The effect of Swedish and American smokeless tobacco extract on periodontal ligament fibroblasts *in vitro*

Gunilla Andersson[2], Åsa Wahlin[1,3], Gunilla Bratthall[1]

Abstract

Use of moist snuff is widespread in Sweden. In 2004 ≈800 000 Swedes were daily users which corresponds to 22 % of the male population and 3 % of the female population. The aim of the present study was to evaluate the effect of Swedish moist snuff extract on PDL fibroblast growth and hard tissue production and compare with moist snuff extract from USA.

Periodontal ligament cells (PDL-cells) were obtained from 3 healthy subjects (1 female 14 years, 2 males 14 and 17 years) from the root surface of premolars extracted for orthodontic reasons. The cells were isolated from explants and grown in Dulbecco's Modified Eagle's Medium® (DMEM) supplemented with 10 % fetal calf serum (FBS) and cultivated in 37°C with 5 % CO_2 in air.

Snuff extract in concentrations 0.3 %, 1 % and 3 % (in DMEM with 1 % FBS) was tested. Cells from each individual were tested three times, each time in triplicate. Photographs were taken at 0 and 24 hours with a digital camera and analysed in terms of growth and morphology. Then the cell suspension was frozen and later thawed for examination of the production of alkaline phosphatase after exposure to different snuff concentrations.

This *in vitro* study has shown that PDL cells from 3 different subjects demonstrated a reduced number of cells at exposure to 3 % of both Swedish and American snuff extract. The production of alkaline phosphatase after 2 hours was similarly reduced from cells exposed to 3 % snuff extract.

Further studies have to be made to understand the effect of smokeless tobacco on periodontal tissues. However, from this study can be concluded that smokeless tobacco has biological effects in terms of reduced PDL cell growth and production of alkaline phosphatase

Key words

Smokeless tobacco, periodontal ligament fibroblasts, cell/tissue incubator, alkaline phosphatase

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Effekt av snusextrakt på paradontalligamenterceller

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Sammanfattning

I Sverige är bruket av vått snus mycket vanligt. År 2004 snusade ≈800 000 svenskar, varav 22 % av männen och 3 % av kvinnorna använde snus regelbundet. Målet med föreliggande arbete var att studera effekten av svenskt vått snusextrakt på parodontalligamentceller (PDL-celler) avseende växt och hårdvävnadsproduktion och jämföra det med vått snusextrakt från USA.

PDL-celler utvanns från 3 friska individer (1 flicka 14 år och 2 pojkar 14 och 17 år) från rotytan på premolarer, som skulle extraheras av ortodontiska skäl. Cellerna isolerades från vävnadsbitar, "explants", och fick tillväxa i Dulbecco's Modified Eagle's Medium® (DMEM) med tillskott av 10 % fetalt kalvserum (FBS) and odlades i 37°C med 5 % CO_2 i luft.

Snusextrakt i koncentrationerna 0,3 %, 1 %, 3 %, testades i DMEM med 1 % FBS.

Celler från varje individ testades 3 gånger, varje gång i triplikat. Foton togs vid 0 och 24 timmar med en digitalkamera och analyserades avseende förändringar i växt och morfologi. Sedan frystes cellsuspensionen och tinades senare för undersökning av produktion av alkaliskt fosfatas efter exponering för olika snuskoncentrationer.

Denna *in vitro*-studie har visat att PDL celler från 3 olika individer uppvisade ett reducerat antal celler vid exponering för 3 % av både svenskt och amerikanskt snusextrakt.

Introduction

Several studies have shown an association between smoking and loss of periodontal attachment and bone destruction (5, 13). Nicotine, which is a major component of cigarette smoking, can be detected on root surfaces of periodontally affected teeth (11). Studies on the effect of nicotine on human periodontal ligament (PDL) fibroblasts show, that nicotine can affect functions of these cells *in vitro* in a dose-dependent manner (7).

Smokeless tobacco in different forms such as chewing tobacco, dry snuff and moist snuff is used worldwide. Use of moist snuff is widespread in Sweden. In 2004 ≈800 000 Swedes were daily users which corresponds to 22 % of the male population and 3 % of the female population (18). The effect of smokeless tobacco on periodontal health has mainly focused on attachment loss manifested as gingival recessions at the site were the tobacco is regularly placed adjacent to the snuff dipper's lesion (2, 4, 10, 17, 19, 20). The recession has been postulated to be a result of mechanical trauma. However, in a more recent *in vitro* study it was shown that smokeless tobacco extract can stimulate peripheral monocyte cells to produce prostaglandins (6). In a study from USA aqueous extracts of chewing tobacco, dry and moist snuff was found to induce inflammatory changes in endothelial cells that may promote recruitment of leukocytes (9). No studies on the effect of extract from Swedish moist snuff on periodontal ligament fibroblasts *in vitro* have been published.

The aim of the present study was to evaluate the effect of Swedish moist snuff extract on PDL fibroblast growth and hard tissue production and compare with moist snuff extract from USA.

Material and methods

Preparation of PDL cells

Periodontal ligament cells (PDL-cells) were obtained from the root surface of premolars extracted for orthodontic reasons from healthy patients, 14-17 years of age. The patients and their parents were informed and the parents gave written consent. The study was approved by the Human Ethical Committee at Lund University, Lund, Sweden.

The teeth were extracted under sterile conditions and washed in phosphate buffered saline (PBS) with 1 % Penicillin Streptomycin solution® (PEST) and 0.5 % Gentamicin (10 mg/ml). Then the periodontal ligament was gently scraped off from the middle third of the root surface to avoid contamination from the gingival and apical tissues. The tissue explants were transferred to 21 cm^2 cell culture dishes with Dulbecco's Modified Eagle's Medium® (DMEM) supplemented with 10 % fetal calf serum (FBS), 1 % PEST and 0.5 % Gentamicin. The explants were covered with sterile glass slips and the dishes were placed in a cell/tissue incubator in 37° C with 5 % CO_2 in air for one week. The cells were allowed to migrate from the explants and after reaching about 50 % confluence the cells were washed twice with PBS, and trypsinated with Trypsin - EDTA solution® (0.5 g trypsin, 0.2 g EDTA/l). Then DMEM was added and the suspension was centrifuged at 1000 rpm for 5 minutes. The pellet was suspended in supplemented DMEM and transferred to cell culture flasks (passage 1). After passage 3 the cells were trypsinated and frozen at -86°C in 69 % DMEM, 20 % FBS, 1 % PEST and 10 % dimethyl sulfoxide, DMSO (HybriMax®), in a concentration of roughly 500 000 cells per ml medium.

Preparation of snuff extract

Swedish moist snuff (Ettan Gothia Tobak AB, Sweden) with a pH of 8.5, water content 55 % and nicotine level of 2.1 % and Kentucky reference snuff (the only available American moist snuff for preparing extract) with a pH of 7.2, water content of 52 % and nicotine level of 2.6 % was used in the preparation of snuff extract. A pH of 8.5 is valid for the three largest brands in Sweden (Ettan, Grovsnus, General). The extracts were made by a standardized procedure (12). A slurry of 100 g snuff in 300 ml distilled water was agitated for 1 hour and then centrifuged at 3500 rpm for 30 minutes. The supernatant was filtered through a 00H filter (Munktell, Stora Grycksbo, Sweden) and then through a 0.2 mm filter (Vacucap 90, Gelman Sciences, KEBO, Stockholm, Sweden). The sterility of the solution was checked by inoculation on agar plates. The extract was stored frozen at -20°C in 2 ml aliquots each of which was subsequently thawed and used in the experiments.

In the extract from Swedish snuff the tobacco specific nitrosamines (TSNA) level was 0.3 µg/ml (0.3 ppm) and the nicotine content 10.6 mg/ml (1.06 %). The corresponding figures for extract from American snuff was a TSNA level of 1.5 µg/ml (1.5 ppm) and a nicotine content 15.8 mg/ml (1.58 %).

In the experiments the snuff extract was diluted in DMEM in the following concentrations: 1µl/ml, 3µl/ml, 10µl/ml, 30µl/ml and 100µl/ml as described below.

Biochemical analyses

The production of alkaline phosphatase after snuff

exposure of PDL cells was determined with an ELISA assay according to *Morishita et al.* (16) and read in a spectrophotometer at 405 nm. The amount of protein was examined according to Bio-Rad Laboratories protein assay (Richmond, CA).

Experimental design

In a *first series of experiments* the effect of different tobacco concentrations on PDL cells from one subject (female 13 years) after different times of exposure was established. The effect of serum free medium was also examined. Cells from passage 3 were used.

PDL cells were plated at 30 000 cells per well and grown in DMEM with 10 % FBS or 4 % bovine serum albumin (BSA). Smokeless tobacco extracts in the concentrations 0.1 %, 3 %, 1 %, 3 % and 10 % together with a negative control (culturing media) were tested. All tests were run in triplicate and photographs were taken at 0, 1, 3, 6, 9, 24 and 48 hours using a digital camera. The photographs were analysed for changes in growth and morphology and the production of alkaline phosphatase 48 hours after snuff exposure in DMEM with 4 % BSA was measured.

Low concentrations 0.1 % and 0.3 % of Swedish and American snuff extract showed an increased number of PDL cells when grown in DMEM with 10 % FBS but not in DMEM with BSA. Vacuolisation was recorded after 24 and 48 hours with 1 % snuff extract in serum free media. This was not recorded in the presence of 10 % FBS. Exposure to 10 % snuff extract caused cell death after 3 hours. After 9 hours all cells were dead.

In this experiment the production of alkaline phosphatase was reduced after exposure to 3 % snuff extract whereas 0.3 % stimulated the production compared to a negative control.

Based on the findings above Swedish and American smokeless tobacco extract in concentrations 0.3 %, 1 % and 3 % (in DMEM with 1 % FBS) was tested. Cells (passage 3) from lower premolars of three different individuals (2 females 14 years, 1 male, 17 years) were used. All experiments were run 3 times, each time in triplicates and performed according to the description below.

Day 0. PDL cells were thawed and diluted with DMEM with 10 % FBS and applied in 9.5 cm^2 microtiter plates. The cells were cultivated in 37°C with 5 % CO_2 in air and the concentrations of cells per well differed between 108 000 - 130 000 in the different runs.

Day 1. The medium was changed to new DMEM with 10 % FBS

Day 2. The medium was changed to DMEM with 1 % FBS

Day 3. The medium was removed from the wells and the different concentrations of smokeless Swedish and Kentucky tobacco was added. DMEM with 1 % FBS was used as a negative control. All wells were photographed in 200 x magnification with a digital camera (Olympus DP 11). The pH of the medium and different snuff concentrations varied between 7.50 and 7.55 for Swedish smokeless tobacco compared to 7.16 - 7.50 for Kentucky reference tobacco.

Day 4. All wells were photographed 24 hours after exposure of tobacco extract.

Then the wells were washed twice with PBS and trypsinated with 1.5 ml trypsin-EDTA solution®, 37°C, to each well. Besides, 3 ml DMEM with 10 % FBS was added to each well, the material was transferred to 15 ml test tubes and centrifuged at 1000 rpm for 5 minutes. The pellets from each concentration were pooled and mixed with 500 µl 0.1 % Triton in diethanolamine buffer (DEA, 1 M/l diethanolamine, 0.5 mM/l Mg Cl_2, pH 9.8) and frozen in -18°C.

For the production of alkaline phosphatase and the amount of protein this cell suspension was thawed, diluted 1:2 with DEA buffer and 1 ml of alkaline phosphate substrate (1mg/ml) was added. The amount of alkaline phosphatase/amount of protein was recorded at baseline and after 30 minutes and 2 hours.

Statistical methods

The comparison against control is made by means of the Wilcoxon signed rank test (two-sided). The comparison between Swedish and American snuff is made by means of the Wilcoxon rank sum test (two-sided). A p-value less than 0.05 is considered statistically significant. Each experiment was used as the unit for analyses.

Results

The photographs were evaluated by 3 calibrated examiners and the result based on the findings of at least 2 observers and 2 wells.

Patient 1, female, 14 years

Control - increased number of cells after 24 hours.

0.3 % snuff extract - unchanged number of cells after exposure to both Swedish and American smokeless tobacco.

1 % snuff extract - unchanged number of cells after

exposure to Swedish snuff extract; increased number of cells after exposure to Kentucky reference snuff.
3 % snuff extract – reduced number of cells after exposure to both Swedish and American smokeless tobacco.

Patient 2, male, 17 years

Control - unchanged number of cells after 24 hours.
0.3 % snuff extract - unchanged number of cells after exposure to both Swedish and American smokeless tobacco.
1 % snuff extract– unchanged number of cells after exposure to both Swedish and American smokeless tobacco.
3 % snuff extract– reduced number of cells after exposure to both Swedish and American smokeless tobacco.

Patient 3, female 14 years

Control - increased number of cells after 24 hours.
0.3 % snuff extract - increased number of cells after exposure to Swedish snuff extract; unchanged number of cells after exposure to Kentucky reference snuff.
1 % snuff extract - unchanged number of cells after exposure to Swedish snuff extract; increased number of cells after exposure to American snuff extract.
3 % snuff extract reduced number of cells after exposure to both Swedish and American smokeless tobacco.

Comparison and conclusion of cell growth

In PDL cells from two subjects cell cultivation for 24 hours without snuff extract (control) showed an increased number of cells, where exposures to 0.3 % snuff extract either were unchanged or increased. Further an increased number of cells were seen also at exposure of 1 % American snuff extract
In PDL cells from one subject there was no change either in the control, the 0.3 % extract exposure or the 1 % exposure.

A reduced number of cells at exposure for 3 % of both Swedish and American snuff extract was demonstrated in PDL cells from all three subjects.

In this experiment with 1 % FBS in the culture medium no clear-cut difference could be demonstrated between Swedish and Kentucky reference snuff in terms of cell growth.

Figr. shows representative photographs of PDL cells after 24 hours incubation with different concentrations of Swedish and Kentucky reference snuff. DMEM with 1 % FBS from the same culture plate was used as a negative control.

Alkaline phosphatase

Exposure to 0.3 and 1 % Swedish and American

Table 1. The production of alkaline phosphatase/amount of protein compared to the control (Ctr) after 30 and 120 minutes' incubation of cells earlier exposed to Swedish (Sw.) and American (Am.) snuff extract.

		Sw.		Sw. Ctr		Am.		Am. Ctr		Sw-Ctr[1]		Am-Ctr[2]	
		Mean	Std	Mean	Std	Mean	Std	Mean	Std	Mean	Std	Mean	Std
Conc.	Time												
0.3%	30	1.01	0.20	0.98	0.16	1.03	0.24	1.01	0.19	0.03	0.15	0.02	0.07
		P-value								0.5781		0.2461	
										0.8590			
	120	3.16	0.48	3.00	0.33	3.14	0.55	3.03	0.39	0.16	0.43	0.11	0.21
		P-value								0.2031		0.1289	
										0.7239			
1%	30	0.97	0.23	0.98	0.16	1.01	0.23	1.01	0.19	-0.01	0.20	-0.00	0.08
		P-value								0.7422		0.8750	
										0.6910			
	120	3.00	0.60	3.00	0.33	3.05	0.46	3.03	0.39	-0.00	0.56	0.02	0.23
		P-value								0.8203		0.9102	
										0.6588			
3%	30	0.87	0.16	0.98	0.16	0.93	0.18	1.01	0.19	-0.11	0.09	-0.08	0.04
		P-value								0.0039		0.0039	
										1.0000			
	120	2.70	0.36	3.00	0.33	2.76	0.42	30.03	0.39	-0.30	0.25	-0.27	0.12
		P-value								0.0039		0.0039	
										0.5959			

[1] Swedish snuff - Swedish control
[2] American snuff - American control

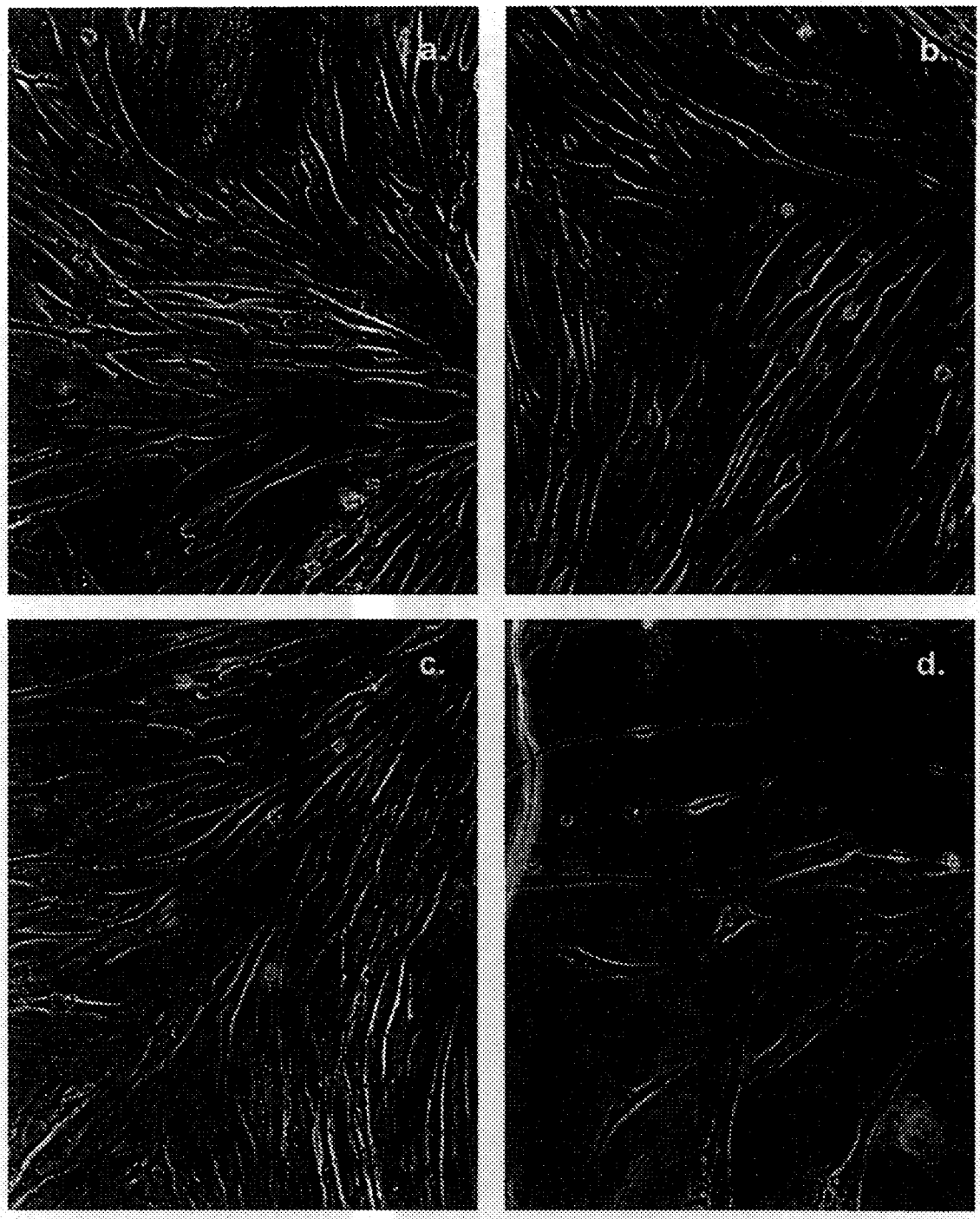
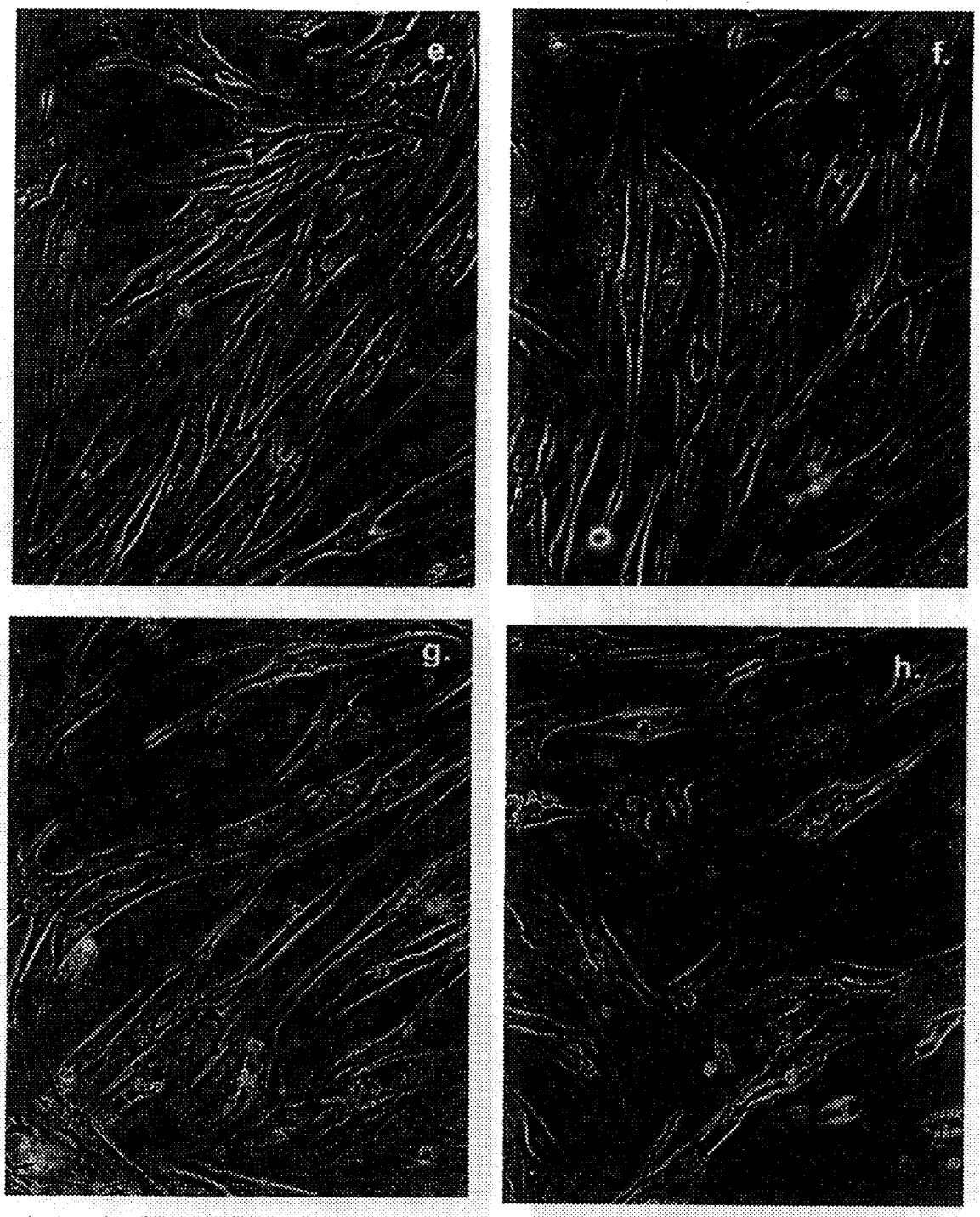


Figure 1. Representative photographs of PDL cells after 24 hours incubation with different concentrations of Swedish and Kentucky reference snuff. DMEM with 1% FBS from the same culture plate was used as a negative control.

a-d. Swedish snuff.
a. negative control b. 0.3 %, c. 1 % and d. 3 % snuff extract



e-h. Kentucky reference snuff
e. negative control, f. 0.3 %, g. 1% and h. 3 % snuff extract.

snuff extract did not show any statistically significant difference in alkaline phosphatase production compared to the control at 30 minutes or two hours incubation. Nor was there any significant difference between Swedish and American snuff extract. Exposure to 3 % snuff extract, however, resulted in a statistically significant reduction in alkaline phosphatase/amount protein both after 30 minutes ($p=0.0039$) and 2 hours ($p=0.0039$) with no difference between Swedish and American snuff extract (Table 1.)

Discussion

In the present study human periodontal ligament (PDL) cells have been exposed to different concentrations of Swedish and Kentucky reference snuff extract. There was no difference between Swedish and Kentucky smokeless tobacco concerning morphological changes and production of alkaline phosphatase from PDL cells in this *in vitro* model.

These results can partly be compared to *Johnson et al.* (14) who examined the effect of an aqueous smokeless tobacco extract (STE) on gingival keratinocyte production of prostaglandin E2 (PGE2) and interleukin-1 (IL-1), mediators involved in periodontal destruction and keratinocyte proliferation. Ten % STE significantly ($p < 0.05$) depressed cell numbers and viability. Lower tobacco concentrations did not affect cell numbers or viability, but significantly ($p < 0.05$) increased PGE2 and IL-1 levels.

Alpar et al. (1) studied the cytotoxicity of nicotine on human primary oral fibroblast cultures and found that the toxic effects of nicotine became irreversible in the range between 10.5 and 15.5 mM, whereas at lower concentrations cells recovered after the withdrawal of nicotine. The acute effects of nicotine on the formation and resorption of bone were examined in cultures of different rat osteogenic cells at concentrations that occur in the saliva of smokeless tobacco users (22) The results suggested that nicotine might have critical effects on bone metabolism.

Nicotine is absorbed into the blood stream via transfer across various oral membranes including the buccal mucosa. Observations by *Furie et al.* (9) suggest that smokeless tobacco may induce inflammatory changes *in vivo* by activating endothelium in a manner that promotes recruitment of leukocytes, which may contribute to tissue damage. A study by *Mavropoulus et al.* (15) showed that acute exposure of snuff induced a rapid increase in gingival blood flow that was higher than the increase in blood pressure, indicating an active vasodilatation. In a recent study on Swedish snuff it was shown that nicotine is one of the substances in snuff that has a biological effect on the oral mucosa (3) which also seems to be valid for PDL cells. The results of the present *in vitro* study have shown that PDL cells from different subjects demonstrated a reduced number of cells at exposure for 3 % of both Swedish and American snuff extract. The production of alkaline phosphatase was similarly reduced from cells exposed to 3 % snuff extract .

In the manufacturing of moist snuff in Sweden a heat treatment process is used while a fermentation process is used in USA. The American snuff has a lower pH but somewhat higher nicotine content than the Swedish product. Since more nicotine is free if the snuff has a higher pH value the biological effects of nicotine on the PDL cells in the two products should be similar as found in the present study. This is also in accordance with in vitro studies from Sweden where the results implied minor or no differences between the snuff extract from Sweden and USA in terms of direct toxicity (21).

The most prevalent effects of smokeless tobacco are localized to the site of placement, in the form of gingival recession and white mucosal lesions. Whether the use of smokeless tobacco may affect periodontal tissues in general is not known, even though a recent epidemiological study has discussed a relationship between smokeless tobacco and periodontal disease. In a US population-based study of 12932 adults participating in the Third National Health and Nutrition Examination Survey the association between smokeless tobacco use and severe active periodontal disease, defined as an individual having at least 1 tooth with 6 mm or more attachment loss and bleeding on the same tooth, was evaluated (8). It was reported that adults currently using smokeless tobacco were twice as likely to have severe active periodontal disease as adults who never used smokeless tobacco. The authors conclude that their results indicate that smokeless tobacco may be an important risk factor for severe active periodontal disease.

Further studies have to be made to understand the effect of smokeless tobacco on periodontal tissues. However, from this study can be concluded that smokeless tobacco has biological effects in terms of reduced PDL cell growth and production of alkaline phosphatase.

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