking cessation studies in adjuvant use with NRT (39). The results from our study must, however, be interpreted with caution, as the study lacks control groups and the sample size was relatively small. There could be several reasons for the fairly successful outcome, including selection of highly motivated subjects, the use of NRT, the use of repeated individual counseling, and the use of motivational feedback through a soft tissue biopsy. Hughes et al. recommended using a 12-month follow-up assessment point because it is more closely associated with life-long abstinence (159). The patients in our

study were reviewed 10 years after termination of the cessation program. Out of the original 50 patients, 40 were located and 10 could not be found; 4 of the located patients were diseased and lost to further follow-up. Telephone interviews were performed with the 36 available healthy patients and 26 of these agreed to a clinical examination. We found that 9 patients had been continuously abstinent during the cessation study and over the following 10 years, and 12 patients were abstinent at the 10 year follow-up, although they had had lapses during the one-year study. Accordingly 21 of the original 50 subjects had unequivocally successful outcomes, and the success rate rose to 42% with the addition of those who were abstinent at 10 years despite early lapses. Fifteen patients had used snuff throughout the entire 10 years and previously during the cessation study (Wallström et al. in manuscript). It is evident that intervention is possible even in a group of snuff dippers with extensive addiction to nicotine and that a fairly stable result can be achieved in the long term. However, the efforts are quite expensive both in time and costs for both researchers and participants in these programs.

Nicotine replacement

Compliance to NRT was relatively high, with 73% users complying from baseline. The mean nicotine consumption (mg/day) in this study during the first 6 weeks was equivalent to the mean nicotine consumption among highly dependent smokers Fagerström Test of Nicotine Dependence (FTND) ≥ 7 (162) in a smoking cessation study (163). The use of adequate doses of nicotine is considered to be very important in achieving abstinence in smokers (164). The greater efficacy of the 4 mg nicotine gum over the 2 mg gum in highly dependent smokers is well documented, and 4 mg gum is accepted for smoking cessation and usually recommended to smokers with a FTND ≥ 7 . Hatsukami et al. published a study on smokeless tobacco cessation in moderate to heavy users comparing the use of 2 mg nicotine gum versus placebo in conjunction with either behavioral treatment or minimal contact and reported unexpectedly that patients assigned to nicotine gum experienced no better success than those on placebo (35). It was concluded that the ineffectiveness of the NRT was most probably attributable to the low dosage. One earlier study reported the same disappointing result when using the 2 mg nicotine gum (34). Benowitz (165) showed that the mean plasma nicotine concentration from snuff and cigarettes are approximately equivalent, as had been found by Holm (117) in a group of Swedish snuff dippers who showed slightly higher nicotine plasma levels compared with smokers. Still, although their nicotine peak blood levels are equivalent, snuff users are exposed to significantly more nicotine due to their continuous use of tobacco over approximately 15 hours per day. The substitution of 4 mg nicotine gum in smokers theoretically compensates for about two thirds of their habitual blood nicotine level (166).

Our results showed that after 3 and 6 months of NRT, 58% and 46% of smokers (respectively) were abstinent. Ebbert et al. obtained comparable results in an ST-cessation trial, but used a 4 mg tablet in a non-placebo-controlled study (37). After 3 and 6 months, 66 % and 47 % of ST users (respectively) were abstinent (7-day points prevalence). In a later randomized placebo-controlled pilot study where 4 mg nicotine lozenges were mailed to the subjects along with a self-help program, there was no statistically significant difference observed between test subjects and controls (167).

NRT has been shown in ST-cessation studies to reduce craving and nicotine withdrawal symptoms, but not to increase abstinence rates with any effectiveness (35, 37). To date there are few ST-cessation studies reporting a higher dosage regime for nicotine replacement, as summarized in a review by Ebbert et al. (168).

We documented an extensive total exposure to nicotine among the participants in our cessation program, which was well in accordance with expectations, since all snuff dippers were classified as “heavy users”. On a pharmacological basis it seemed logical to compare these snuff users to highly nicotine-dependent smokers and to recommend that all subjects use the 4 mg nicotine gum. The better long-term success in this study compared to earlier studies on ST cessation is most probably due to abstinent snuff users benefiting from the higher dose of nicotine. The important part was that the level of nicotine substitution was adequate. The plasma nicotine level reached with the chewing gum the same level as previously obtained with the sublingual tablet (163). An alternative method of treatment in smokeless tobacco cessation worth studying is the effect of combining the nicotine patch and the nicotine gum, since data from studies in smokers has shown promising results (169); the sublingual tablet could also contribute to an increased efficacy of nicotine substitution, as reported previously (37).

The inclusion of a placebo group, a low NRT dose group, or both could have given information on whether or not the high nicotine content was in fact the determining factor for the relatively successful outcome.

Intervention

This intervention design, with a face-to-face counseling recall program for reinforcement and relapse prevention, has been shown effective in a meta-analysis of smoking cessation programs (146, 156, 170). Therefore, we adapted the method (156) in the present study by using a single cessation technique supplemented with a biopsy, medical examinations at five recurrent visits (weight, blood pressure, heart rate, cholesterol, oral screening and measurements of CO), and random tests for cotinine. Motivational information was combined with the subject’s own inspection of the former snuff lesion. The overall finding by the investigators of the effectiveness of repeated professional examinations and care to reinforce cessation success was consistent with previous findings (171). A significant problem in the treatment design was its heavy use of resources as the investigator spent 3.5 hours per subject for individual counseling and treatment. In addition, the importance of the biopsy of the snuff-induced lesion taken at quit-day should not be ignored, but it requires a different sort of setting and competence for meaningful interpretation. Following the mucosal biopsy the subjects suffered from moderate pain, swelling, and irritating stitches that contributed to their readiness to refrain from applying snuff.

The use of the nicotine gum seems important, not only for its delivery of nicotine, but also for the local tactile sensation on the oral mucosa. For some subjects the feeling of having the gum under the lip resembled the feeling of a snuff- quid, and that feeling seemed important. For others active chewing of the nicotine gum as a form of oral distraction was crucial to their success, a finding that has previously been reported (172). Hjalmarson et al. (173), however, argued that the form of delivery plays only a marginal role.

A problem when designing a functional intervention program for snuff users is the high percentage of former smokers and the proportion of subjects with mixed habits. In a ST-cessation intervention for college athletes, 4% started smoking in their attempt to quit using ST (174). Although habitual or occasional smoking was an exclusion criterion, 10 subjects in our snuff cessation study reported smoking (8 occasionally, 2 regularly) in the failure group at follow-up.

In the success group craving for nicotine was still present in 20% of the subjects, confirming the strong addictive potential of nicotine. Long-term dependence on nicotine gum has also been reported (175, 176). Findings in a multicenter intervention study by Murray et al. involving 3,094 participants with NRT, showed 5% still using nicotine 2 mg gum after 4 years (177). This indicates that there are two related problems; highly nicotine-dependent subjects might benefit from a treatment period longer than 6 to 12 months in order to achieve and maintain complete abstinence, and at the same time they risk developing into long-term users of NRT products. This is supported by the fact that in the failure group of the present study, 16% were still on NRT at 12 months' follow-up, although they were not using tobacco. One way to address these problems and achieve a better outcome could be to redesign the cessation program by prolonging the counseling in order to promote tapering. In reviewing 10 years after the snuff cessation study we found 42% of participants abstinent. This shows that though the design was adequate, it was not sufficient, supporting the notion that a redesign of the program should be considered. From a health perspective it is better to continue using pharmaceutical nicotine than commercial snuff products because the commercial products contain TSNA and other potentially dangerous chemicals (178). However, there are indications that prolonged NRT is not without risk either (179).

ST users as a group show a higher BMI than smokers, although nicotine increases metabolism and is considered to contribute to smokers' lower BMI. After tobacco cessation both smokers and ST users show weight gain, but ST users attain the highest weight levels because they start from a higher weight level (104). This is in line with our findings that showed almost all subjects gained weight during the study, which was correlated with a significant increase in diastolic blood pressure in the success group, as well as in total cholesterol values. Increased BMI, blood pressure, and cholesterol are definite drawbacks and the health benefits of cessation therefore might be questionable. The significant increase in metabolic risk factors might be due to the increased BMI, but this must be examined further in studies that include behavioral factors (104, 180). These results further show the need for a redesign of the cessation program.

Reversibility

The overall clinical and histomorphological picture improved in subjects after 6 months of abstinence; however, at the site of snuff application, 40% of the participants still exhibited lesions, although these were less severe, and the area of the affected mucosa was reduced. Only one lesion had the same clinical picture as at baseline, and the remaining lesions shifted to a less severe clinical appearance.

By contrast, it has been reported that tissue changes from snuff use are completely reversible after quitting, although only 16 out of 29 subjects met the criteria of

abstinence between 3 and 6 months (46). What we report is that all subjects were confirmed tobacco abstinent prior to the second biopsy. A comparison of the data of these two studies shows that the age of the subjects and the clinical severity of the lesions at baseline were approximately the same. However, the mean exposure time was 10 years longer ($p=000.1$) in the present study. We suggest that in addition to the difference in number of abstinent subjects, the differences between the two studies with regard to clinically healed lesions are related to the snuff exposure data.

Another contributing factor to our finding may be the fact that 75% 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 suggested (179). This indication was partly substantiated by our own study exposing the oral mucosa to lozenges (181). In this safety study, we exposed the sublingual mucosa to 2 to 4 mg nicotine hourly for 3 months followed by a tapering period of 3 months. This resulted in reversible superficial transient lesions at the site of application, verified by biopsies after cessation of the nicotine administration (181). Tobacco is one of the main etiological factors for initiation of oral squamous cell carcinoma and nicotine is one of the major alkaloids in tobacco. This finding should indicate caution regarding extensive and prolonged use of oral NRT in connection with tobacco cessation. Nicotine activates a proto-oncogene, FOXM1, which has been shown to play an important role in oncogenesis. The gene is up-regulated as an early event in human squamous cell carcinoma and it is enhanced by nicotine during malignant cell transformation (179). This suggests that administration of NRT should still be undertaken, but with caution, with due regard to dose and duration, and with careful monitoring of the oral condition, especially in patients with premalignant and malignant oral lesions.

The histomorphological picture of the snuff-induced lesions at baseline was consistent with previous reports, i.e. they showed increased epithelial and keratin thickness, ortho- or parakeratinization, and inflammatory reaction of varying severity with only occasional slight dysplasia (4, 11, 158, 182).

Biopsies obtained 6 months after snuff cessation showed microscopically a significant reduction of the keratin layer, a decreased total epithelial thickness, and a persistent chronic subepithelial inflammation. This is consistent with our previous findings (20), where rats exposed to snuff in a surgically created lip canal exhibited slight hyperplastic epithelium with little or no keratinization and slight inflammatory reaction. By contrast, Larsson et al. (46) classified biopsies from 16 abstinent subjects as normal according to the Kramer classification (183).

In summary, our findings show that after long and extensive snuff use, snuff-induced lesions do not resolve completely, either histologically or clinically. We suggest that these patients be reviewed closely, especially in light of the significant risk that ex-snuff users run of developing oral cancer (94). Because of the possible involvement of nicotine in malignant cell transformation, special attention should also be given to those using NRT as a cessation aid but not following the recommendations for nicotine use.

We studied the effect on the floor of the mouth of nicotine exposure from a sublingual nicotine tablet. The clinical type of lesion that appeared and its extension were

registered as well as the patient's subjective symptoms. The application of the tablet resulted during the first 3 months in reversible, mainly limited, clinically hyperplastic or hyperkerotatic lesions, but no severe reaction in 8 subjects, all of whom were in the group $FTQ \geq 7$. The total exposure to nicotine over the period was significantly higher among subjects with lesions than in subjects with no lesions. The floor of the mouth did not exhibit any clinical changes after the tapering period at 6 months. Since all lesions had resolved during the tapering period we concluded that they were probably related to the lozenges, i.e. to nicotine and mechanical irritation. Little information is available in the literature regarding the local effects of nicotine on the oral mucous membrane. In the biopsy that was obtained after 6 months no serious changes were seen microscopically. Unfortunately, since the biopsy was optional, only one third of the subjects participated. According to the records most of the application sites exhibited healthy mucosa during the study and this corresponds well with the pathological reports. In a report on the use of nicotine nasal spray the same conclusions were made after biopsy of the nasal mucosa (184). Generally there is a higher incidence of mucosal lesions among tobacco users than among non-tobacco users due to the impact of toxic components in the tobacco (185). The dominant mucosal diagnosis that was seen was smokers' melanosis, which is consistent with earlier reports (186). Smoking cessation or decrease in smoking had a positive effect on the mucosal lesions seen at baseline, so we could conclude that the tablet did not aggravate or induce any significant new pathological changes. The methods used to study mucosal reactions in this study seem to be relevant and neither expensive nor time consuming.

Although the primary objective was not smoking cessation, the study was carried out under conditions of smoking cessation. In order to keep as many subjects as possible throughout the study period, complete abstinence was not stressed, but spontaneous abstinence with or without slips was high. The success rate was significantly higher in the $FTQ \geq 7$ group. The explanation for this could be that the subjects with the lower FTQ did not substitute sufficiently with nicotine, as they were recommended only 2 mg/hour and often used less. Alternatively these subjects were not nicotine dependant but rather had a habitual behaviour which was not easily changed and most probably not with NRT.

It is evident from this study that the tablet produced nicotine plasma levels sufficient to maintain abstinence and depress withdrawal symptoms. Smoking withdrawal symptoms were kept at baseline level or below.

Substituting nicotine in tablet form was earlier considered to be similar to using nicotine gum (187). A new administration form, such as the sublingual tablet, was needed not only as a more appealing way to administer nicotine and thus increase treatment compliance, but also as a recognized and familiar method for the physician to treat medical conditions. We know that the effective substitution level is 50% of the habitual dose, and it is essential to successful abstinence to recommend adequate dosage, but a common problem with the use of nicotine gum is underdosage through non-compliance. The addition of the recognized medication treatment of prescribed tablets facilitates compliance and increases the patient's chance of success. Another possible advantage of the tablet is that absorption of nicotine through the mucosal membrane in the floor of mouth is much more effective and faster than in buccal mucosa or skin due to the rich vascular supply in the mouth (188). When smoking, the nicotine plasma concentration

peaks within five minutes. NRT normally needs 20 to 30 minutes to reach a plasma level where withdrawal symptoms are alleviated (18). Comparisons between the tablet and 2 mg nicotine gum showed no difference in plasma concentration after 30 minutes, but the tablet concentration peaks 5 minutes faster (187). The nicotine tablet has also been shown to deliver 8% to 10% higher maximal plasma concentrations and 25% to 75% higher area under the curve than the gum in single-dose studies (189). Theoretically, then, the tablet might be expected to give faster relief of cravings and withdrawal symptoms than the gum.

The subjective adverse experiences of the tablet were mild and tolerable. Only 10% of the subjects reported a burning or stinging sensation in the mouth, either at the site of application or at the tip of the tongue, or in the throat. These adverse events have previously been reported with use of the gum (27, 190) and are recognized as nicotine related. Dry lips were experienced by some patients, and that had not been reported among gum users. This event might be explained by frequent licking of the lips, thereby exposing the lips to nicotine. When the treatment ended, this also resolved.

Although oral tobacco products lack the toxicants associated with combustion, they include around 30 known carcinogens (66), some of which are TSNAs. The TSNAs that have been most strongly linked to cancer are NNK and NNN. These TSNAs are formed during the curing, processing and aging of tobacco, as well as during consumption, and they are present in both burned and unburned tobacco. According to the IARC these TSNAs are considered Group 1 carcinogens (1). They cause tumors of the oral cavity, esophagus, pancreas, and lungs in laboratory animals. The quantities of TSNAs in some noncombusted oral tobacco products manufactured in Sweden and in the USA have decreased over time due to changes in production, which were clearly observable after the snuff products had been analyzed, data on the nitrosamines content published, and the risks stressed (69, 70). In Study 1, we investigated *in vitro* the effects of individual TSNAs and alkaloids and SS as a whole, and we observed that water soluble extract from SS significantly inhibited mitogen-stimulated T cell proliferation induced by accessory cells from rat oral epithelium. When T cells and oral epithelial cells were pre-exposed to high concentrations of the extract there was a significant irreversible inhibition of T cell proliferation. However, the SS derivative alkaloids and TSNAs in concentrations similar to those in SS had no significant effect. Furthermore, no mitogenic properties were shown by SS extract, alkaloids, or TSNA. Several cell types and *in vitro* toxicity methods have been used to evaluate the toxicity of smokeless tobacco (191). In toxicity experiments with human keratinocytes and fibroblasts, Wang et al. found that smokeless tobacco extracts promoted fibroblast growth, but conversely suppressed growth of keratinocysts (192). Interestingly they reported a stimulation of keratinocyte proliferation at low concentrations of ST extracts (192). This supports the findings from our study where NNN showed a tendency to be stimulatory in an *in vitro* assay with T cells and rat oral epithelial cells (193). There is clear evidence that ST extracts suppress cell viability and proliferation *in vitro*. However, it is important to note that in some studies the stimulatory potential of substances in SS extract has been observed (19). This should be considered when discussing the cancerogenic potential of SS. It has also been shown that SS extract induces significant increases in intracellular oxidized states of human oral keratinocytes which may be inhibited by the use of antioxidants such as vitamin C and E (194). Reactive oxygen species are known to

cause genetic damage that may lead to the development of cancer cells (195). The carcinogenic potential of ST may in part be attributed to this mechanism.

Primary prevention

The dental profession has the advantage of regularly meeting a large part of the tobacco-consuming population who still have no or only minor clinical symptoms of tobacco-related disease, i.e. “healthy tobacco users”. The dental team has unique resources to contribute to public health improvement by taking an active part in tobacco prevention and cessation assistance, and it also has great potential to contribute to primary prevention of a large number of fatal and disabling diseases. These contributions to public health and prevention should be considered an important mission for the profession. Knowledge and skills of basic tobacco cessation treatment should be a compulsory part of the education of dental professionals. It is also vitally important that the dentist screen the oral cavity, document mucosal changes, take steps to establish a correct diagnosis, initiate treatment, and enter the patient into a recall system. The observations of tobacco-related lesions or disease should lead to implementation of a cessation program. The advantages of the dental setting for facilitating tobacco cessation have been summarized in two reviews (171, 196) that show long-term abstinence rates increase when interventions are conducted by dental professionals. It is important that further research regarding intervention, prevention, and health promotion also take place in the dental setting. The collaboration of dental clinicians and researchers is necessary to ensure that our patients will get the most effective evidence-based care. The cost-effectiveness of preventive efforts by the dental team also requires further study as in most countries they are rarely compensated by the health systems.

8 Summary and Conclusion

This thesis on harm and harm reduction in smokeless tobacco (ST) users has addressed various topics. Investigations of ST, nicotine, and TSNA *in vitro* have been combined with clinical and histological analyses. Also, an interventional study has explored the reversibility of snuff lesions and the use of NRT in a cessation program.

Snuff cessation conducted together with NRT in a professional setting is a promising method for achieving a tobacco-free state, and compliance to treatment was high regardless of outcome. Almost all subjects gained weight during the study, which correlated with a significant increase in both diastolic blood pressure in the success group and cholesterol values in the sample as whole.

In a prospective study, tissue samples from snuff dippers with an extensive exposure to snuff who were still using NRT on a daily basis 6 months after cessation were neither clinically nor histomorphologically completely normal.

Daily repeated sublingual exposure to nicotine for three months appears to be a safe form of administration with mild and transient effects in individuals devoid of clinical

lesions. Mild and tolerable adverse events attributable to the nicotine gum treatment were encountered.

No mitogenic properties were shown in SS extract, various alkaloids, or TSNA, other than a tendency for NNN to be stimulatory in an *in vitro* assay with T cells and rat oral epithelial cells. SS extract can evoke an immunosuppressive effect on T cell proliferation using cells from oral epithelium as accessory cells. This effect was more pronounced when the complete SS extract was employed compared with when single components were used.

In conclusion, long-term tobacco exposure is most likely to cause injury to different defense systems including the immune system of the oral mucosa. It can also be concluded that the sublingual nicotine tablet is a safe form of nicotine administration in a cessation program. The nicotine lozenges used in this study did not cause harm to the oral mucosa in the long term.

The findings that oral mucosa at site of ST application do not recover completely suggest that these patients should be reviewed closely, especially since it has been reported that ex-snuff users run a significant risk of developing oral cancer. Because there is a possible involvement of nicotine in malignant cell transformation, special attention should be given to subjects using NRT as a cessation aid.

Although ST may represent a measure of harm reduction in comparison with smoking tobacco, the use of ST involves exposure to a very addictive drug and toxicants that we know cause disease to various extents. As professionals we must advocate cessation of both smoking and ST among our patients and not use ST for smoking cessation.

9 Summary in Swedish

Immunotoxikologiska effekter av snus på den accessoriska kapaciteten hos orala epitelceller på råttor.

Bengt Hasséus, Mats Wallström, B-G Österdahl, Jan-M Hirsch, Mats Jontell

Immunotoxic effects of smokeless tobacco on the accessory cell function of rat oral epithelium. Eur J Oral Sci 1997; 105: 45-51

Snus ger upphov till toxiska reaktioner i munslemhinnan vilket dokumenterats i ett flertal kliniska studier. Toxikologiska studier har också gjorts på djur som visat att snus kan ha en viss positiv immunostimulerande potential och negativ effekt på i blod cirkulerande NK-celler. I föreliggande studier i cellkulturer (*in vitro*) undersöktes hur extrakt av svenskt snus, fem tobaksspecifika nitrosaminer (TSNA) och nikotin påverkar immunokompetenta råttcellers funktion.

Munslemhinna, cervikala lymfkörtlar och mjältar togs ut från grupper om 15 råttor som avlivats med koldioxid och cervical dislokation.

Suspensioner av epitelceller från munslemhinna, Langerhans celler, T- och mjältceller bereddes genom enzymatisk behandling av vävnaderna.

I inledande experiment användes mjältceller för att dosbestämma lämpliga koncentrationer inför toxicitetstester. Mjältcellerna odlades i cellodlingsbrunnar tillsammans med ett mitogen (con A) och olika koncentrationer av snus, orala epitelceller inklusive Langerhans celler tillsammans med T-celler och con A. Studien utformades så att test gjordes i en s.k. crossover design med förbehandling med snusextrakt eller TSNA av de olika celltyperna och att snusextrakt eller TSNA inkluderades under cellodlingsperioden.

Experimenten visade att det skedde en koncentrationsberoende hämning av celltillväxten av snusextrakt samt om orala epitelceller, inklusive Langerhans celler, förbehandlades med snusextrakt. TSNA i de koncentrationer som finns i munhålan i samband med snusning testades dels med förbehandling och dels med substanserna närvarande under cellodlingsperioden utan att signifikant effekt kunde påvisas. Nikotin var möjligt att testa endast i en koncentration 1:10 000 av vad finns närvarande i munnen under snusning då justering till fysiologiskt pH för cellodling inte kunde göras. Inga signifikanta skillnader kunde påvisas.

Studien visar att snusextrakt har en immunosuppressiv effekt på immunceller från råttor *in vitro*.

Långtidsexponering av nikotin på munslemhinna.

Mats Wallström, Lars Sand, Fredrik Nilsson, Jan-M Hirsch

The long-term effect of nicotine on the oral mucosa. Addiction 1999; 94 (3): 417-423.

Trots att nikotinersättningsmedel för att användas i munnen i samband med tobaksintervention har funnits i ett 20-tal år är tillgänglig dokumentation av dess effekter på munslemhinna otillräcklig. Denna studie avsåg att studera effekten på munbottenslemhinnan av en 2 mg resorberbar nikotintablett i samband med

rökavvänjning. Trettio rökare med minst en konsumtion av 10 cigaretter per dag, inkluderades i studien och erhöll 2 mg nikotintablett att applicera under tungan under 3-6 månader. Slemhinnan bedömdes kliniskt och fotograferades vid varje besök. Efter 3 månaders behandling följt av en avtrappning till 6 månader togs på frivillig basis ett vävnadsprov från applikationsstället för analys.

Resultatet visade på 10 läsioner i munslemhinnan bland totalt 8 patienter. Alla förändringarna noterades under de 6 första veckorna som var läkta efter 3 månader utom en läsion som var utläkt vid 6 månaders uppföljningen. Den histologiska bedömningen av vävnadsprover tagna efter 6 månader visade normal slemhinna.

De subjektiva besvären var lindriga och övergående.

Författarna konkluderade att nikotinersättningsmedel applicerat på munbottens slemhinna under en 6 månadersperiod är en säker administrationsform.

Ett avvänjningsprogram för snusare med långvarig exponering för svenskt snus. En 1-års uppföljning.

Mats Wallström, Gunilla Bolinder, Bengt Hasséus & Jan-M Hirsch

A cessation program for snuff-dippers with long-term, extensive exposure to Swedish moist snuff: A 1-year follow-up study. Acta Odontologica Scandinavica 2010 Early online.

Snusare är ofta starkt beroende av nikotin. Rökavvänjning med hjälp av nikotinersättningsmedel har visat sig effektivt i många studier. För snusare finns det få utprovade program för tobaksstopp med hjälp av nikotinersättning. Arbetets syfte var att utforma och testa ett avvänjningsprogram för snusare med en konsumtion på minst 2 dosor i veckan under mer än 10 år. Till en öppen kontrollerad studie rekryterades 50 snusare genom annonsering. Programmet bestod av individuell rådgivning under 1 år, medicinska kontroller av vikt, blodtryck samt puls och kolesterol samt undersökning av munhålan med fotografering av snusläsionerna.

Vid första besöket togs en biopsi från snusläsionen som analyserades mikroskopiskt. Samtliga rekommenderas 4 mg nikotintuggumi under följande 3 månaders period samt en därpå en 3 månaders avtrappning. Försökspersonerna rapporterade eventuell uppnådd tobaksfrihet, vilket verifierades med analys av nikotinmetaboliten kotinin i saliv samt kolmonoxid (CO) mätning av utandningsluften.

Vid 3, 6 och 12 månader var 58, 46 och 30% både snus och rökfria. Dessa siffror är jämförbara med motsvarande uppnådda resultat vid rökavvänjningsstudier där man också använt nikotinersättning. Slutsatsen blev att snusavvänjning med hjälp av nikotinersättning i en individuell terapiform är en fungerande metodik.

Reversibiliteten av snusinducerade läsioner – En klinisk och histomorfologisk studie

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The Reversibility of the Snuff-Induced Lesion - a Clinical and Histomorphological Study Submitted.

Hos 50 snusare som deltog i en snusavvänjningstudie avsåg vi att studera eventuell utläkning av snuslesioner efter det att snusningen upphört. Som hjälp vid att sluta

rekommenderades nikotinersättningsmedel i form av 4 mg nikotintuggummi. Vid studiens början togs foto samt ett vävnadsprov från snusläsionen. Efter 6 månader var 26 snusare tobaksfria sedan start. Från dessa 20 togs ett nytt vävnadsprov från platsen för snusapplikation att jämföra med det ursprungliga provet. Kliniska förändringar dokumenterades fotografiskt. Medelåldern för de 20 exsnusarna var 40,6 år (SD \pm 9.3) och den sammanlagda genomsnittliga tiden som de snusat 22,1 år (SD \pm 6.2) med en genomsnittlig konsumtion av 4,8 (SD \pm 2.8) dosor snus per vecka.

Kliniskt kunde man notera att 8 individer uppvisade förändringar där snuset tidigare applicerats. Av dessa använde 75% fortfarande nikotintuggummi. Fyra individer använde nikotinersättningen som ett tuggummi och 2 simulerade snusning genom att lägga tuggummit under läppen. Histomorfologiskt kunde man notera att medelepitel-tjocklek och keratintjocklek samt inflammation hade minskat, men hos vissa hade dessa förändringar ökat i storlek. Hos snusare med omfattande och långvarigt bruk kunde man notera att slemhinnan efter 6 månader inte hade normaliserats utan det kvarstod förändring både kliniskt och histomorfologiskt. Förändringar var mer uttalade hos dem som fortfarande använde nikotinersättningsmedel som hjälp vid sin snusavvänjning.

Sammanfattning

I en *in vitro* studie på munslemhinne-celler och immunokompetenta celler från råttor har vi studerat effekten av extrakt av svenkt snus, nikotin och cancerframkallande nitrosaminer i olika koncentrationer. Vidare har vi studerat effekten av nikotin och snus på munslemhinna både kliniskt och histologiskt. Vi har utarbetat och testat ett interventionsprogram för snusare där stöd och rådgivning samt nikotinläkemedel ingått samt studerat slemhinnans återhämtning efter snusstopp.

In vitro visades att det skedde en koncentrationsberoende hämning av celltillväxten av snusextrakt samt om orala epitelceller, inklusive Langerhans celler, förbehandlades med snusextrakt. TSNA i de koncentrationer som finns i munhålan i samband med snusning testades dels med förbehandling och dels med substanserna närvarande under cellodlingsperioden utan att signifikant effekt kunde påvisas. Studien visade att snusextrakt har en immunosuppressiv effekt på immunceller från råttor *in vitro*.

När slemhinnan i munbotten utsätts för nikotin dagligen under 6 månader kan man notera att vissa förändringar uppstår men att dessa var av övergående natur. Totalt noterades 8 slemhinneförändringar tidigt under behandlingen men alla som var utläkta efter 3 månader förutom en som var läkt vid 6- månaders kontrollen. Måttliga subjektiva besvär i form av sveda och irritation registrerades, samtliga av övergående natur. Efter 6 månader togs vävnadsprover från 1/3 av individerna, som samtliga visade på normal slemhinna.

Snusavvänjning i vårdmiljö med hjälp av nikotinersättningsmedel visar sig vara en lovande metod för att uppnå tobaksfrihet. Oavsett om tobaksfrihet eller inte uppnåddes använde flertalet nikotinersättning under behandlingsperioden. Samtliga deltagare gick upp i vikt, vilket i lyckandegruppen korrelerade med en stegring av diastoliskt blodtryck samt förhöjt kolesterol.

Från gruppen snusare med ett långvarigt snusbruk togs vid studiens start ett vävnadsprov från snusförändringen, därefter ett andra prov när man hållit upp i 6 månader. Kliniskt kunde konstateras att slemhinnan inte hade normaliserats efter 6 månaders snusstopp, vare sig kliniskt eller histologiskt.

Sammanfattningsvis kan man säga att långvarigt snusbruk orsakar skada på kroppens försvar inklusive det orala immunologiska försvarssystemet. Att använda sig av nikotin tablett under tungan i samband med rökavvänjning är en säker administrationsform under 6 månader. Nikotintabletten orsakade inte någon permanent skada på munslemhinnan. Vi noterade att slemhinnan efter långvarigt snusbruk inte normaliserades efter snusstopp utan uppvisade kvarstående förändringar även efter 6 månaders snusuppehåll. Det finns rapporter om risk för cancerutveckling hos före detta snusare varför dessa individer bör följas upp även efter snusstopp. Dessutom finns rapport om att nikotin kan vara en del i tumörutveckling, vilket gör att det kan finns ytterliggare anledning att följa upp individer som använder sig av nikotinprodukter. Även om snus anses vara en säkrare form av tobakkonsumtion än rökning, exponeras man för en starkt vanebildande drog som innehåller ca 3000 kemikalier flera välkända cancerframkallande ämnen (TSNA) som på sikt påverkar ohälsotalet och kan ge sjukdom. Som vårdgivare måste vi därför förespråka och ingripa för att skapa tobaksfrihet hos våra patienter, såväl rökare som snusare, och poängtera att snus *inte* är en rökavvänjningsprodukt.

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