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Adverse effects of Sudanese *toombak* vs. Swedish snuff on human oral cells

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BACKGROUND: The high incidence of oral cancer in Sudan has been associated with the use of *toombak*, the local type of smokeless tobacco. However, its specific effects on human oral cells are not known. We aimed to investigate the effects of *toombak* on primary normal human oral keratinocytes, fibroblasts, and a dysplastic oral keratinocytic cell line, and to compare them with the effects induced by Swedish snuff.

METHOD: Aqueous extracts were prepared from moist *toombak* and Swedish snuff and added in serial dilutions on *in vitro* monolayer cultured cells. Cell viability, morphology and growth, DNA double-strand breaks (γH2AX staining), expression of phosphatidylserine (Annexin V staining), and cell cycle were assessed after various exposure time periods.

RESULTS: Significant decrease in cell number, occurrence of DNA double-strain breaks, morphological and biochemical signs of programmed cell death were detected in all oral cell types exposed to clinically relevant dilutions of *toombak* extract, although to a lesser extent in normal oral fibroblasts and dysplastic keratinocytes. G2/M-block was also detected in normal oral keratinocytes and fibroblasts exposed to clinically relevant dilutions of *toombak* extract. Swedish snuff extract had less adverse effects on oral cells, mainly at non-clinically relevant dilutions.

CONCLUSION: This study indicates a potential for *toombak*, higher than for Swedish snuff, to damage human oral epithelium. Dysplastic oral keratinocytes were less sensitive than their normal counterparts, suggesting that they might have acquired a partially resistant phenotype to *toombak*-induced cytotoxic effects while still being prone to DNA damage that could lead to further malignant progression.

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Introduction

There is a global increase in the incidence and mortality rates of head and neck cancer (1, 2). The incidence varies, however, world-wide, with developing countries showing a higher incidence compared with the developed ones (1). The social habits of tobacco use and alcohol consumption are incriminated as the major factors involved in the aetiology of this disease (3–6). The incidence rate of oral cancer was found particularly high in Sudan (11.60 for males and 6.91 for females) (7). This has been shown to be strongly associated with the extensive use of the Sudanese smokeless tobacco, locally known as *toombak* (8). Chemical analysis has revealed that *toombak* contains at least 100-fold higher concentrations of the tobacco specific *N*-nitrosamines than the commercial smokeless tobacco (snuff) brands from US and Sweden (9). It has been suggested that the ageing process of *toombak* at elevated temperatures and fermentation with sodium bicarbonate leads to significant increase of the tobacco specific *N*-nitrosamines. These tobacco-specific *N*-nitrosamines are by far the most powerful and most abundant carcinogens in snuff (10, 11). Their concentration in the saliva of *toombak* dippers is about the same as the concentrations that had induced tumours of the cheek and palate of rats after swabbing of the oral cavity (12). However, there is still a paucity of scientific data concerning the putative adverse biological effects induced by *toombak* on human oral mucosa. The very few studies published so far have highlighted the need for in-depth epidemiological studies and further laboratory research to prove that *toombak* chewing causes oral adverse effects. Further basic knowledge seems also necessary for taking

preventive steps regarding *toombak* use and standardization of the manufacturing procedure (8).

On the other hand, there is an extensive literature on the biological effects of US and Swedish brands of moist snuff, although with controversial results (13–15). The question on the putative harmful effects of Swedish snuff on oral tissues is still being debated (16–18). A recent report from the National Health Institute of Sweden was critical about the health promotion idea of encouraging smoking substitution with the use of smokeless tobacco, concluding that the carcinogenic risk of both Swedish and US snuff can not be excluded (19).

In this context, the present study aimed to: (i) investigate *in vitro* the biological effects of a *toombak* aqueous extract on primary normal human oral keratinocytes (NOK), fibroblasts (NOF), and a dysplastic oral keratinocytic cell line (DOK), and (ii) compare these effects with the biological effects of a Swedish snuff extract.

Materials and methods

Toombak and Swedish snuff extract preparations

Moist *toombak* was prepared from sun-dried tobacco leaves of the plant *Nicotiana rustica* and sodium bicarbonate (locally known as *natron*), both brought from Sudan by one of the authors (SOI). A Sudanese native manufacturing protocol was followed (9). In brief, four parts dry leaf powder (80 g) were mixed slowly with one part sodium bicarbonate (20 g) dissolved into 100 ml dH_20. The mixture was let overnight in the incubator at 37°C. Analysis of this mixture (run at the National Food Administration, Uppsala, Sweden) revealed that the moist *toombak* prepared in the laboratory had a level of nitrosamines comparable with the native Sudanese snuff samples: 91.5% dry matter (DM), 232 μg/g DM *N*′-Nitrosonornicotine (NNN), 427 μg/g DM 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), 14.9 μg/g DM N′-nitrosoanatabine (NAT), and 23.7 μg/g DM *N*′-nitrosoanabasine (NAB), with a total content of tobacco specific nitrosamines of 697 μg/g DM (9). Aqueous extracts were prepared from the moist *toombak* and the Swedish moist snuff (Ettan, Gothia Tobak AB). For preparation of the extracts, 100 g of moist *toombak* or Swedish snuff were mixed with 300 ml phosphate buffered saline (PBS) pH 7.2–7.4, incubated at 37°C for 1 h, and stirred at each 10 min (20). The mixture was first centrifuged at 450 *g* for 10 min at 4°C. The supernatant was centrifuged again at 13 000 *g* for 1 h at 4°C. The second supernatant was collected, aliquoted in 2 ml ampoules, and stored at −70°C for later use. Analysis of the 10-fold diluted extract showed the following composition for the *toombak* extract: 49 μg/ml NNN, 83 μg/ml NNK, 5.2 μg/ml NAT, 6.7 μg/ml NAB and 3 mg/ml nicotine; and for the Swedish snuff extract: 0.42 μg/ml NNN, 0.63 μg/ml NNK, 0.006 μg/ml NAT, 0.004 μg/ml NAB and 1.7 mg/ml nicotine. For exposure of cell cultures, serial dilutions were made from the thawed supernatant after adjusting its pH (to 7–7.2) and filtrating through a low protein binding filter with 0.2 μm pores. Although the concentrations of nicotine and tobacco-specific nitrosamines in saliva can vary widely between users, based on publically available literature, their range of concentrations is as follows: 3–620 μg/ml nicotine and 0.5–20 μg/ml NNN in saliva of *toombak* dippers (21); 73–1560 μg/ml nicotine and 0.025–0.42 μg/ml NNN in saliva of Swedish snuff dippers (22). In the view of these clinical data, 10^2-fold and lower dilutions of our extracts would have clinical relevancy.

Cell culture and handling

Primary NOK and NOF were isolated from superfluous tissues of clinically healthy buccal mucosa from adult volunteers undergoing surgical removal of wisdom teeth. The samples were taken after informed consent. The study was approved by the regional ethics committee. Cells were isolated through a multistep procedure combining enzymatic digestion and mechanical dissolution of cells, essentially as previously described (23). NOK were cultured in keratinocyte serum-free medium (KSFM) supplemented with 1 ng/ml EGF, 25 μg/ml BPE, 20 μg/ml L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B (all from GibcoBRL, Grand Island, NY, USA). NOF cells were cultured in DMEM (Gibco BRL) supplemented with 10% FBS (Gibco BRL), 20 μg/ml L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B. The commercially available human (caucasian) dysplastic oral keratinocytic cell line (DOK cell line, accession no. 94122104) (24) was obtained from the European Cell Culture Bank. DOK cells were routinely maintained in DMEM supplemented with 10% FCS, 20 μg/ml L-glutamine, 5 μg/ml hydrocortisone (Sigma, St Louis, MO, USA). For the exposure experiments the cells were used in their 1st to 3rd passage (split ratio 1:4) for NOK, 2nd to 5th passage (split ratio 1:4) for NOF, and 29th to 32th (split ratio 1:6) passages for DOK.

Evaluation of morphology by phase contrast microscopy

Cells (5×10^3 cells/cm^2) were seeded in six-well plates, allowed to plate and enter the logarithmic phase of growth for 24 h. The cells were then exposed to various dilutions (range 10–1 000 000-fold) of *toombak* and Swedish snuff extracts in their routine medium. Staurosporine (Sigma), an agent shown to induce apoptosis in various cell types including primary oral cells, was used at a concentration of 0.5 μM as positive control for morphological changes that accompany apoptosis in cultured cells. Medium with high calcium concentration (1.8 M) was used as positive control for morphological changes that accompany cell differentiation in NOK. An evaluation of cell morphology was performed using phase contrast microscopy (Axiovert 25 inverted microscope; Carl Zeiss MicroImaging GmbH, Göttingen, Germany). Serial photographs were taken at regular time points after exposure, in randomly selected areas, at 400-fold magnification, with a camera (Powershot G2 digital camera; Canon, Tokyo, Japan) mounted on the microscope. Morphological changes suggestive of cell death were characterized by rounding up and cell

shrinkage, blebbing and blistering of the cell membrane, increased nuclear brightness and nuclear shrinkage. Morphological changes suggestive of keratinocyte cell differentiation were characterized by cell flattening, increased cellular area and increased cytoplasmic brightness. The experiment was repeated six times in duplicate, each time with cells isolated from different patients for NOK and NOF.

Cell viability assay
Cells (5×10^3 cells/cm^2) were seeded in 96-well plates, allowed to plate for 24 h, and then exposed to various dilutions (range 10–100 000 000-fold) of *toombak* and Swedish snuff extracts in their specific culture medium. The medium was changed after 24 h with 100 μl transparent DMEM-Ham's F12 medium (Gibco BRL) in which 10 μl of the WST-1 reagent (Roche Diagnostics GmbH, Mannhein, Germany) was added. The cells were incubated for 3 h and afterwards the absorbance of the samples was measured against a background control as a blank using a microplate (ELISA) reader at a wavelength of 440 nm, with a reference length of 600 nm. The experiment was repeated twice, in triplicate. The median lethal dose, LD50, was determined using the dose–response curves obtained after plotting the percentage of surviving cells relative to the control against extract concentration/dilution in Sigma Plot program (SPSS Science Software GmbH, Erkrath, Germany).

Cell cycle analysis (flow cytometry)
NOF and NOK cells (5×10^3 cells/cm^2) were seeded in 6 cm dishes, allowed to plate and enter into the logarithmic phase of growth for 24 h. The cells were then treated with various dilutions of *toombak* and Swedish snuff extracts (range 10–100 000-fold) in their routine medium. After 48 h of exposure the culture medium was collected and the cells were treated with 0.25% trypsin/0.05% EDTA (Sigma) for 5 min, at 37°C. The detached cells were mixed with the conditioned medium collected before and then centrifuged at 200 *g* for 7 min. After washing in PBS with 2% FCS, the cell pellet was fixed for 30 min at 4°C in 70% ethanol. The cells were resuspended in 250 μl PBS supplemented with propidium iodide (40 μg/ml) and RNAse A (0.1 μg/ml) (all from Molecular Probes Europe, Leiden, the Netherlands) after an additional washing with PBS. The cells were kept in the dark for 1 h at 4°C and then analysed for DNA content in a fluorescence-activated cell sorter, FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA) using a 488-nm laser excitation wavelength and 585-nm emission. Routinely, cell debris and doublets were excluded by gating. In each sample, 30 000 events (cells) were included for analysis using FlowJo DNA analysis software (Tree Star Inc, Ashland, OR, USA). The experiment was repeated three times in duplicate.

Determination of DNA double-strand breaks
Phosphorylation of histone H2AX at serine-139 (γH2AX) occurs in response to DNA double-strand breaks (DSB) damage, it can be visualized microscopically after antibody labelling, and it is used to locate and quantify the low-level DNA damage induced by various genotoxic agents (25). Thus it was used in this study to detect any potential DNA damage induced by the extracts tested. For this purpose, 5×10^3 cells/cm^2 were seeded on glass cover slips and allowed to plate and enter into the logarithmic phase of growth for 24 h. The cells were then exposed to various dilutions (range 10–1 000 000-fold) of *toombak* and Swedish snuff extracts in their routine medium. Camptothecine, an agent shown to induce DSB in various cell types, was used at a concentration of 6 μM as positive control for expression of γH2AX foci in cultured cells. After 1 h the cells were fixed in 4% buffered formaldehyde for 5 min, washed in PBS and permeabilized with 0.1% Triton X. Three percent bovine serum albumine (BSA) was used as a blocking agent for 30 min. Mouse monoclonal anti-γH2AX antibody (Upstate Biotechnology Inc., Waltham, MA, USA) (2 μg/ml, diluted in blocking buffer) was then added for 1 h. After washing with PBS, a secondary goat anti-mouse Alexa Fluor 488-conjugated antibody (Molecular Probes) was added and left for 1 h in dark. The cells were afterwards washed five times in PBS with 5% Tween and mounted in Vectashield Mounting Medium with DAPI and analysed using a fluorescence microscope.

Chromatin condensation assay
Cells (5×10^3 cells/cm^2) were seeded in 24-well plates, allowed to plate and enter into the logarithmic phase of growth for 24 h, and then exposed to various dilutions (range 10–1 000 000-fold) of *toombak* and Swedish snuff extracts in their routine medium. A DNA stain (Hoechst 33342 from Molecular Probes, final concentration 8.1 μM) was added in each well, and the plates incubated for 15 min at 37°C in a humidified atmosphere supplemented with 5% CO_2. The cells were then analysed in a Leica IRB inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany) for fluorescence. Cells with condensed and intensely fluorescent or fragmented nuclei were scored as dead, while those with diffusely and weakly fluorescence nuclei were scored as normal (26). Cells that expressed an intermediate fluorescence pattern were scored as normal. The experiment was repeated three times in duplicate.

Annexin V assay
Cells (5×10^3 cells/cm^2) were seeded in 8-well chamber slides (Lab-Tek Permanox^{TM}; NUNC, Thermo Fisher Scientific, Roskilde, Denmark), allowed to plate and enter into the logarithmic phase of growth for 24 h, and then exposed to various dilutions (range 10–1 000 000-fold) of *toombak* and Swedish snuff extracts in their routine medium. The cells were then exposed to Alexa 555 conjugated Annexin–V (Roche) for 15 min in the incubator, the chambers were removed, the remaining slides were rinsed in Hepes buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 5 mM $CaCl_2$ in distilled water) and the cells fixed in 4% buffered formalin for 5 min. The cells were mounted in Vectashield Mounting

Medium with DAPI (Vector Laboratories Ltd, Peterborough, UK), and analysed using a fluorescence microscope.

Selective plasma membrane permeability assay
Cells (5×10^3 cells/cm^2) were seeded in 24-well plates, allowed to plate and enter into the logarithmic phase of growth for 24 h, and then exposed to various dilutions (range 10–1 000 000-fold) of *toombak* and Swedish snuff extracts in their routine medium. The cells were then treated with three fluorescent DNA stains; Hoechst 33342 (8.1 μM), propidium iodide (1.5 μM) and YO-PRO-1 (0.1 μm) (Molecular Probes) for 20 min on ice and then analysed for fluorescence. Cells staining positive with all three dyes were considered necrotic, those staining positive with YO-PRO-1 and Hoechst 33342 were considered apoptotic (27), whereas cells positively stained only with Hoechst 33342 were considered normal.

Evaluation of morphology by transmission electron microscopy (TEM)
Cells (5×10^3 cells/cm^2) were seeded in 6 cm dishes, allowed to plate and enter into the logarithmic phase of growth for 24 h, and then exposed to various dilutions (range 10–100 000-fold) of *toombak* and Swedish snuff extracts in their routine medium. After 24 h of exposure, the culture medium was collected and the cells were treated with 0.25% trypsin/0.05% EDTA (Sigma) for 5 min, at 37°C. The detached cells were mixed with the conditioned medium collected before and then centrifuged at 200 *g* for 7 min. The cell pellet was fixed overnight at 4°C in 0.1 M Na-cacodylate buffer, pH 7.4, containing 2% glutaraldehyde (all from Sigma). Samples were then rinsed in PBS, post-fixed in 1% osmium tetraoxide (Sigma) in PBS (30 min), dehydrated using graded ethanols, and embedded in epoxy resin. Ultrathin sectioning as well as double staining with uranyl acetate and lead citrate (Sigma) was also performed essentially as previously described (28). Specimens were examined using a transmission electron microscope (JEOL 1230; Jeol Ltd, Tokyo, Japan), and the micrographs processed using an Arcus II scanner (Agfa-Gevaert N.V, Mortsel, Belgium).

Determination of 6-day growth curves half maximal inhibitory concentration (IC50)
Cells (5×10^3 cells/cm^2) were seeded in six well plates, allowed to plate and enter into the logarithmic phase of growth for 24 h, and then exposed to various dilutions (range 10–1 000 000-fold) of *toombak* and Swedish snuff extracts. The extracts were diluted in the culture medium that was replenished every second day, so the cells were continuously exposed for 6 days. Cell numbers were determined every 24 h by counting cells in three predetermined areas of 0.015 mm^2 of each well until confluency. The experiment was repeated three times in duplicate.

Statistical analysis
Data were expressed as mean ± standard deviation (SD) or standard error of the mean (SEM). Statistical analysis with Student's-t and ANOVA tests were performed using the SPSS program version 14.0.1 (SPSS Inc., Chicago, IL, USA), and with the level of significance set at 5%.

Results

Toombak but not Swedish snuff extract decreased the viability of oral cells at clinically relevant dilutions
At clinically relevant, 100-fold dilutions of *toombak* extract, but only at 10-fold dilutions of Swedish snuff extract (less diluted than the clinically relevant 100-fold dilution), morphological features suggestive of cell death were observed by phase contrast microscopy in all cell types. These changes consisted of cell rounding up and plasma membrane blebbing. At later time points (12–24 h) these cells detached from the cell culture surface (Fig. 1). These morphological changes were compatible with those observed in presence of staurosporine (0.5 μM), a substance known to induce apoptosis. A significant decrease in metabolically active/viable cells was detected by the WST assay in NOK exposed to as low as 1000-fold dilutions of *toombak* extract, and in NOF and DOK down to 100-fold dilutions only (Fig. 1). The median lethal doze/dilution (LD50) for *toombak* extract was found to be 25.2-fold lower than the LD50 for the Swedish snuff extract for NOK, 4.5-fold lower for NOF and 2.5-fold lower for DOK (Fig. 1).

Both toombak and Swedish snuff extracts induced G2/M growth arrest of normal oral cells at clinically relevant dilutions
Normal oral cells exposed to 100-fold dilutions of *toombak* extract, but only to 10-fold dilution of Swedish snuff extract for 48 h showed a significantly higher sub-G1 fraction of events, as cells exposed to staurosporine 0.5 μM, supportive of presence of dead cells or DNA-containing debris from dead cells (data not shown). Cell cycle analysis performed on normal oral cells exposed to 1000-fold dilutions of *toombak* or 100-fold dilution of Swedish snuff for 48 h revealed that a significantly higher proportion of cells were in G2/M phase when compared with non-treated controls (Fig. 2). Further dilution of the extracts did not cause any significant effect on cell cycle parameters (Fig. 2).

Toombak but not Swedish snuff extract induced DNA double-strand breaks in oral cells at clinically relevant dilutions
Non-exposed normal oral cells had a low background of nuclear γH2AX, with only few cells expressing γH2AX either homogeneously or in small foci (Fig. 3). By contrast, non-exposed DOK cells had a higher endogenous level of nuclear γH2AX possibly related to a higher frequency of endogenous breaks transiently produced during replication in culture, as shown by others (29). In NOK cells exposed for 24 h to 100 and 1000-fold dilutions of *toombak*, but only at 10-fold dilution of the Swedish snuff extract, a significant increase in the expression of γH2AX was observed

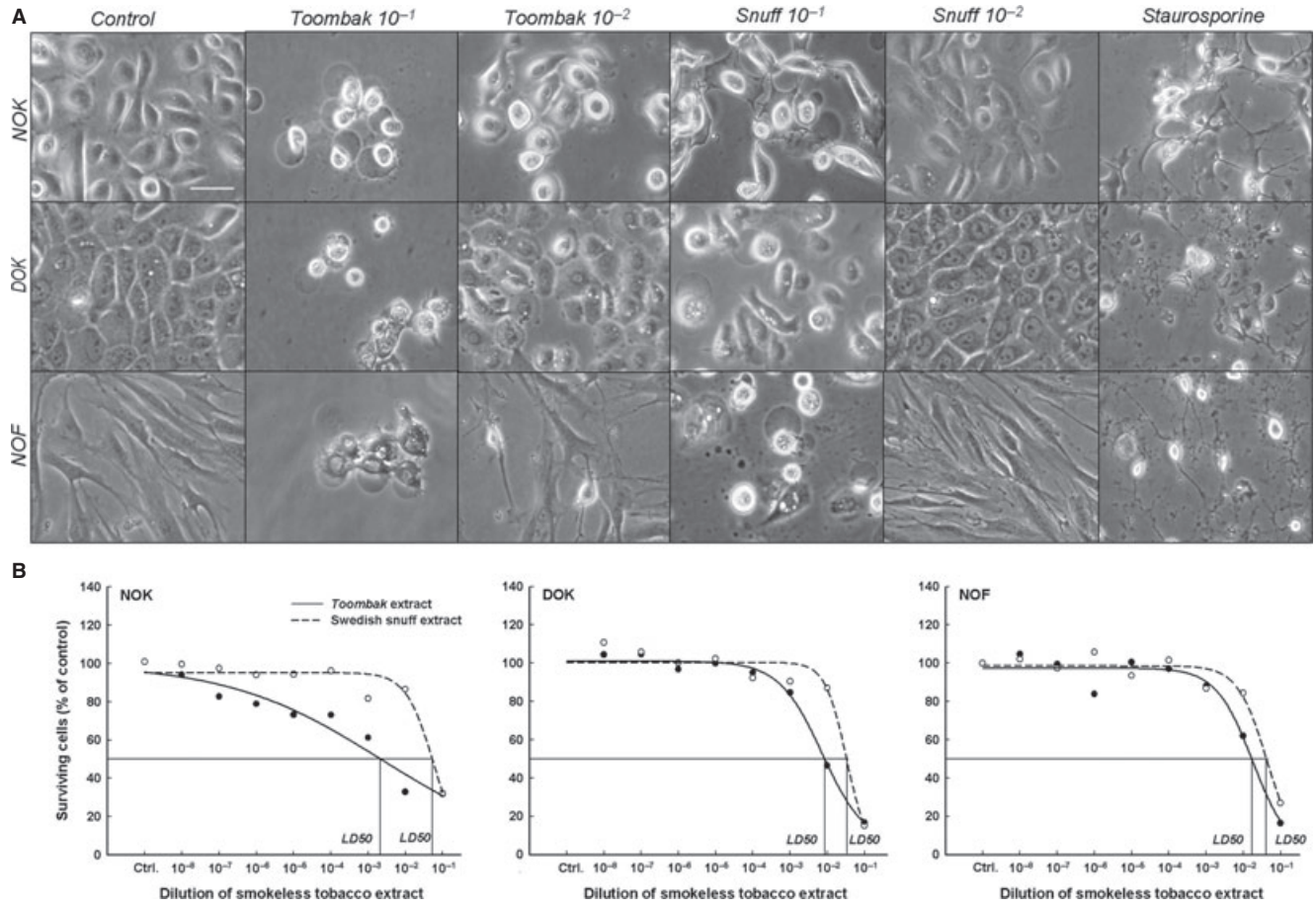

Figure 1 Effects of *toombak* and Swedish snuff extracts on cell morphology and viability of human oral cells. Panel A – phase contrast morphology: cells (normal oral keratinocytes – NOK, normal oral fibroblasts – NOF, and dysplastic oral keratinocytic cell line – DOK) were exposed to various dilutions of *toombak* and Swedish snuff extracts in their routine medium and observed after 24 h at phase contrast microscope. Staurosporine (0.5 μM) was used as control for morphological changes that accompany apoptotic cell death in cultured cells. Morphological changes detectable by phase microscopy as suggestive of cell death were characterized by rounding up and cell shrinkage, blebbing and blistering of the cell membrane, increased nuclear brightness and nuclear shrinkage. Panel B – cell viability/metabolically active cells detected using WST assay: the cells were exposed to various dilutions of *toombak* and Swedish snuff extracts for 24 h. For determination of LD50, dose–response curves were obtained after plotting the percentage of survival cells relative to control against extract. The data represent mean ± SD from three separate batches of primary cells. The experiments were run in duplicates, and the data normalized using Sigma Plot program.

(Fig. 3 and Table 1). A significantly higher fraction of NOF and DOK cells expressing γH2AX was also observed after exposure to *toombak* at 100-fold dilutions (Fig. 3). In the positive cells, the number of γH2AX foci per nucleus, which indicates the frequency of double DNA strand breaks, was consistently higher after exposure to *toombak* than after exposure to the Swedish snuff extract, suggesting a lower potency of the latter in inducing DNA damage. A lower number of foci per nucleus were consistently observed in fibroblasts compared with both normal and dysplastic keratinocytes.

Toombak but not Swedish snuff extract induced apoptotic cell death in oral cells at clinically relevant dilutions

Fluorescent DNA staining (Hoechst 33342) showed presence of intensely stained nuclei indicating chromatin condensation in all cell types exposed for 24 h to *toombak* extract at 100-fold dilutions, but only at the most concentrated, the 10-fold dilution of the Swedish snuff extract (Table 2, Fig. 4). The intensely stained nuclei presented a range of morphological changes from only a marginal bright halo indicating peripheral chromatin condensation (the earliest change observed), to brightly stained, totally condensed nuclei (uniform chromatin condensation seen especially in NOK), small bright nuclear fragments (fragmented nuclear chromatin, observed mainly in DOK), or bright dots within the nuclei (chromatin clumping observed mainly in NOF).

A significant number of all cell types ($P < 0.05$ when compared with controls) exposed to 100-fold dilutions of *toombak* extract, but only to 10-fold dilution of Swedish snuff extract showed plasma membrane positivity to Annexin V already after 1 h of exposure (Table 3, Fig. 4). The percentage of Annexin V or Yo-Pro-1 positive cells exposed to either *toombak* extract or staurosporine was almost double than the percentage of cells having condensed nuclei as detected by Hoechst 33342 staining (Tables 2 and 3). Part of the YO-PRO-1 positive cells was also propidium iodide positive (Fig. 4 Panels M-P), suggesting either that the

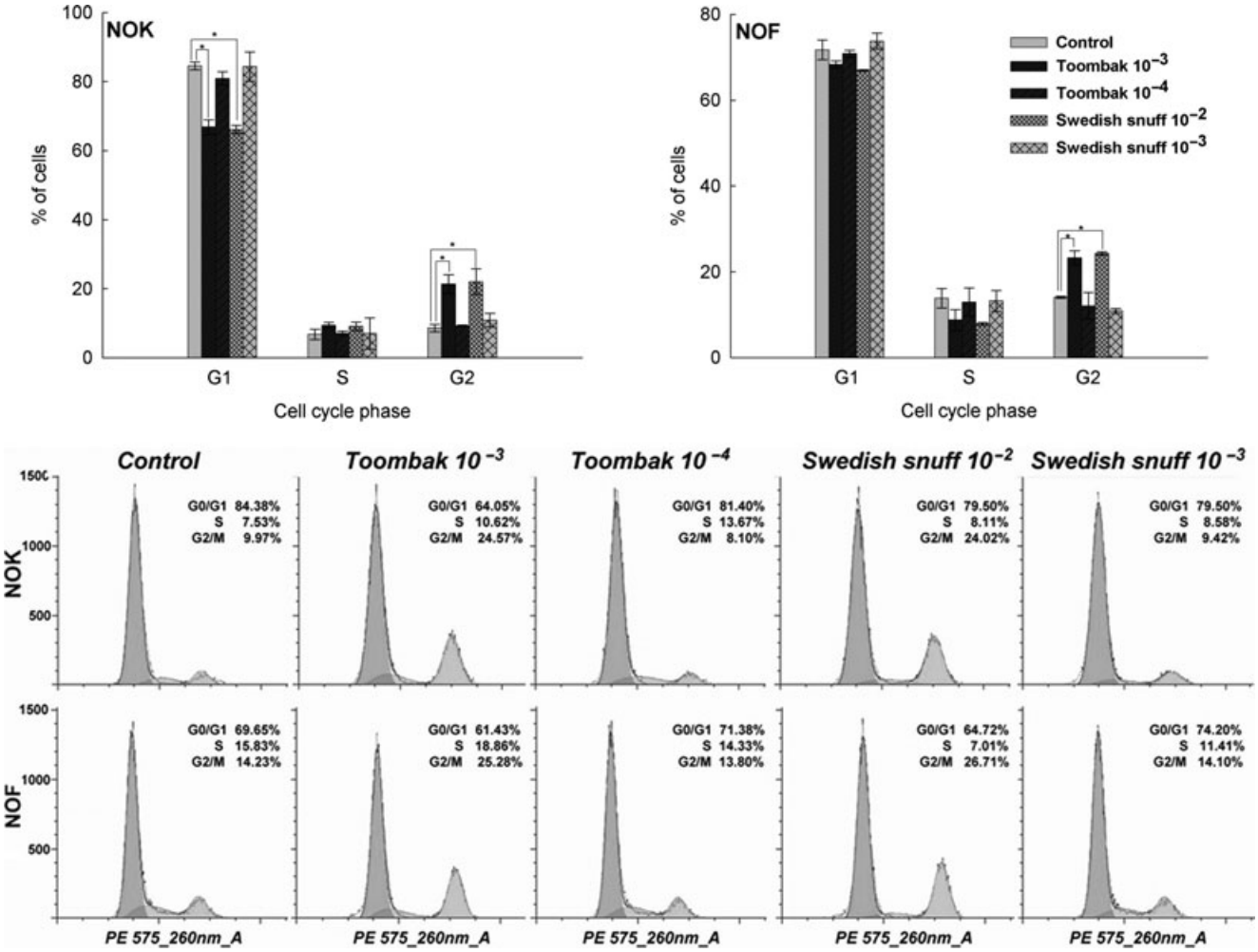

Figure 2 Cell cycle analysis of normal oral cells exposed to *toombak* and Swedish snuff extract. Cells treated with various dilutions of *toombak* and Swedish snuff extracts in their routine medium and harvested after 48 h of exposure. The percentage of cells in each of the various cell cycle phases (G1, S, or G2/M) was determined using propidium iodide staining and flow cytometry. Representative examples of NOK and NOF cells are shown. The data in the graphs represent the mean ± SD of three or more independent experiments. Significant differences in extract exposed cells treated vs. control cells are shown by the asterisk (*).

double positive cells died through other type of cell death than apoptosis or that they were in a late apoptotic phase ('secondary necrosis'). The latter suggestion is more likely to reflect the experimental settings, as the *in vitro* cultured cells are not normally phagocytized. Thus, after some time in culture, their membrane is so severely damaged that it may become permeable for stainings not normally taken by apoptotic cells *in vivo*.

Consistent with these observations, transmission electron microscopy showed signs of clumped or peripherally condensed chromatin, cell shrinkage, loss of microvilli with membrane blebbing, and polarization of the cellular organelles towards one side of the cell at clinically relevant dilutions of *toombak* (Fig. 5). Scattered normal oral cells with the above mentioned signs were detected using electron microscopy after a 24-h exposure to 100 and 1000-fold dilutions of *toombak,* and only to 10-fold dilutions of Swedish snuff. NOK exposed to further dilutions of *toombak* extract and the clinically relevant (100-fold) dilution of Swedish snuff extract flattened and became larger in diameter after 24 h of exposure (Fig. 5), and started to stratify in monolayer culture after day 6 of exposure (data not shown). These features were similar to NOK exposed to high concentrations of calcium, suggesting epithelial cell differentiation rather than cell death occurring at these dilutions.

Toombak showed more prominent cumulative adverse effects than the Swedish snuff extract on normal oral cells, but less pronounced on dysplastic oral keratinocytes

After 6 days of culture*, toombak* extract significantly decreased the number of NOK and NOF at as low as 10 000-fold dilutions, but the number of DOK down to only 1000-fold dilutions (Fig. 6). Swedish snuff extract significantly decreased the number of cells at 100-fold dilutions in NOK, but only at 10-fold dilution in DOK and NOF (Fig. 6). Of note was the significantly higher number of cells detected in NOF exposed to the lowest (10^6-fold) dilution of Swedish

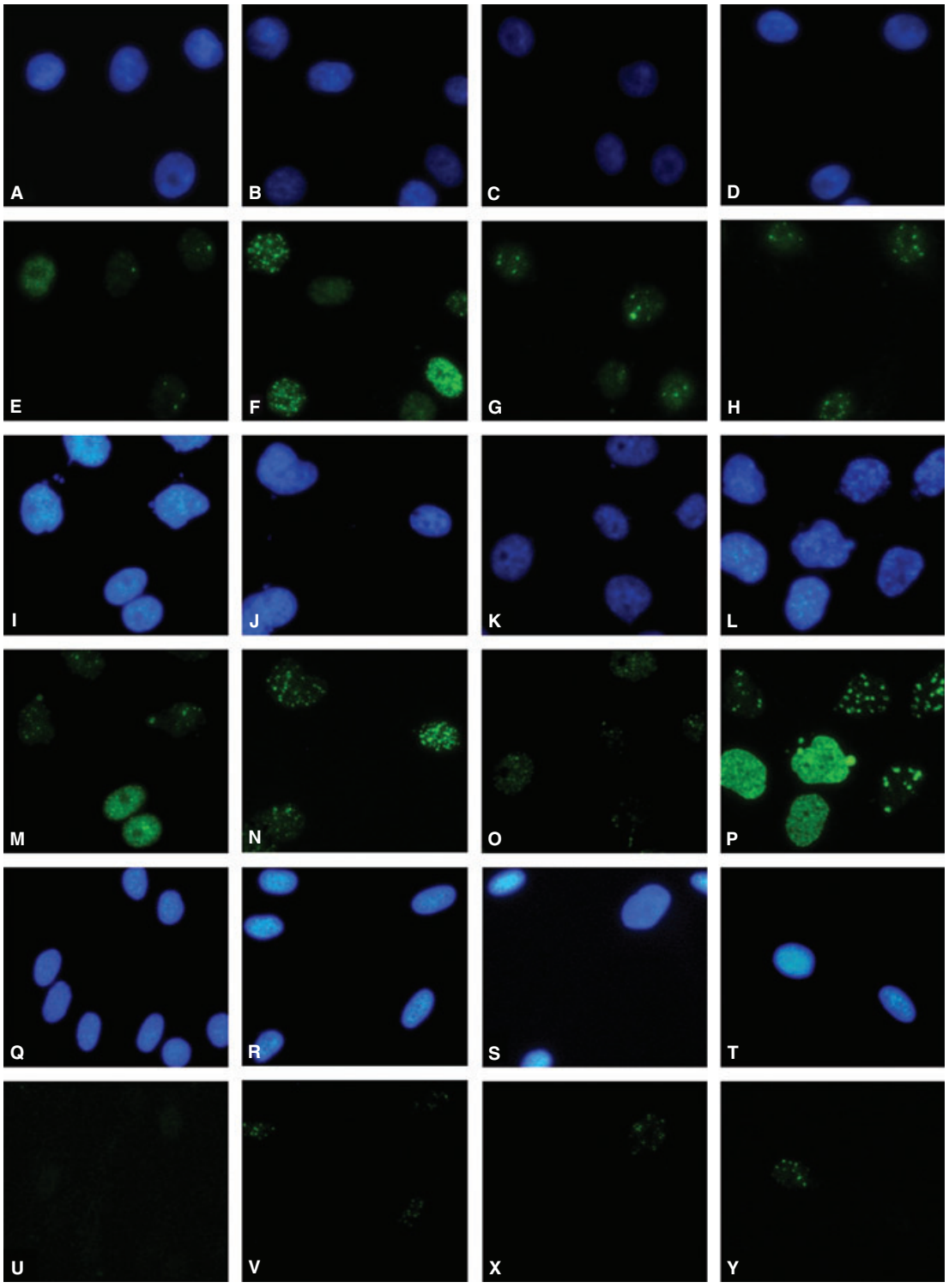

Figure 3 Detection of DNA double-strand breaks by anti-γH2AX staining in oral cells exposed to *toombak* and Swedish snuff extract. Normal oral keratinocytes (Panels A–H), dysplastic oral keratinocytes (Panels I–P) and normal oral fibroblasts (Panels O–Y) were exposed to 100-fold dilutions of *toombak* extract (Panels B, F, J, N, R, V) and 100-fold dilutions of Swedish snuff extract (Panels C, G, K, O, S, X) in their routine medium, stained with anti-γH2AX antibody and examined for the expression of nuclear foci by fluorescence microscopy. Camptothecine, an agent shown to induce double-strand breaks in various cell types, was used at a concentration of 6 μM as positive control for expression of γH2AX foci in cultured cells (Panels D, H, L, P, T, Y).

snuff extract (Fig. 6). The adverse effects of *toombak* could not be attributed to nicotine or NNN alone, as the addition of these components to the culture medium at concentrations equivalent to those of the 10-fold dilution of *toombak* extract did not significantly alter the growth of oral cells.

Table 1 Percentage of nuclei with γH2AX foci in oral cells exposed to *toombak* and Swedish snuff extracts for 1 h (staining with anti-γH2AX antibody)

Exposure	*Fold-dilution*	*NOK*	*DOK*	*NOF*
Control	–	5.41 ± 2.00	6.66 ± 6.66	0.78 ± 0.78
Toombak extract	10	55.43 ± 10.30*	66.66 ± 14.92*	47.43 ± 12.13*
	100	22.32 ± 7.32*	34.66 ± 9.37*	27.22 ± 8.33*
	1000	17.64 ± 5.88*	10.57 ± 4.25	1.64 ± 1.64
Swedish snuff extract	10	18.18 ± 2.66*	8.28 ± 4.93	25.83 ± 6.34*
	100	7.21 ± 3.28	5.41 ± 3.24	1.20 ± 1.20
Camptothecine	6 μM	18.18 ± 2.66*	73.80 ± 13.22*	55.32 ± 11.22*

Data are presented as mean and standard deviation ($n = 3$).
*Statistical significance when compared with the control ($P < 0.05$).

Table 2 Percentage of nuclei with chromatin condensation in oral cells exposed to *toombak* and Swedish snuff extracts for 24 h (staining with Hoechst 33342)

Exposure	*Fold-dilution*	*NOK*	*DOK*	*NOF*
Control	–	1.11 ± 1.11	6.66 ± 6.66	1.19 ± 1.19
Toombak extract	10	39.58 ± 27.08*	36.66 ± 3.33*	21.97 ± 6.59*
	100	20.19 ± 4.80*	24.28 ± 4.28*	17.42 ± 0.75*
	1000	3.84 ± 3.84	3.57 ± 3.57	2.63 ± 2.63
Swedish snuff extract	10	19.64 ± 7.57*	24.28 ± 4.28*	22.61 ± 5.95*
	100	1.02 ± 1.02	2.63 ± 2.63	2.08 ± 2.08
Staurosporine	0.5 μM	20.83 ± 4.16*	23.80 ± 13.46*	25.00 ± 8.33*

Data are presented as mean and standard deviation ($n = 3$).
*Statistical significance when compared with the control ($P < 0.05$).

Discussion

This study shows that an aqueous extract of *toombak* is able, at clinically relevant concentrations, to induce adverse effects in normal human oral keratinocytes and fibroblasts through both apoptotic cell death and G2/M cell cycle block. To the best of our knowledge, there are no such previous studies on the putative cytotoxic effects of *toombak* on oral cells, although possible harmful effects of *toombak* on oral mucosa have been previously suggested by various epidemiological studies (7, 8, 30, 31).

On the other hand, the biological effects of various commercially available, highly standardized brands of smokeless tobacco, mainly from US and Scandinavia, have been investigated by numerous studies, although with controversial results. The previous studies have shown that an aqueous extract of moist snuff could stimulate normal epidermal keratinocyte and fibroblast proliferation (32), enhance the growth and culture longevity of normal oral keratinocytes (33), increase DNA synthesis in DMBA-transformed golden Syrian oral keratinocytes (34), and even induce oral cavity tumours in rats (3 of 32) treated with snuff extract (12). On the contrary, other studies have shown cytotoxic effects like growth inhibition in primary epithelial cultures from mouse tongue (35) and periodontal ligament fibroblasts (36), or cell death through apoptosis in hamster cheek pouch cell cultures (37) and normal human oral keratinocytes (38). The differences between the results of these studies may be attributed to the use of different tobacco brands, as well as different cell types and species. Different methods of extraction and quantification of the extract concentration are also likely to play a role, and a correlation between the concentrations of snuff extract used in various studies is difficult to achieve as various methods for obtaining the extracts are described. Thus, to accurately compare the biological effects of two different types of smokeless tobacco (Sudanese *toombak* vs. Swedish snuff), in this study the extracts were obtained at the same time following the same protocol. To mimic in an experimental setting what is happening daily during the *in vivo* habit of snuff dipping, we have kept the two different types of smokeless tobacco in a PBS solution at 37°C for 1 h. This was done in an attempt to reproduce *in vitro* the extraction of the smokeless tobacco components as it takes place *in vivo,* at body temperature (37°C) and in a buffering medium (that is saliva *in vivo*, and PBS *in vitro*). The time of extraction was randomly chosen to be of 1 h, that is within the range of the published time for snuff dipping reported to range from few minutes to several hours (39). The cells were exposed to a wide range of dilutions of the *toombak* and Swedish snuff extract, those of *toombak* including dilutions with the same concentration of nitrosamines and nicotine as found in the saliva of *toombak* dippers (8).

Our results are in concordance with a previous suggestion that snuff extract might have dual effects (31), and we show it here for normal human oral fibroblasts. The aqueous extract of Swedish snuff inhibited the growth rate of NOF at a 10-fold dilution

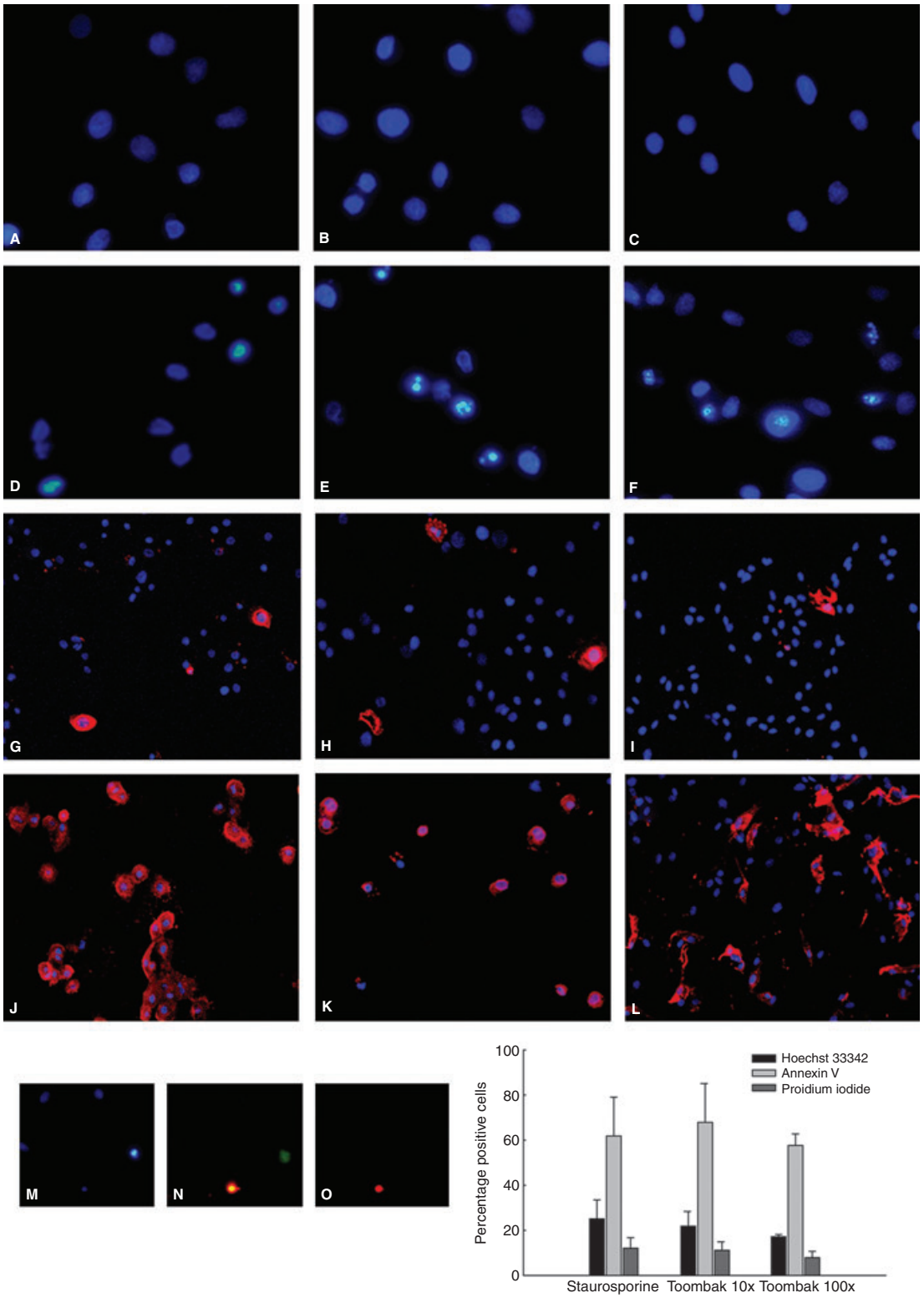

Figure 4 Detection of chromatin and membrane changes induced by *toombak* and Swedish snuff extracts on oral cells. Hoechst (panels A–F) and Annexin V (panels G–L) staining of controls and oral cells exposed to *toombak* are shown. A comparison between Hoechst 33342 (panel M), Yo-Pro-1 (panel N) and propidium iodide (panel O) stainings as assays used for quantification of cell death in NOF exposed to *toombak* extract is shown (panel P).

while increasing it at a 1 000 000-fold dilution (Fig. 6). However, although the continuous exposure for 6 days to snuff extracts might be an overestimation of the *in vivo* settings and effects, the aqueous extract of Swedish snuff showed, at clinically relevant concentrations, certain adverse effects on NOK, inhibiting their

Table 3 Percentage of Annexin V positive cells exposed to *toombak* and Swedish snuff extracts for 1 and 24 h

		NOK		*DOK*		*NOF*	
Exposure	*Fold-dilution*	*1 h*	*24 h*	*1 h*	*24 h*	*1 h*	*24 h*
Control	–	8.06 ± 1.84	5.14 ± 0.94	4.41 ± 1.45	3.00 ± 1.56	2.55 ± 0.94	2.36 ± 0.92
Toombak extract	10	49.61 ± 13.18*	80.42 ± 8.06*	58.28 ± 7.79*	81.36 ± 9.82*	35.25 ± 6.67*	67.77 ± 17.46*
	100	34.47 ± 1.94*	24.22 ± 0.97*	34.85 ± 7.17*	22.27 ± 12.84*	14.78 ± 2.54*	57.68 ± 5.24*
	1000	7.68 ± 0.57	7.20 ± 0.65	3.67 ± 1.91	7.53 ± 3.64	2.41 ± 0.59	3.38 ± 0.60
Swedish snuff extract	10	16.94 ± 2.22*	67.3 ± 7.62*	34.97 ± 8.96*	61.61 ± 13.08*	20.72 ± 3.16*	64.44 ± 2.93*
	100	2.42 ± 0.36	3.75 ± 0,73	5.75 ± 0.47	6.40 ± 2.83	3.92 ± 0.5	5.14 ± 1.40
	1000	3.42 ± 1.19	1.98 ± 0.58	2.10 ± 1.24	3.29 ± 0.78	1.34 ± 0.74	2.51 ± 0.37
Staurosporine	0.5 μM	19.73 ± 4.56*	23.67 ± 3.20*	58.67 ± 10.01*	39.87 ± 12.10*	17.29 ± 3.68*	61.75 ± 10.88*

Data are presented as mean and standard deviation ($n = 3$).
*Statistical significance when compared with the control ($P < 0.05$).

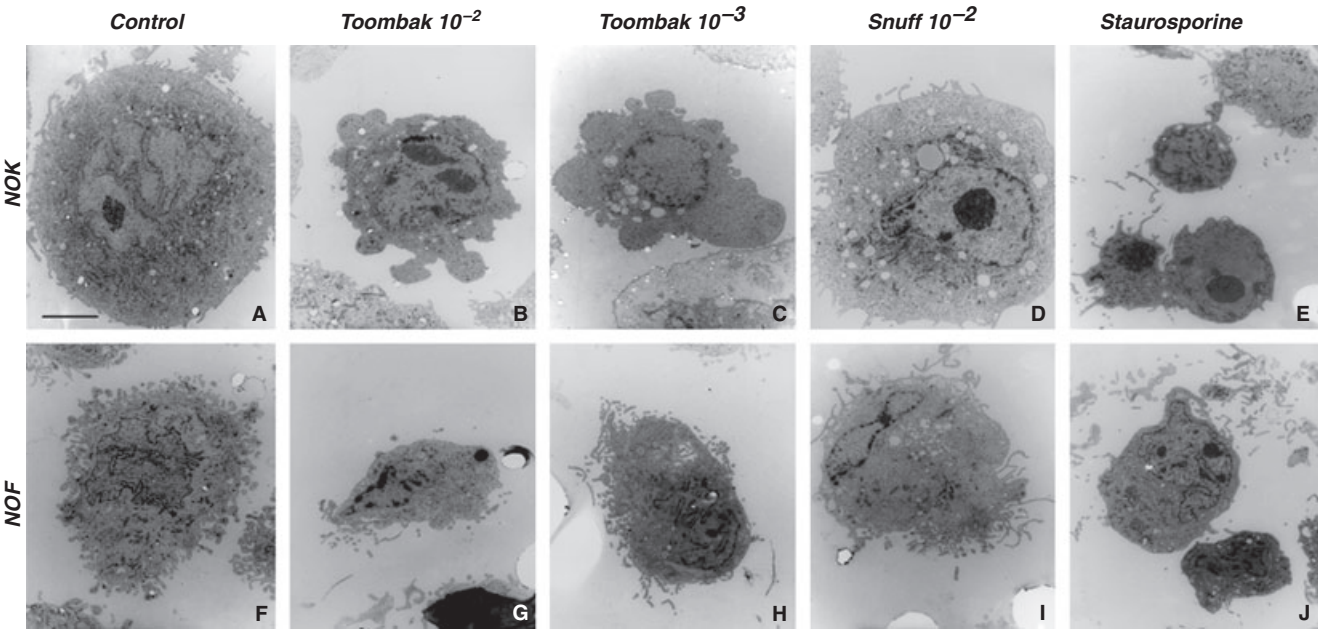

Figure 5 Sub-cellular morphological changes (transmission electron microscopy) induced by *toombak* and Swedish snuff extracts on oral cells. Representative pictures of cells exposed to *toombak* and Swedish snuff extracts are shown. Chromatin marginalization, loss of microvili and membrane blebbing are observed by electron microscopy in NOK exposed to *toombak* 100 and 1000-fold dilutions and staurosporine. Chromatin marginalization and polarization of the organelles is observed in NOF exposed to *toombak* 100 and 100-fold dilutions, Swedish snuff 100-fold dilutions and staurosporine.

growth after this long and continuous exposure. Nevertheless, the adverse effects of Swedish snuff were far less prominent than the effects of *toombak* extract. *Toombak* extract had significant adverse effects on NOK and NOF cells at clinically relevant and higher dilutions than the Swedish snuff extract and this could be attributed, at least partially to apoptotic cell death and a G2/M cell cycle block. Morphological changes of cell shrinkage, membrane blebbing, and nuclear condensation, together with the externalization of phosphatidylserine as early as 1 h after exposure, permeabilization to the fluorescent dye YO-PRO-1 and chromatin condensation after 24 h of exposure were compatible with cell death by apoptosis (40). Further investigations showed that both extracts were able to induce DNA damage under the form of double-strand DNA breaks in NOK, as identified by the expression of γH2AX foci, but again *toombak* extract was more potent than the Swedish snuff extract. Of note are the observations that DOK cells were resistant to the induction of DNA double-strand brakes by the Swedish snuff extract, but not by the *toombak* extract. This suggests that the transformed oral keratinocytes are less prone to further transformation and progression towards malignancy induced by Swedish snuff but not by *toombak*.

A common feature of primary normal oral cells, both NOK and NOF, was that the degree of cell death induced by *toombak* extract showed some variability from strain to strain. Some cell strains were more sensitive than others, presenting signs of cell death up to 10 000-fold dilutions of *toombak*. On the other

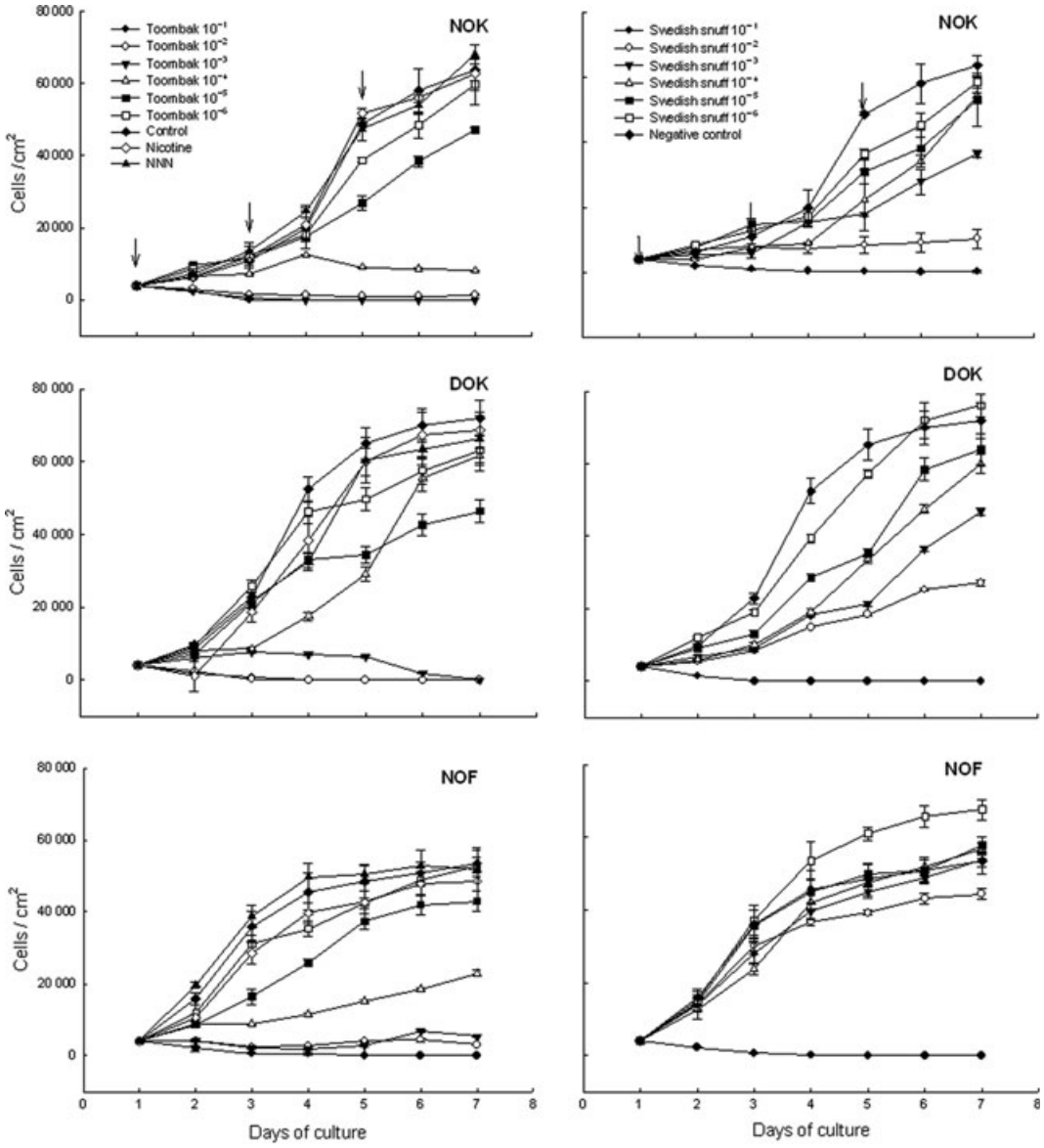

Figure 6 Cumulative adverse effects of *toombak* and Swedish snuff extracts on oral cells after 6 days of exposure. Cells were exposed to various dilutions of *toombak* and Swedish snuff extracts for 6 days and cell counts were performed daily in three different microscopical fields. The data represent mean ± SD from three separate batches of primary cells. The experiments were run in duplicates, and the data normalized using Sigma Plot program.

hand, the response of the DOK cells showed much less variability in the sensitivity towards *toombak* extract and they were less sensitive to the growth inhibitory effects induced by *toombak* extract than their normal counterparts (Fig. 6). Together with the observation that DOK cells had a better ability to preserve their viability in presence of the *toombak* extract when compared with NOK cells (Fig. 1), these observations might indicate that the dysplastic keratinocytes might have had acquired some resistance to the cellular effects induced by putative carcinogens as *toombak*. This could favour their growth and expansion over their normal counterparts after multiple exposures in time. However, further studies are necessary to persuade these observations. Another aspect that would need further consideration would be to elucidate the identity of the fraction(s) responsible for the adverse effects described in this study.

In conclusion, this study shows that an aqueous extract of *toombak* induces DNA damage, G2/M cell cycle block and cell death in normal human oral cells more efficiently than the Swedish snuff and at clinically relevant dilutions, and suggests that the dysplastic keratinocytes had acquired a partially resistant phenotype to these adverse effects. Together with the chemical description and the epidemiological data for the two types of snuff, these results point to a much higher potential of *toombak* to induce abnormal development of normal oral mucosa than the Swedish snuff.

References

1. Moore SR, Johnson NW, Pierce AM, Wilson DF. The epidemiology of mouth cancer: a review of global incidence. *Oral Dis* 2000; **6**: 65–74.
2. Moore SR, Johnson NW, Pierce AM, Wilson DF. The epidemiology of tongue cancer: a review of global incidence. *Oral Dis* 2000; **6**: 75–84.
3. Johnson NW, Warnakulasuriy S, Tavassoli M. Hereditary and environmental risk factors; clinical and laboratory risk matters for head and neck, especially oral, cancer and precancer. *Eur J Cancer Prev* 1996; **5**: 5–17.
4. Proia NK, Paszkiewicz GM, Nasca MA, Franke GE, Pauly JL. Smoking and smokeless tobacco-associated human buccal cell mutations and their association with oral cancer – a review. *Cancer Epidemiol Biomarkers Prev* 2006; **15**: 1061–77.
5. Warnakulasuriya S. Smokeless tobacco and oral cancer. *Oral Dis* 2004; **10**: 1–4.
6. Critchley JA, Unal B. Health effects associated with smokeless tobacco: a systematic review. *Thorax* 2003; **58**: 435–43.
7. Idris AM, Ahmed HM, Mukhtar BI, Gadir AF, el-Beshir EI. Descriptive epidemiology of oral neoplasms in Sudan 1970–1985 and the role of toombak. *Int J Cancer* 1995; **61**: 155–8.
8. Idris AM, Prokopczyk B, Hoffmann D. Toombak: a major risk factor for cancer of the oral cavity in Sudan. *Prev Med* 1994; **23**: 832–9.
9. Idris AM, Ibrahim SO, Vasstrand EN, et al. The Swedish snus and the Sudanese toombak: are they different? *Oral Oncol* 1998; **34**: 558–66.
10. Hoffmann D, Harley NH, Fisenne I, Adams JD, Brunnemann KD. Carcinogenic agents in snuff. *J Natl Cancer Inst* 1986; **76**: 435–7.
11. Warnakulasuriya KA, Ralhan R. Clinical, pathological, cellular and molecular lesions caused by oral smokeless tobacco–a review. *J Oral Pathol Med* 2007; **36**: 63–77.
12. Hecht SS, Rivenson A, Braley J, DiBello J, Adams JD, Hoffmann D. Induction of oral cavity tumors in F344 rats by tobacco-specific nitrosamines and snuff. *Cancer Res* 1986; **46**: 4162–6.
13. Merne M, Heikinheimo K, Saloniemi I, Syrjanen S. Effects of snuff extract on epithelial growth and differentiation *in vitro*. *Oral Oncol* 2004; **40**: 6–12.
14. Jansson T, Romert L, Magnusson J, Jenssen D. Genotoxicity testing of extracts of a Swedish moist oral snuff. *Mutat Res* 1991; **261**: 101–15.
15. Nilsson R. A qualitative and quantitative risk assessment of snuff dipping. *Regul Toxicol Pharmacol* 1998; **28**: 1–16.
16. Zatterstrom UK, Svensson M, Sand L, Nordgren H, Hirsch JM. Oral cancer after using Swedish snus (smokeless tobacco) for 70 years – a case report. *Oral Dis* 2004; **10**: 50–3.
17. Hirsch JM, Johansson SL, Vahlne A. Effect of snuff and herpes simplex virus-1 on rat oral mucosa: possible associations with the development of squamous cell carcinoma. *J Oral Pathol* 1984; **13**: 52–62.
18. Hirsch JM, Johansson SL, Thilander H, Vahlne A. Effect of long-term application of snuff and herpes simplex virus 1 on rat oral mucosa. Possible association with development of oral cancer. *IARC Sci Publ* 1984; **57**: 829–36.
19. Cnattingius S, Galanti R, Grafstroöm S, et al. *The health risks of the Swedish snuff*. Stockholm: National Institute of Public Health, Statens Folkhasoinstitut, 2005.
20. Vondracek M. *Toxicity of smokeless tobacco in human oral epithelium with emphasis on carcinogen metabolism and regulation of programmed cell death*. Stockholm: Karolinska Institutet, 2002.
21. Idris AM, Nair J, Friesen M, et al. Carcinogenic tobacco-specific nitrosamines are present at unusually high levels in the saliva of oral snuff users in Sudan. *Carcinogenesis* 1992; **13**: 1001–5.
22. Hoffmann D, Adams JD. Carcinogenic tobacco-specific N-nitrosamines in snuff and in the saliva of snuff dippers. *Cancer Res* 1981; **41**(11 Pt 1): 4305–8.
23. Costea DE, Dimba AO, Loro LL, Vintermyr OK, Johannessen AC. The phenotype of *in vitro* reconstituted normal human oral epithelium is essentially determined by culture medium. *J Oral Pathol Med* 2005; **34**: 247–52.
24. Chang SE, Foster S, Betts D, Marnock WE. DOK, a cell line established from human dysplastic oral mucosa, shows a partially transformed non-malignant phenotype. *Int J Cancer* 1992; **52**: 896–902.
25. Sedelnikova OA, Pilch DR, Redon C, Bonner WM. Histone H2AX in DNA damage and repair. *Cancer Biol Ther* 2003; **2**: 233–5.
26. Boe R, Gjertsen BT, Doskeland SO. Vintermyr OK. 8-Chloro-cAMP induces apoptotic cell death in a human mammary carcinoma cell (MCF-7) line. *Br J Cancer* 1995; **72**: 1151–9.
27. Idziorek T, Estaquier J, De Bels F, Ameisen JC. YOPRO-1 permits cytofluorometric analysis of programmed cell death (apoptosis) without interfering with cell viability. *J Immunol Methods* 1995; **185**: 249–58.
28. Boe R, Gjertsen BT, Vintermyr OK, Houge G, Lanotte M, Doskeland SO. The protein phosphatase inhibitor okadaic acid induces morphological changes typical of apoptosis in mammalian cells. *Exp Cell Res* 1991; **195**: 237–46.
29. Banath JP, Macphail SH, Olive PL. Radiation sensitivity, H2AX phosphorylation, and kinetics of repair of DNA strand breaks in irradiated cervical cancer cell lines. *Cancer Res* 2004; **64**: 7144–9.
30. Idris AM, Ahmed HM, Malik MO. Toombak dipping and cancer of the oral cavity in the Sudan: a case-control study. *Int J Cancer* 1995; **63**: 477–80.
31. Elbeshir EI, Abeen HA, Idris AM, Abbas K. Snuff dipping and oral cancer in Sudan: a retrospective study. *Br J Oral Maxillofac Surg* 1989; **27**: 243–8.
32. Wang Y, Rotem E, Andriani F, Garlick JA. Smokeless tobacco extracts modulate keratinocyte and fibroblast growth in organotypic culture. *J Dent Res* 2001; **80**: 1862–6.
33. Murrah VA, Gilchrist EP, Moyer MP. Morphologic and growth effects of tobacco-associated chemical carcinogens and smokeless tobacco extracts on human oral epithelial cells in culture. *Oral Surg Oral Med Oral Pathol* 1993; **75**: 323–32.
34. Rubinstein I. Smokeless tobacco potentiates VIP-induced DNA synthesis and inactivates NEP 24.11 in oral keratinocytes. *Am J Physiol Cell Physiol* 2000; **278**: C391–6.
35. Gijare PS, Rao KV, Bhide SV. Effects of tobacco-specific nitrosamines and snuff extract on cell proliferation and activities of ornithine decarboxylase and aryl hydrocarbon hydroxylase in mouse tongue primary epithelial cell cultures. *J Cancer Res Clin Oncol* 1989; **115**: 558–63.
36. Andersson G, Wahlin A, Bratthall G. The effect of Swedish and American smokeless tobacco extract on periodontal ligament fibroblasts *in vitro*. *Swed Dent J* 2006; **30**: 89–97.

37. Mangipudy RS, Vishwanatha JK. Role of nitric oxide in the induction of apoptosis by smokeless tobacco extract. *Mol Cell Biochem* 1999; **200**: 51–7.
38. Bagchi M, Balmoori J, Bagchi D, Ray SD, Kuszynski C, Stohs SJ. Smokeless tobacco, oxidative stress, apoptosis, and antioxidants in human oral keratinocytes. *Free Radic Biol Med* 1999; **26**: 992–1000.
39. Idris AM, Nair J, Ohshima H, et al. Unusually high levels of carcinogenic tobacco-specific nitrosamines in Sudan snuff (toombak). *Carcinogenesis* 1991; **12**: 1115–8.
40. Martin SJ, Reutelingsperger CP, McGahon AJ, et al. Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. *J Exp Med* 1995; **182**: 1545–56.

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