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Factors effecting the induction of rat forestomach hyperplasia induced by Swedish oral smokeless tobacco (snus)

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ABSTRACT

Long term exposure to oral smokeless tobacco may induce lesions in the oral cavity characterized by a hyperplastic epithelium. The possible role of nicotine and the physical properties of oral tobacco for developing these lesions, as well as of dysplasia and neoplasia is unclear. Low nitrosamine Swedish snus as well as non-genotoxic butylated hydroxyanisole induces increased cellular proliferation in the rat forestomach epithelia. Using this model, we report here on the effects of nicotine, pH, and particle size. Snus with different properties had no impact on oxidative stress as determined by 8-oxo-7,8-dihydro-2′-deoxyguanosine, or on interleukin IL-1b. Whereas BHA boosted IL-6, probably due to the presence of nicotine. there was no significant enhancement of cell divisions with increasing particle size, although in individual samples the variations in proliferation rates increased greatly with increasing particle size. Conforming to human experience, the enhanced cell proliferation caused by snus was found to be completely reversible. A cacao bean extract had a protective action similar to that previously found for blueberries. The main cause of the observed tobacco induced cell proliferation could be mechanical irritation, possibly in combination with nicotine, whereas within the studied range, pH did not affect the rate of cell division.

1. Introduction

During the last 3-4 decades male smoking-related deaths have gradually decreased in Sweden. This development can to a major extent be attributed to the fact that smoking has to a large extent been replaced by nicotine delivery by a smokeless tobacco called “snus”. In spite of the fact that about 20% of the adult male population uses snus regularly, there is no convincing epidemiological evidence linking snus with an elevated risk for cancers in the oral cavity, esophagus or stomach (Lewin et al., 1998; Schildt et al., 1998; SCENIHR, 2008, p.11; Lee, 2011). Further, it has not been possible to verify the claim, that Swedish snus causes pancreatic cancer (Lee, 2011; Bertuccio et al., 2011; Araghi et al., 2017). In addition, in Sweden there has also been a drastic reduction in other smoking related diseases (Foulds et al., 2003; Lee, 2011, 2013).

The failure to detect an elevated cancer incidence in users of snus is compatible with the fact that the levels of tobacco specific nitrosamines (TSNA) are up 10,000 times lower than in impure carcinogenic Sudanese oral tobacco (Idris et al., 1991, 1994; Österdahl et al., 2004; WHO, 2009). Further, when comparing the expected levels of promutagenic TSNA-induced DNA damages induced by snus with the “normal” background of the same lesions, no increase in cancer risk can be expected (Nilsson, 2011, 2017). It has not been possible to induce tumors in experimental animals using internationally recognized methodology (Grasso and Mann, 1998).

After long term exposure snus may, nevertheless, induce local oral lesions known as “snuff dipper's lesions”, leucoplakias, or snuff-induced keratosis, characterized by a hyperplastic epithelium with vacuolization and keratinization. Acanthosis and slight inflammation may also be present accompanied by an increased mitotic rate (Larsson et al., 1991). It should be distinguished from smoking induced preneoplastic forms of leucoplakia displaying cellular atypia/dysplasia (Axéll, 1993; Axéll et al., 1976; Andersson and Axéll, 1989). These dysplastic lesions are typically induced by betel chewing, or by other impure tobacco products used in South East Asia and Sudan (Zain et al., 1997).

The smokeless tobacco keratosis induced by modern Swedish snus is reversible after cessation of exposure, and has a very low probability for malignant transformation (Larsson et al., 1991; Roosaar et al., 2006).

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Abbreviations

BHA	Butylated hydroxyanisole
BrdU	Bromodeoxyuridine
EFSA	European Food and Safety Authority
IgG	Immunoglobulin G
IL	Interleukin
8-oxodG	8-oxo-7,8-dihydro-2′-deoxyguanosine
PI	proliferation index
TSNA	Tobacco specific nitrosamines

Nevertheless, a carcinogenic risk associated with snus lesions in some particularly susceptible individuals cannot be ruled out, although the risk would be too low for reliable detection by conventional epidemiological methods in view of residual confounding by smoking and alcohol. However, these lesions represent a clinical problem in themselves. Snus users with leukoplakias are sometimes referred for various, unnecessary clinical work ups, including surgical biopsy.

A number of potent carcinogens induce tumors in the rodent forestomach, which is also susceptible to the irritative or corrosive action of several non-genotoxic compounds. In the latter case, stimulation of cell division induced by cytotoxicity and regenerative hyperplasia represents important underlying mechanisms (Clayson et al., 1991). In a previous study we found that 4–6 weeks' exposure to snus administered by oral intubation resulted in marked enhancement of hyperplasia and cell divisions as assayed by bromodeoxyuridine (BrdU) in the rat stomach epithelium (Allen et al., 1978). After 6 weeks' exposure, blueberries (bilberries), and an extract from the common milk thistle were found to exert a highly significant inhibition of cell proliferation induced by snus in the rat forestomach epithelium (Nilsson et al., 2016).

The possible role of nicotine and the physical properties of various kinds of oral tobacco for development of oral dysplasia and neoplasia have been extensively debated (Grasso and Mann, 1998; Andersson and Warfvinge, 2003; Nilsson et al., 2016; Singh et al., 2018).

Although nicotine *per se* is not considered to be a carcinogen (EFSA, 2009), Sanner and Grimsrud (2015) have speculated that nicotine promotes the induction of neoplasia by indirect mechanisms. The aim of this experimental study was to investigate the influence of nicotine content, pH, granularity as well as post treatment recovery on the induction of rat forestomach hyperplasia induced by oral smokeless tobacco (Swedish snus). In addition, the protective action of an extract from cacao beans and blueberries was investigated.

2. Materials and methods

2.1. bromodeoxyuridine (BrdU)

BrdU, 99%; Sigma Aldrich Co. USA) was purchased from Uni-Chem, Belgrade. Flat-faced cylindrical matrices containing 50 mg of BrdU with a diameter of 5.6 mm were prepared at the Faculty of Pharmacy, Belgrade University, by a direct compression technique using an eccentric tablet press (Korsch EK-0, Korsch, Berlin, Germany).

2.2. Butylated hydroxyanisole (BHA)

BHA (99%) was obtained from Sigma/Aldrich Co., USA. It was dissolved in corn oil (Sigma, Aldrich), and mixed with pulverized commercial pellet (Veterinarian Institute Subotica, Serbia) to a final concentration of 2%, and used as such.

2.3. Snus products

Snus with different physical and chemical characteristics was obtained from Swedish Match AB. The concentration of selected impurities are given below (Table 1) (Rutqvist et al., 2011). The levels for nitrosamines and benzo(a)pyrene (B(a)P) conform to the limits recommended by WHO (2 mg/kg for NNN plus NNK and 5 μg/kg for B(a)P; WHO, 2009).

Table 1
- Impurities in Swedish snus. Average observed levels in Swedish Match's products (for year 2009).

Component	Content	Component	Content
Nitrite (mg/kg)	2.0 (mg/kg)	Cadmium (mg/kg)	0.6 (mg/kg)
TSNA total	1.6 (mg/kg)	Lead	0.3 (mg/kg)
NDMA[a]	0.7 (μg/kg)	Arsenic	0.1 (mg/kg)
B(a)P	1.1 (μg/kg)	Nickel	1.3 (mg/kg)

[a] NDMA, N-nitrosodimethylamine.

The following batches of experimental snus with different properties were used in this study of the impact of physical properties on cellular proliferation in the rat forestomach:

1 pH 8.5 (nicotine, 2.5%) mixed particle size (< 315, 315–500, and > 500 μm)
2 pH 8.5 (nicotine, 1.2%) mixed particle size (< 315, 315–500, and > 500 μm)
3 pH 8.5 (nicotine, 0.75%) mixed particle size (< 315, 315–500, and > 500 μm)
4 pH 7.5 (nicotine, 1.2%) mixed particle size (< 315, 315–500, and > 500 μm)
5 pH 9.5 (nicotine, 1.2%) mixed particle size (< 315, 315–500, and > 500 μm)
6 pH 8.5 (nicotine, 1.2%) coaarse particle size (> 500 μm)
7 pH 8.5 (nicotine, 1.2%) medium particle size (315–500 μm)
8 pH 8.5 (nicotine, 1.2%) fine particle size (< 315 μm)
9 pH = 8.5 (nicotine 0.75%), commercial snus (General) mixed particle size (< 315, 315–500, and > 500 μm)

The prescribed pH levels were achieved by adding appropriate amounts of sodium carbonate to the experimental snus blends. The prescribed nicotine concentrations of 0.75% and 1.2% were achieved by using two different raw tobacco blends with the corresponding nicotine contents. The nicotine concentration of 2.5% was achieved by adding appropriate amounts of nicotine salt to the 1.2% blend. Both pH and nicotine content in the finished experimental snus products was confirmed analytically.

The variation of particle size distribution was achieved by sieving of the ground, raw tobacco. The intermediate size experimental blend was the one with the size distribution that most closely resembles that found in most commercial snus brands. The typical particle size was in the order of 2–400 μm and almost no particles exceeded 600 μm. The typical particle size in the “small particle size” blend was in the order of 50–150 μm with nearly no particles exceeding 300 μm. The “large particle size” blend had a typical particle size in the order of 500–700 μm but with a large variation ranging from 5 to 1000 μm.

For the other investigations commercial snus (General, Swedish Match) was used.

The tobacco was thoroughly homogenized with drinking water in a blender, mixed in a vortex blender, and used as such. At high concentrations the tobacco slurry separated into two phases, which was inappropriate for intubations. 10 g tobacco homogenized in 100 ml of H_2O was found to be appropriate. 4 animals per group are given by intubation of 1.5 ml snus suspensions 3 times a week for 6 weeks.

2.4. Blueberries (Vaccinium myrtillus)

As supplied by Frostab AB, Arvidsjaur, wild fresh berries were collected in the north of Sweden and freeze dried at the Institute for Plant Breeding at Balsgård, Swedish University of Agricultural Sciences,

Kristianstad. The berries were ground, and 10 g of the powder was mixed with 100 ml drinking water and used in the current study.

2.5. Cacao extract

Roasted cacao beans from Swisslion Takovo, Gornji Milanovac, were ground into a powder, of which 10 g was suspended in drinking water and heated 15–20 min at 50 °C to produce a liquor. The liquor was filtered through sterile gauze, and mixed with equal volumes of a slurry of pulverized tobacco and co-administered in the same gavage.

2.6. Chemical analysis

The total phenolic content of blueberries was performed using UPLC, where gallic acid was used for calibration. The total amount of polyphenols was about 20.2 mg/g dry weight.

2.7. Animals and treatments

2.7.1. Experimental snus

Seven weeks old male inbred Wistar rats (250 ± 10 g) from separate litters obtained from a colony at the Vinča breeding facility, were maintained under standard conditions, group-housed (4 per cage) on chip bedding with free access to food (pelleted commercial diet from the Veterinarian Institute at Subotica, Serbia) and tap water, regular 12 h light/12 h dark cycle and constant temperature (21 ± 2 °C) and humidity. Each individual animal was labeled with a unique identification code printed with markers on the earlobes and tail. Body weights were measured 3 times per week, and the animals observed daily for signs of toxicity and behavioral changes. Volumes of 1.5 ml containing the tested agents described below were intragastrically intubated 3 times a week, for 6 weeks, using reusable stainless steel feeding needles, 3 mm ball diameter (Cadence Inc. USA).

Flat-faced cylindrical matrices containing 50 mg of BrdU with a diameter of 5.6 mm were prepared by a direct compression technique using an eccentric tablet press Korsch (Korsch EK-0, Korsch, Berlin, Germany). The BrdU matrices were subcutaneously implemented in dorsal neck region under chloral hydrate anesthesia (Allen et al., 1978).

2.7.2. Reversibility of snus induced changes

In addition to 6 control animals, 18 male Wistar rats were administered 1.5 ml snus (10 g snus homogenized in 100 ml of drinking water) by intragastric intubation 3 times a week for 6 weeks. 6 animals were sacrificed on day 43, and the proliferation index, inflammatory markers and micronuclei were determined. After cessation of snus exposure, the remaining animals were put on a standard diet. Subsequently, 6 animals were sacrificed on the weeks 12th and 18th and the same analyses were repeated.

2.7.3. Protection of blueberries and cacao

Seven weeks old male Wistar rats were intragastrically intubated 3 times a week with slurry of snus supplemented with blueberries or cacao as described in section 2.4 and 2.5, for 10 weeks. Animals were sacrificed and target tissue analyzed as previously described, section 2.7.

10 g Samples of different types of Swedish snus were homogenized in 100 ml drinking water (ca. 0.5 g/kg bw), and 10 groups of 6 rats were treated with aliquots with characteristics described under “2.2 Snus products” including BHA for 6 weeks, a group given BHA, as well as an untreated control group. Sacrifice was done on day 43.

All procedures for treatment of the animals were approved by the Ethical Committee for the Use of Laboratory Animals of the Vinča according to the guidelines issued by the EU registered Serbian Laboratory Animal Science Association implementing the European Communities Council Directive of 24 November 1986 (86/609/EEC) as well as the rules for good laboratory practice established by EU and OECD. All animal experiments are under the guidance of an authorized veterinarian specialized in the conduction of animal experiments, and the principal investigators and technicians are authorized to perform experiments in animals.

2.8. Blood sampling

Rat blood samples were allowed to clot for 1 h under refrigeration. The samples were centrifuged for 20 min at 1000 g, and the serum divided in 4 cryotubes 0,5 ml each, and kept at −80 °C until measurement.

2.9. Autopsy and tissue preparation

The animals were sacrificed by decapitation 24 h after the last gavage treatment, and the stomachs with the parts of upper small intestine were removed. The forestomachs were separated (near the limiting ridge) and cut along the minor curvature. During target tissue separation, the animals' abdominal and thoracic organs were inspected for any pathological changes. After separation, forestomachs were washed in 0.9% aqueous sodium chloride, spread on paraffin substrate with pins and covered with 10% neutral buffered formalin for one week's fixation. Tissue blocks of the forestomach were cut transversely into serial sections 5 μm in thickness from the forestomach middle region. Two consecutive sections in the range of 250 mm between each level, were taken for analysis. The tissue sections were stained with hematoxylin and eosin for histological analysis and immunohistochemically for the incorporation of BrdU into DNA for analysis of cell proliferation. The analysis was done at magnifications 10x and 20x.

Because the forestomach-esophageal junction and the middle region were found to exhibit the highest levels of incorporation in our previous study (Nilsson et al., 2016), sections from the middle region were selected for the BrdU analysis.

2.10. Cell proliferation study

After deparaffinization in xylene, dehydration with a series of decreasing concentrations of ethanol, and rehydration in drinking water, the forestomach sections were stained immunohistochemically (Pharmingen™ BD Biosciences®, San Jose, CA, USA). Basically, the procedure involves the use of a monoclonal antibody against BrdU (anti-BrdU murine IgG), which is detected with a secondary antibody (biotinylated goat anti-mouse IgG) linking the primary antibody to a label (streptavidin-HRP) as the detection system, and 3,30-diaminobenzidine as chromogen for visualization of BrdU incorporation (BD Biosciences, 2014). The forestomach tissue sections incubated with normal serum instead of the primary antiserum were used for control staining. The sections were counterstained with Mayer's hematoxylin (Merck) for analysis under light microscope. Proliferating BrdU positive cells are found in the basal layer and granulose layers of squamous epithelium.

The number of immunoreactive cells in the squamous stratified epithelium was determined using a computer-supported imaging system connected to a light microscope with an objective magnification of 10x. BrdU positive cells were expressed per mm^2 of epithelium. The area of the epithelium was calculated using the following expression:

$$P = p \times d^2/10^6$$

Here, P is the surface area, p is the number of grid points that lie in the epithelium, and d is the size of the square network at a magnification of 200 (10x objective). The number of proliferating (BrdU) cells was expressed per mm^2 of epithelium, and calculated according to the expression $N = n/P$, where n is the number of BrdU positive cells on the presented surface, and P is the examined surface area of epithelium. From each animal tissue sections were scored in the forestomach-

middle region. In addition to the number of BrdU positive cells/mm^2, the ratio between dividing cells and the total number of cells per unit area (proliferation index, PI), expressed as percentage is given. This enabled a comparison to be made between the two manners of presentation.

2.11. Induction of micronuclei

6 rats were given commercial snus for up to 10 weeks. Micronuclei were scored in the forestomach tissue prepared as described in section 2.9.

For each sample 1000 BrdU stained cells were scored and micronuclei were recorded using a Zeiss microscope (400*X* or 1000*X*). The criteria for identifying micronuclei were as follows:1) BrdU positive staining with intensity similar to that of the nucleus (2) less than one-third of the diameter of the associated nucleus located outside the nucleus; (3) texture similar to that of the nucleus.

2.12. Effects on interleukins

Interleukins were determined in blood samples collected on day 43. For measurement of IL-1β, the Quantikine Elisa Immunoassay was used, a quantitative sandwich enzyme immunoassay that contains a polyclonal antibody specific for rat IL-1β, pre-coated onto a microplate, HRP conjugated secondary antibody and a colorimetric detection substrate. Concentration was determined spectrophotometrically according to standard curve using Tecan Sunrise microplate reader. For determination of IL-6, the Quantikine Elisa Immunoassay was used, Immunoassay was used, the design of which is similar to that for IL-1β.

2.13. Effects on oxidative stress

For measurement of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) in serum, the HT 8-oxo-dG ELISA Kit (Trevigen®, Gaithersburg, MD, USA), was used, which employs an anti-8-oxo-dG monoclonal mouse antibody, an HRP conjugated secondary antibody, and a colorimetric detection substrate. Concentration was determined spectrophotometry (Tecan Sunrise microplate reader).

2.14. Statistical analysis

The results are expressed as the mean ± SD. Values were compared using the nonparametric Mann-Whitney *U* test in the program SPSS 10 for Windows. The biostatisticians J.S. Fry and P.N. Lee, London, were consulted for an in depth analysis of the apparently complex association between nicotine concentration and PI. In addition to parametric statistics (F-test) and a Pearson correlation coefficient, the Spearman nonparametric correlation coefficient based on the ranks of the values, as well as the Kendall tau correlation coefficient were used.

3. Results

3.1. Body weight (BW) gain

There were no significant gross pathological alterations in controls or in the treated animals, nor was any evidence of an inflammatory response detected. As demonstrated in Table 2, the BW gain of the rats indicated no treatment related excessive general toxicity that could have been induced e.g. by nicotine. For samples 7 and 8 with 1.2% nicotine the BW gains in treated rats were actually higher than in controls. The small decrease in BW gain in the sample with 2.5% is statistically not significant. The observed variations for these small groups of animals were not significant (Mann-Whitney test), were within the normal range, and could not be related to the physical properties of the different batches.

3.2. Effects on proliferation of nicotine, pH and particle size

The following conclusions may be drawn with respect specific snus characteristics (Table 2):

pH in the range 7.5–9.5 did not influence PI, and the levels of 8-oxo-dG or IL-1β were unaffected. The data for IL-6 were characterized by high inter-individual variations. However, whereas BHA treatment increased this cytokine, the levels were significantly depressed by treatment with snus, where the value for sample 3 was exceptionally low.

For nicotine some differences at different concentrations were evident using both parametric and non-parametric analyses. Based on all 48 data points, parametric statistics (F-test) gave a significant positive correlation ($p = 0.0019$) with the increase in PI per percent nicotine, estimated as 5.514 (SE 1.678). This was confirmed using a Pearson correlation coefficient. However, an additional analysis using a Spearman non-parametric correlation coefficient based on the ranks of the values, the p value was no longer significant at $p = 0.1257$. Nor was it significant ($p = 0.1542$) using a Kendall tau correlation coefficient. Nevertheless, in comparison with snus containing 0.75%, the PI for samples with 2.5% nicotine was higher, although there was no significant increase between 0.75% and 1.2%.

The trend for augmented rates of cell divisions with increasing coarseness was statistically not significant, although the variations in PI in individual samples increased greatly with increasing particle size (Fig. 1). In contrast to snus with fine particles (< 315 μm) as well as BHA treatment, administration of coarse snus resulted in an uneven distribution of foci with high, respective low rates of cell proliferation (Fig. 2).

3.3. Induction of micronuclei (MN)

See Table 4.

Table 2
Body weight (BW) of the animals at the beginning and the end of 6 weeks of treatment with BHA or batches of snus with different characteristics.

Experimental batch No. (nicotine %; pH)	BW (g) 0 weeks of treatment	BW (g) 6 weeks of treatment	BW gain (g) at 6 weeks
Controls	255 ± 5	430 ± 22	165
BHA	255 ± 5	413 ± 40	158
1 (2.5%; pH 8.5) mixed particle size	247 ± 3	405 ± 35	158
2 (1.2%; pH 8.5) mixed particle size	243 ± 6	370 ± 36	127
3 (0.75%; pH 8.5) mixed particle size	250 ± 9	418 ± 31	168
4 (1.2%; pH 7.5) mixed particle size	250 ± 10	395 ± 36	145
5 (1.2%; pH 9.5) mixed particle size	243 ± 8	375 ± 38	132
6 (1.2%; pH 8.5) coaarse particle size	250 ± 5	385 ± 30	135
7 (1.2%; pH 8.5) medium particle size	250 ± 5	440 ± 17	190
8 (1.2%; pH 8.5) fine particle size	255 ± 0	445 ± 18	190
Commercial snus (0.75%; pH 8.5) mixed particle size	255 ± 5	400 ± 26	145

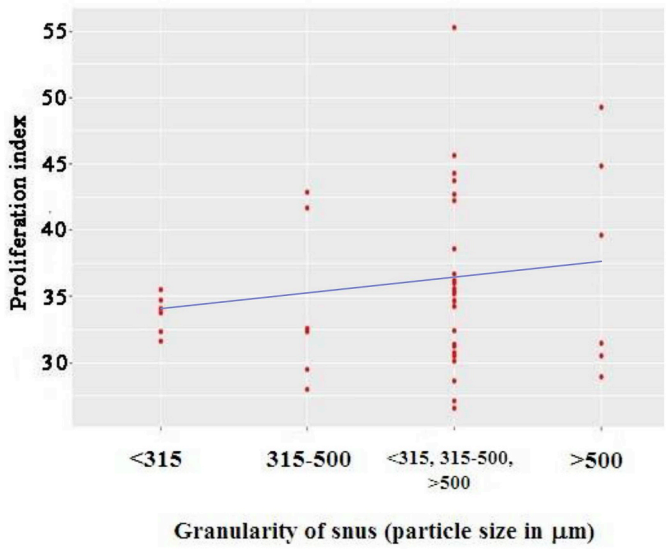

Fig. 1. Effect of tobacco particle size in the range < 315 to > 500 µm on the proliferation index (PI) in epithelial sections of the rat forestomach.

3.4. Protective effects

The protective effect of blueberries described before (Nilsson et al., 2016) could be verified in this study. In addition, an extract from cacao beans proved to be equally efficient in suppressing forestomach hyperplasia induced by commercial snus (Table 5, Fig. 3).

3.5. Reversibility

Already after 6 weeks recovery post-treatment, there was a significant reduction in the number of dividing cells, and after 12 weeks the values were identical with those seen for controls (Table 6). These results agree with the corresponding microphotographs (Fig. 4, magnification 40x).

4. Discussion

Based on promotion of cell division (proliferation index, PI) in the rat forestomach, the impact of Swedish smokeless tobacco (snus) was investigated systematically with respect to reversibility, nicotine content, pH, particle size, oxidative stress, the inflammatory interleukins IL-1β and IL-6. This seems to be the first study of this kind to be published. The non-genotoxic BHA as well as all snus samples induced cellular proliferation in the forestomach epithelia, and whereas pH did not seem to affect the rate of cell division, mechanical irritation, possibly in combination with nicotine, could represent main causes.

A clear cut result was obtained in the experiments related to reversibility (Fig. 5; Table 6). In line with human experience (Larsson et al., 1991), the enhanced cell proliferation caused by snus was found to be completely reversible. Further, there was a concordance between the effects on cell proliferation as measured by the uptake of BrdU in DNA and the impact on relevant molecular growth factors and biomarkers for cellular signaling. The results provide good evidence for the relevance of the animal model used.

Mainly because it has been relatively easy to analyze, 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo dG) is the most extensively studied marker of oxidative DNA damage. The absence of any impact on oxidative stress as monitored by 8-oxo dG, as well as on the levels of the proinflammatory cytokine IL-1β, was noteworthy. One possible explanation for the absence of an effect on 8-oxo dG could be that the forestomach tissue mass relative to total body mass was not rather adequate to detect oxidative stress biomarker in blood.

In users of smokeless tobacco an increased frequency of micronuclei (MN) in exfoliated cells from the oral cavity epithelia has been reported (Livingston et al., 1990; Trivedi et al., 1993; Ozkul et al., 1997). Unfortunately, the TSNA content of the tobacco used was not provided. The Swedish snus with a very low level of TSNA used in our study did not enhance the frequency of MN. The inhibition of MN induction caused by BHA can probably be ascribed to its antioxidant properties.

When combining three different characteristics of the tested snus (Table 3), this resulted in few data points for the batches with a low respective high nicotine content, as well as for snus with fine, intermediary and coarse granulation. The resource demanding nature of these studies posed a limit to the number of different types of snus that could be processed. There was a statistically non-significant trend for enhanced rates of cell divisions with increasing coarseness, There was a trend for enhanced rates of cell divisions with increasing coarseness, although the variations in PI in individual samples increased greatly with increasing particle size (Fig. 1). Whereas the SD of the PIs for finely granulated snus (particle size < 315 µm), as well as for BHA, were similar to that found for controls, the SDs were very large for snus with mixed particle size and granular size greater than 500 µm. An uneven distribution of focal centers with high and low rates of cell proliferation no doubt resulted in the highly variable PI values (Fig. 2). Scoring dividing cells in combined homogenates from different sections of the epithelium might provide a more coherent picture.

EFSA (2009) has concluded that nicotine is not a carcinogen, but a higher pH in smokeless tobacco has been reported to increase nicotine uptake (Pickworth et al., 2014). Sanner and Grimsrud (2015) have speculated that nicotine may enhance the induction of cancer by endogenous conversion to tobacco specific nitrosamines (TSNA), and/or by acting as a promoter. In comparison with the TSNA levels present in snus, not to mention the doses of TSNA required in bioassays to induce tumors, the actual conversion of nicotine to NNK (and NNN) is negligible in experimental animals (Carmella et al., 1997) as well as in humans (Hatsukami et al., 2004; Stepanov et al., 2009). Further, our study as well as *in vivo* data from adequately performed studies on promotion (Murphy et al., 2011) provide little evidence to support the claims of Sanner and Grimsrud (2015).

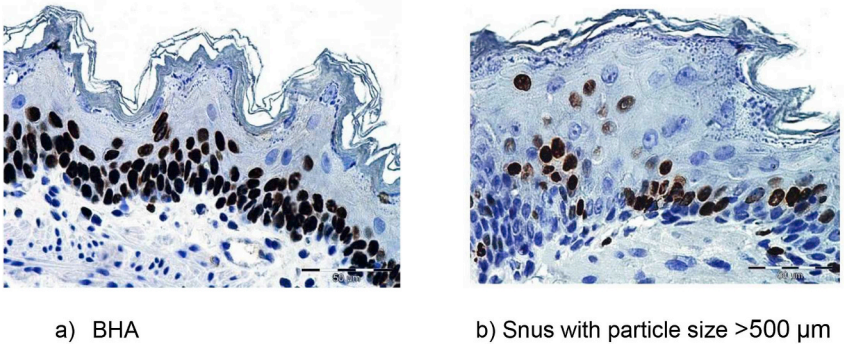

Fig. 2. Microphotographs showing the effects of treatment with (a) BHA for 6 weeks as compared to treatment with (b) commercial snus for 6 weeks with a particle size > 500 µm. Black-brown colored cells represent proliferative (BrdU incorporated) cells. (Magnification 40x). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 3
Proliferation indices (PI), levels of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG), interleukins IL1 *beta* and IL6 for the various types of experimental snus administered for 6 weeks. Bromodeoxyuridine (BrdU) incorporation was determined in the forestomach mid region. Butylated hydroxyanisole (BHA) served as positive control.

Experimental batch No. (nicotine %; pH)	*PI*	*8-oxo-dG. nM*	*IL-1β pg/mL*	*IL-6 pg/ml*
Controls	*23.37 ± 3.1a*	*0.88 ± 0.12*	*50.66 ± 4.82*	*45.40 ± 14.49*
1 (2.5%; pH 8.5) mixed particle size	*43.05 ± 7.04b*	*1.09 ± 0.45*	*47.73 ± 2.21*	*25.50 ± 4.59*
2 (1.2%; pH 8.5) mixed particle size	*33.64 ± 2.26c*	*1.18 ± 0.07*	*54.91 ± 2.77*	*18.83 ± 3.37*
3 (0.75; pH 8.5) mixed particle size	*35.14 ± 2.39d*	*1.34 ± 0.98*	*49.11 ± 1.03*	*7 ± 1.55*
4 (1.2%; pH 7.5) mixed particle size	*32.16 ± 2.27*	*1.03 ± 0.04*	*46.45 ± 1.88*	*23.08 ± 6.03*
5 (1.2%; pH 9.5) mixed particle size	*34.8 ± 8.4*	*0.82 ± 0.16*	*49.53 ± 2.75*	*25.83 ± 8.9*
6 (1.2%; pH 8.5) coarse particle size	*37.43 ± 8.45e*	*1.03 ± 0.16*	*50.55 ± 0.96*	*41.2 ± 3.37*
7 (1.2%; pH 8.5) medium particle size	*31.62 ± 6.29*	*0.54 ± 0.13*	*49.71 ± 2.43*	*14.83 ± 5.54*
8 (1.2%; pH 8.5) fine particle size	*33.67 ± 1.47*	*0.76 ± 0.03*	*49.91 ± 0.36*	*18.0 ± 0*
Commercial snus (0.75%; pH 8.5) mixed particle size	*37.24 ± 2.69f*	*1.06 ± 0.18*	*49.4 ± 1.76*	*32.5 ± 11*
BHA	*73.12 ± 2.61g*	*1.13 ± 0.02*	*55.8 ± 1.39*	*64.33 ± 3.18h*

[a] $p < 0,01$, control vs. Experimental batches 1-8.
[b] $p < 0.001$, Experimental batch 1 vs. Experimental batches 2–5, 7 and 8.
[c] $p < 0.01$, Experimental batch 1 vs. Experimental batches 5 and 6.
[d] $p < 0.05$, Experimental batch 3 vs. Experimental batch 6.
[e] $p < 0.01$, Experimental batch 6 vs. Experimental batches 4 and 7.
[f] $p < 0.025$ Commercial snus vs. Experimental snus 1.
[g] $p < 0.001$, BHA vs. Experimental batches 1-8.
[h] $p < 0.01$, BHA vs. Experimental batches 1-8.

Table 4
Induction of micronuclei (MN) in the rat forestomach epithelium after treatment with commercial snus for 4, 6, 8 and 10 weeks in comparison with controls and BHA (10 weeks).

Duration of treatment	Incidence of MN per 1000 BrdU stained cells (SNUS)	Incidence of MN per 1000 BrdU stained cells (BHA)
0 (Control)	0,0028	0,0038
4 weeks	0,0036	
6 weeks	0,0036	
8 weeks	0,004	
10 weeks	0,0036	0,0018

The MN frequencies were low upon exposure to snus, and there was no significant trend (Man-Whitney test). The depression induced by BHA also was statistically insignificant ($p < 0.248$).

In comparison with snus containing 0.75% nicotine there was a small, but statistically significant increase of PI for exposure to snus with 2.5% nicotine, although no significant impact on PI could be found in the range 0.75% and 1.2%. However, it may be premature to draw definite conclusions about the dose-effect relationship in view of the fact that 36 animals were treated with snus with nicotine 1.2%, but only groups of 6 animals were exposed each to 0.75% and 2.5%. Under our experimental conditions, pH in the range 7.5–9.5 did not influence PI, and there was no indication of increased oxidative stress as measured by 8-oxo dG.

Snus-induced hyperplasia could be expected to be accompanied by an inflammatory response. For this reason the cytokines interleukin 1 and 6 were monitored. However, the levels of inflammatory cytokine IL-1β were not affected. The positive control BHA enhanced the production of IL-6, and it was expected that tobacco would have a similar action, but the effect was found to be the opposite. This can most probably be related to the presence of nicotine in snus. Thus, it has been reported that nicotine suppresses IL-6 production from vascular endothelial cells (Tarantula et al., 2010), as well as in lipopolysaccharide activated human coronary artery endothelial cells and pulmonary monocytes (Patton et al., 2006).

Dietary cacao flavanols improve major hallmarks of cardiovascular function including blood pressure and blood cholesterol, and the positive effects in humans have been unequivocally established and recognized by EU (EFSA, 2014; EU, 2015). We have demonstrated that cacao, in addition, has a protective effect against snus-induced cell proliferation in the rat forestomach which equals that of the blueberries from northern Sweden (Fig. 3; Table 5), which had a considerably higher concentration of polyphenols than e.g. found in cultivated blueberries (Sellappan et al., 2002).

5. Conclusions

Low nitrosamine Swedish snus, as well as non-genotoxic butylated hydroxyanisole (BHA), induce increased cellular proliferation in the rat forestomach epithelia. Snus with different properties, such as pH, nicotine and particle size, had no significant impact on oxidative stress as determined by 8-oxo-7,8-dihydro-2′-deoxyguanosine, or on interleukin IL-1β. Whereas BHA boosted IL-6, it was depressed for all snus samples, probably due to the presence of nicotine. There was a statistically non-significant trend of increased cell divisions with increasing particle

Table 5
Protective effects against hyperplastic effects induced by commercial snus in epithelial sections of the rat forestomach by blueberries and a cacao extract, and measured as proliferation index (PI). Bromodeoxyuridine (BrdU) incorporation was determined in the forestomach mid region. Butylated hydroxyanisole (BHA) served as positive control.

Group	PI	8-oxo dG nM	IL-1β pg/mL	IL-6 pg/mL
Snus 10 weeks	42.15 ± 8.16	1.57 ± 0.44	78.71 ± 5.4	76.52 ± 9.69
Snus + Blueberries	26.52 ± 5.52[a]	0.93 ± 0.13	52.27 ± 2.56	38.00 ± 21.94
Blueberries	16.68 ± 0.86	1.02 ± 0.16	55.69 ± 2.53	26.5 ± 3.83
Snus + Cacao	26.92 ± 0.89[a]	1.25 ± 0.07	53.54 ± 1.95	32.08 ± 13.09
Cacao	27.99 ± 1.28	1.05 ± 0.18	54.13 ± 3.04	22.67 ± 4.5
BHA 10 weeks	82.94 ± 7	1.30 ± 0.01	51.86 ± 2.10	45.5 ± 8.28

[a] $p < 0.001$, significantly lower than snus 6 weeks.

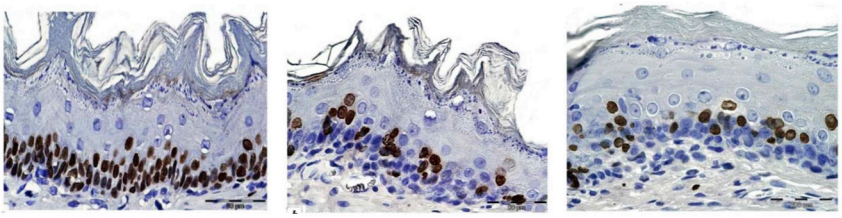

Fig. 3. Microphotographs showing the effects of treatment with commercial snus for 10 weeks with and without and protective compounds. Black-brown colored cells represent proliferative (BrdU incorporated) cells. (magnification 40x). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 6
– Reversibility of hyperplastic effects induced by commercial snus in epithelial sections of the rat forestomach upon 6 weeks treatment. Recovery was measured as changes in the proliferation index (PI) after 12 weeks recovery. Bromodeoxyuridine (BrdU) incorporation was determined in the forestomach mid region. Butylated hydroxyanisole (BHA) served as positive control.

Group	PI	8-oxo dG. nM	IL-1β pg/mL	IL-6 pg/mL
Controls	23.27 ± 0.84	0.88 ± 0.12	50.66 ± 4.82	45.4 ± 14.49
Snus 6 weeks	37.24 ± 2.69[a,b]	1.06 ± 0.18	49.4 ± 1.76	32.5 ± 11
Recovery 6 weeks	29.31 ± 4.17[c]	0.94 ± 0.09	53.07 ± 3.39	131.20 ± 71.66[d]
Recovery 12 weeks	23.59 ± 3.06	1.26 ± 0.31	51.07 ± 0.97	30.30 ± 19.87
BHA 6 weeks	73.12 ± 2.61	1.13 ± 0.02	55.80 ± 1.39	64.33 ± 3.18

[a] $p < 0.01$, Snus 6 weeks' vs. recovery 6 weeks.
[b] $p < 0.001$, Snus 6 weeks vs. recovery 6 weeks, control and BHA 6 weeks.
[c] $p < 0.01$, Recovery 6 weeks vs. recovery 12 weeks.
[d] $p < 0.001$, Recovery 6 weeks vs. control, snus 6 weeks, recovery 12 weeks and BHA 6 weeks.

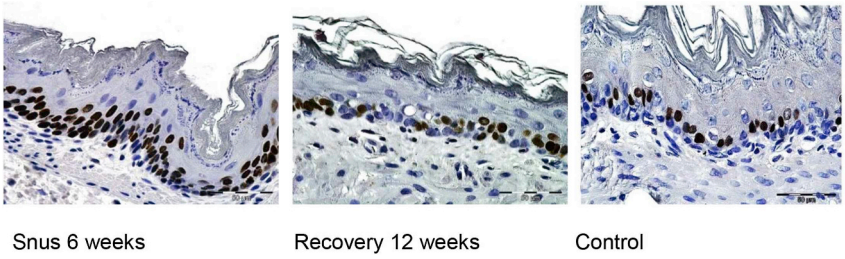

Fig. 4. Microphotographs showing 12 week's recovery post-treatment with commercial snus for 6 weeks. Black-brown colored cells represent proliferative (BrdU incorporated) cells. (magnification 40x). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

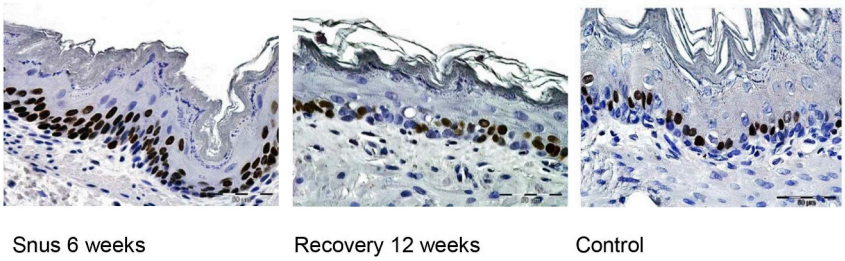

Fig. 5. Microphotographs showing 12 week's recovery post-treatment with commercial snus for 6 weeks (magnification 40x).

coarseness, although the variations in proliferation index increased greatly with increasing particle size, evidently due an uneven distribution of foci with a high rate of cell proliferation. In comparison with snus containing 0.75%, the rate of cell proliferation was higher for samples with 2.5% nicotine, although there was no significant increase between 0.75% and 1.2%. Administration of snus for up to 10 weeks resulted in a moderate increase in the frequency of micronuclei.

In line with human experience, the enhanced cell proliferation caused by snus was found to be completely reversible. A cacao bean extract had a protective action similar to that previously found for blueberries.

The main cause of the observed tobacco induced cell proliferation seems to be mechanical irritation, possibly in combination with nicotine.

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Transparency document

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