Nicotine derived genotoxic effects in human primary parotid gland cells as assessed in vitro by comet assay, cytokinesis-block micronucleus test and chromosome aberrations test

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Abstract

Genotoxic effects of nicotine were described in different human cells including salivary gland cells. Based on the high nicotine concentration in saliva of smokers or patients using therapeutic nicotine patches, the current study was performed to evaluate the genotoxic potential of nicotine in human salivary gland cells.

Therefore, primary salivary gland cells from 10 patients undergoing parotid gland surgery were exposed to nicotine concentrations between 1 μM and 1000 μM for 1 h in the absence of exogenous metabolic activation. The acinar phenotype was proven by immunofluorescent staining of alpha-amylase. Genotoxic effects were evaluated using the Comet assay, the micronucleus test and the chromosome aberration test. Cytotoxicity and apoptosis were determined by trypan blue exclusion test and Caspase-3 assay.

Nicotine was able to induce genotoxic effects in all three assays. The chromosome aberration test was the most sensitive and increases in numerical and structural (chromatid-type and chromosome-type) aberrations were seen at 1 μM, whereas increases in micronuclei frequency were detected at 10 μM and DNA damage as measured in the Comet assay was noted at >100 μM. No cytotoxic damage or influence of apoptosis could be demonstrated.

Nicotine as a possible risk factor for tumor initiation in salivary glands is still discussed controversially. Our results demonstrated the potential of nicotine to induce genotoxic effects in salivary gland cells. These results were observed at saliva nicotine levels similar to those found after oral or transdermal exposure to nicotine and suggest the necessity of careful monitoring of the use of nicotine in humans.

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1. Introduction

Nicotine is the major alkaloid of tobacco products and is known for causing strong addiction depending on the form of application (Benowitz et al., 1988). Besides investigations regarding the role of tobacco-related diseases, there has been a major focus on the specific effects of nicotine itself in several diseases in recent decades. The physiological effects of nicotine are mediated by the nicotinic acetylcholine receptors (nAChR) on neuronal and non-neuronal cells. Furthermore, the influence of nicotine on angiogenesis has been shown by increased proliferation and migration of endothelial cells. Additionally, nicotine supports angiogenesis by its potential to induce morphological alterations in endothelial cells, which is necessary in later stages of angiogenesis, similar to the vascular endothelial growth factor (VEGF) (Heeschen et al., 2001). This pro-angiogenic potential is induced by the α7 homomeric type of nAChR at the physiological nicotine plasma levels reported in smokers (Lee and Cooke, 2012). Besides endothelial cells, the pro-mitogenic potential of nicotine has also been investigated in different human cancer cell lines, e.g., non-small cell lung cancer...
cells, breast cancer cells and pancreatic cancer cells. In these various cells nicotine was found to promote proliferation and invasion, mediated by the α7 subunit of nAChR (Dasgupta et al., 2006, 2009). Considering these effects and its involvement in different signaling pathways such as Akt, Ras and JAK-2/STAT-3 (Arredondo et al., 2006; West et al., 2003; Egleton et al., 2008; Schuller, 2007), nicotine is assumed to play a key role in the regulation of the complex cellular cascades with the potential to promote tumor progression and metastasis.

Data concerning genotoxic effects of nicotine are contradictory. Nicotine failed to increase mutations in the Salmonella mutagenicity assay or the frequency of sister-chromatid exchanges (SCE) in Chinese hamster ovary (CHO) cells in concentrations up to 1000 μg/ml (~5 mM) with and without metabolic activation (Doellittle et al., 1995). Other findings reported a significant increase in SCE frequency and chromosome aberrations in CHO cells after treatment with nicotine concentrations between 125 and 1000 μg/ml (~0.75–5 mM; Trivedi et al., 1990). In human gingival fibroblasts, nicotine is shown to strongly induce micronuclei and to inhibit apoptosis at a nicotine concentration of 1 μM (Argentin and Cicchetti, 2004). Using the Comet assay, genotoxic effects caused by nicotine in human tissue have been published for spermatozoa at 0.75 mM (Arabi, 2004), for nasal mucosa at 2 and 4 mM (Sassen et al., 2005) and tonsillar tissue and lymphocytes at 0.125–4 mM (Kleinsasser et al., 2005).

Nicotine is present in the saliva of smokers due to deposition in the oral cavity and then dissolving in saliva during smoking. Nicotine concentration in heavy smokers was reported to reach values more than 2 μg/ml (10 μM) (Hukkanen et al., 2005; Teneggi et al., 2002).

After intravenous administration of nicotine in non-smoking healthy volunteers the plasma and saliva concentrations of nicotine were determined. Saliva nicotine levels were up to 8.8-fold higher as compared to plasma levels (Curvall et al., 1990). In addition, transdermally administered nicotine in non-smokers was shown to accumulate in saliva at a 10-fold higher concentration compared to nicotine plasma levels. Maximum concentration of saliva nicotine levels were measured between 91 and 231 ng/ml, corresponding to 0.5 and 1.4 μM. The cellular mechanism for accumulation of nicotine in saliva was explained by ion-trapping due to a slight intracellular acidification in salivary acinar cells during active secretion of saliva (Lindell et al., 1996).

The minor salivary glands in the lips and palate are located within the mucosa and are thereby exposed to nicotine dissolved in saliva in the oral cavity. With rising pH values in saliva, the amount of nicotine in a non-ionized form increases and is able to penetrate oral mucosa cells by passive diffusion (Adrian et al., 2006). Binding of nicotine to oral fibroblasts and rapid absorption has been reported previously (Hanes et al., 1991). As reviewed in 2007, one reason for the high incidence of oral cancer in South Asia may be the combination of tobacco products with betel quid chewing, resulting in more alkaline saliva with higher rates of nicotine absorption (Warnakulasuriya and Rathan, 2007).

The above mentioned pharmacokinetic mechanisms are based on a high exposure of acinar salivary glands cells to nicotine. We could demonstrate significant genotoxic effects of nicotine in freshly isolated parotid gland cells exposed to a nicotine concentration of 0.25 mM for 1 h, and in mini-organ cultures of human parotid glands after repetitive exposure using the Comet assay (Ginzkey et al., 2009, 2010).

The aim of the present study was to add data to these prior observed results using screening methods, involving a battery of tests. This battery includes the Comet assay, cytokinesis-block micronucleus test and chromosome aberration test. For the current investigation, primary cultures of salivary gland epithelial cells derived from human parotid glands were established and exposed to increasing nicotine concentrations.

2. Materials and methods

2.1. Cell cultures

2.1.1. Primary culture of salivary gland epithelial cells derived from human parotid glands

Primary culture of salivary gland epithelial cells was developed according to a protocol described by Ping et al. (2005) with minor modifications. Regular pieces from parotid glands were dissected distantly from tumors during surgery in 10 patients suffering from benign adenoma and transferred to the laboratory. Histologic findings in all cases demonstrated a complete tumor resection. Therefore, specimens of the parotid glands in the laboratory only consisted of healthy acinar cells. No further determination of a possible growth of cancerous cells in the culture was performed. Specimens were cut into small cubes with a volume of 1 mm³ and cultivated in uncoated 24-well plates (BD Biosciences, Heidelberg, Germany) with 250 μl bronchial epithelium growth medium (BEG, PromoCell, Heidelberg, Germany) containing supplement and 1% penicillin–streptomycin (Biochrom, Berlin, Germany) at 37 °C and 5% CO₂ atmosphere as described previously (Ginzkey et al., 2010). Medium was changed every 2nd day.

Within 2 weeks, epithelial cell outgrowth from tissue specimens was observed. Within another 10 days, a confluent monolayer of cells was formed in each well. For further cultivation, cells were harvested by trypsinization for 3–5 min with 0.25% trypsin (Biochrom, Berlin, Germany), followed by addition of fetal calf serum (FCS, Linaris, Wertheim, Germany) to stop enzymatic reaction and three washing steps with PBS (Roche Diagnostics, Mannheim, Germany). Cells were resuspended in BEG medium containing supplement and 1% penicillin–streptomycin and cultured in T-25 cell culture flasks (Greiner Bio-One GmbH, Frickenhausen, Germany) and cells were used for further experiments when reaching ~90% confluency.

Data of smoking habits, alcohol consumption, drug intake and occupation were recorded from the patient charts. The study was approved by the Ethics Commission of the Medical Faculty, Julius-Maximilian-University Wuerzburg, and all participants gave written informed consent.

2.1.2. Human bronchial epithelial cell line BEAS-2B

The human bronchial epithelial cell line BEAS-2B (Sigma-Aldrich, Taufkirchen, Germany) was used as a negative control for immunofluorescent staining against alpha-amylase. Cells were cultured as monolayer at 37 °C and 5% CO₂ in coated T-25 flasks in BEG medium. For coating, 0.01 mg/ml fibronectin, 0.03 mg/ml collagen (both BD Biosciences, Heidelberg, Germany) and 0.001 mg/ml bovine serum albumin (Sigma–Aldrich) were used and flasks were stored at 4 °C. For harvesting, cells were trypsinized with 0.25% trypsin for 3–5 min and washed twice with PBS.

2.1.3. Preparation of freshly isolated cells of human parotid gland

Freshly prepared parotid gland cells were used as positive control for immunofluorescent staining against alpha-amylase. Pieces from parotid glands were minced with a scalpel in a common petri dish followed by enzymatic digestion with protease type XIV from Streptomyces griseus (6.25 mg/ml), hyaluronidase from bovine testis (1.25 mg/dl, both Sigma–Aldrich) and collagenase P (1.25 mg/dl, Roche, Mannheim, Germany) for 1 h in a shaking water bath as described previously (Ginzkey et al., 2009). After stopping the enzymatic reaction with fetal calf serum and filtration through
sterile gauze, the cell suspension was washed in PBS and resuspended in BEG medium.

2.2. Immunofluorescent staining of x-amylase

Cells from different cultures were harvested, centrifuged on glass slides and fixed with methanol for 4 h at −20 °C. Cells were blocked with 10% FCS in PBS. After washing in PBS, cells were incubated with a polyclonal rabbit-anti-alpha-amylase antibody (1:100, Sigma–Aldrich) in PBS containing 0.1% TritonX-100 (Sigma–Aldrich) for 1 h. After three washing steps with PBS, cells were incubated for 1 h with a secondary antibody (Alexa® Fluor 488 goat anti-rabbit, 1:1000, Sigma–Aldrich) for 1 h in PBS at room temperature (RT). After washing, in a third step cells were incubated with 4',6-diamidino-2-phenylindole (DAPI, 5 mg/ml, Sigma–Aldrich) for 3–5 min at RT for staining of cell nuclei. Microscopy was performed with a fluorescence microscope (Leica Microsystems, Wetzlar, Germany).

2.3. Exposure to nicotine

2.3.1. Comet assay and cytokinesis-block micronucleus test

Monolayer cells were harvested from cell culture flasks after incubation with 0.25% trypsin, washing with PBS and resuspension in BEG medium. 150,000 Cells were dispensed to 6-well plates in 2 ml BEG medium. After 3–4 h to allow adhesion to the bottom of the wells, exposure to nicotine at the concentrations 1, 10, 100 and 1000 μM in BEG medium at 37 °C and 5% CO2 for 1 h was performed. Liquid (−) nicotine was obtained from Sigma–Aldrich (purity >99%, CAS-no. 54-11-5). Before use, nicotine was diluted in buffered BEG medium for required concentrations. BEG medium alone served as negative control, and methyl-methane-sulfonate (MMS, Sigma–Aldrich) at the concentration of 100 μM as positive control. All assays were repeated once in each of the 10 patients.

2.3.2. Chromosome aberration test

For the chromosome aberration (CA) test harvested cells were sowed on sterile glass slides in special cell culture dishes. Exposure to different nicotine concentrations, negative and positive controls were performed as described above.

2.3.3. ELISA

For the ELISA test harvested cells were dispensed to 6-well plates and exposed to nicotine as described above for 1 h. For the positive control, exposure was performed with MMS at a concentration of 800 μM for 24 h to induce apoptosis.

2.4. Alkaline single cell microgel electrophoresis (comet) assay

After exposure to nicotine, cells were harvested by incubation with trypsin, washed in PBS and incubated in BEG medium. The alkaline comet assay was used as described previously (Ginzkey et al., 2009). Slides were analyzed with a DMLB fluorescence microscope (Leica Microsystems, Wetzlar, Germany) with a high-resolution CCD camera (model KP-M1AP, Hitachi Ltd., Tokyo, Japan) and comet analyses were performed by an image analysis system (Komet 5.5, Kinetic Imaging, Liverpool, UK). After blinding, two slides per individual with 50 cells (total of 100 cells) for every test sample were counted and analyzed for the following parameters to quantify the induced DNA damage: % DNA in tail (DT), tail length (TL) and Olive tail moment (OTM), which is the product of the median migration distance and the percentage of DNA in tail (Olive et al., 1993). The figures and statistical evaluations were based on the OTM.

2.5. Cytokinesis-block micronucleus test

After exposure to nicotine, cells were washed with PBS and incubated with BEG medium with supplement containing 2.5 μg/ml cytochalasin B (Sigma–Aldrich) for 48 h. Cells were harvested (see above) and resuspended in RPMI.

The assay was performed as described previously (Koehler et al., 2010). For each sample, cells were centrifuged on 2 glass slides and fixed in methanol at −20 °C for 2 h. Staining was performed by incubation in acridine orange staining solution (15 mmol/l Na2 HPO4 × 2H2O, 15 mmol/l KH2PO4 × H2O, 6.25 mg/ml acridine orange; Serva, Heidelberg, Germany) for 5 min, followed by washing twice in Soerensen buffer (15 mmol/l Na2HPO4 × 2H2O, 15 mmol/l KH2PO4 × H2O; Serva).

Analysis of cells was performed using a fluorescence microscope (Zeiss, Jena, Germany) according to the criteria described by the Human Micronucleus project (Fenech et al., 2003). After blinding, 1000 cells were counted and the number of binucleated cells, multi-nucleated cells, apoptotic cells, cells during mitosis, and necrotic cells were determined. In a second count of the same slides, the frequency of micronuclei in 1000 binucleated cells was determined.

2.6. Chromosome aberration test

The chromosome aberration test was performed as described previously (Hackenberg et al., 2011). After exposure to nicotine, cells were washed and incubated with 0.1 μg/ml colcemid (Gibco, Eggenstein, Germany) in BEG medium containing supplement for 1.5 h, followed by treatment with 0.4% KCl in PBS for 25 min at 37 °C. Fixation was performed using a 3:1 mixture of methanol and acetic acid (Carnoy’s solution) three times at −20 °C. The slides were then air-dried, placed on a 90 °C heating plate for 30 min and stored at 60 °C overnight. Cells were stained with 3% Giemsa solution (pH 6.4; Merck Biosciences, Schwalbach, Germany) for 2 min, followed by rinsing in water and air drying.

Analysis of structural and numerical chromosome aberrations was performed by a competent analyst trained in the testing laboratory standard operating procedures after blinding. Two slides per individual from the same culture were evaluated with an inverted light microscope. Fifty metaphase cells per individual were observed and chromosome and chromatid aberrations were recorded according to the International System for Human Cytogenetic Nomenclature (ISCN, 1985).

2.7. Measurement of caspase-3 activity and cell death

After cell harvesting and exposure to BEG medium alone, nicotine or MMS, viability of cells was determined by the trypan blue exclusion test as described previously (Ginzkey et al., 2009). Viable cells were detected based on the ability to exclude the dye, whereas non-viable cells were blue due to defects in the cell membrane.

The caspase-3 activity of the cell lysates was measured using the enzyme-linked immuno-sorbent assay (ELISA) technique. Therefore, cells were washed with PBS after exposure and cultured in BEG medium with supplement for 4 h at 37 °C and 5% CO2. The supernatant was discarded and cells were harvested after trypsin incubation. After incubation with cell lysis buffer for 45 min on ice, the cell pellet was stored at −80 °C. Antibodies, cytokine standard, cell lysis buffer and avidin horseradish peroxidase for the Caspase-3 ELISA assay were obtained from BD Biosciences (Heidelberg, Germany) and the assay was performed in accordance with the manufacturer’s protocol.
2.8. Analysis and statistics

Mean values of scored cells for each concentration in the different assays were used within treatment groups for concentration-dependent effects according to the Friedman test, and the $p$-value was set to 0.05. For analysis of statistical differences between the treatment groups and respective controls, the Wilcoxon signed-rank test was performed. The $p$-value was adjusted to 0.0125 according to the Bonferroni correction for multiple comparisons.

3. Results

3.1. Alpha-amylase synthesis of salivary gland epithelial cells

Epithelial monolayer cell cultures generated from specimens of parotid gland tissue were investigated with regards to the synthesis of alpha-amylase to prove the salivary gland phenotype of the cells. Therefore, immunofluorescent staining of monolayer cultures with an anti-alpha-amylase antibody was performed.

Using a fluorescence microscope, no positive staining of amylase was determined in the cell line BEAS 2b (Fig. 1A–C) serving as negative control, whereas strong fluorescence was measured in freshly isolated parotid gland cells (Fig. 1D–F) serving as positive control. After staining of the monolayer culture of salivary gland cells, a similar staining pattern with cytoplasmatic expression of alpha-amylase could be shown (Fig. 1G–I). DAPI was used for staining of cell nuclei.

3.2. Patient characteristics

From all patients ($n = 10$) data regarding smoking habits, alcohol consumption, daily drug intake and occupation were recorded. 4 males and 6 females were included in the study and mean age was 51.3 years (range 25–82). Mean consumption of cigarettes was $11.5 \pm 12.5$ pack years, four patients did never smoke or had frequent passive exposure to smoking. None of all patients reported of any other consumption of tobacco products or nicotine replacement therapy. Mean consumption of alcohol was $2.5 \pm 4.3$ g per day. None of the patients reported of an intake of immunosuppressive drugs or had undergone chemotherapy. Detailed data is given in Table 1. No statistical correlation regarding smoking habit or alcohol consumption was seen between the results of comet assay, CBMN test, chromosome aberration test and caspase-3 assay (data not shown).

3.3. Comet assay

Salivary gland cells in a monolayer culture from 10 patients were exposed to nicotine at concentrations of 1, 10, 100 and 1000 $\mu$M for 1 h. DNA migration was measured by the alkali version of the Comet assay to determine single-strand breaks (SSB), alkali labile sites, and incomplete excision repair sites.

Looking at the OTM, the mean value of the negative control was $0.31 \pm 0.12$ (S.D.). An increase in DNA damage was detected according to a rising nicotine concentration ($p < 0.01$, Friedman test, Fig. 2).

Comparison between groups using the Wilcoxon test showed no increase at 1 $\mu$M (mean OTM $0.43 \pm 0.22$) and 10 $\mu$M ($0.38 \pm 0.14$), whereas a significant increase in DNA migration could be measured at nicotine concentrations of 100 $\mu$M ($0.47 \pm 0.18$; $p = 0.01$) and 1000 $\mu$M ($0.62 \pm 0.33$; $p < 0.01$). MMS as positive control induced strong DNA damage (mean OTM $8.39 \pm 4.05$, data not shown in graph).

3.4. Cytokinesis-block micronucleus test

Exposure to nicotine for 1 h was followed by the CBMN test in all 10 patients. Medium served as negative control and MMS as
positive control. The micronuclei (MN) frequency of the negative control was 0.76% (SD ± 0.4) of 1000 binuclear cells counted. A concentration-dependent increase in the MN frequency was detected with the Friedman test (\( p < 0.05 \)). Compared with the negative control, no increase could be detected with the Wilcoxon signed-rank test at nicotine concentrations of 1 \( \mu \)M (0.99% ± 0.6; \( p > 0.0125 \)), 10 \( \mu \)M (1.27% ± 1.0; \( p > 0.0125 \)) and 100 \( \mu \)M (0.97% ± 0.5; \( p > 0.0125 \)), whereas a significant increase was measured at 10 \( \mu \)M (1.23% ± 0.6; \( p < 0.0125 \); data presented in Fig. 3). The positive control MMS was capable of inducing a strong increase in MN frequency (2.69 ± 2.4; \( p < 0.0125 \)). No increase or decrease in the frequency of apoptotic cells could be detected with the CBMN test, data not shown in graph.

### 3.5. Chromosome aberrations

After exposure of salivary gland cells to nicotine and MMS for 1 h, the chromosome aberration (CA) test was performed to evaluate possible genotoxic effects at the chromosomal and chromatid level. The assay was repeated in all 10 patients. The structural aberrations consisted of chromatid-type and slightly more chromosome-type aberrations. For statistical calculations all structural aberrations of chromatid- and chromosome-type (breaks, fragments, exchanges, dicentric and ring) were taken into account. A distinct increase in CA was measured with the Friedman test (\( p < 0.001 \)). Compared to the negative control using the Wilcoxon signed-rank test, rising CAs were determined beginning at a nicotine concentration of 1 \( \mu \)M (\( p < 0.0125 \)). Further significant increases for the concentrations 10 \( \mu \)M, 100 \( \mu \)M and 1000 \( \mu \)M could be measured as well (\( p < 0.0125 \), see Fig. 4). Detailed values are presented in Table 2. No polyploidy or cells with pulverized chromosomes or >10 aberrations per cells were detected. Numeri-

### Table 1

Characteristics from all 10 patients: m: male, f: female; smoking habit is given in pack years (py) (1 pack year = 365 days × 20 cigarettes/day; e.g.: 30 py = 1 pack/day for 30 years or = 2 packs/day for 15 years etc.). Alcohol consumption is given in grams per day (g/d).

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Gender</th>
<th>Age</th>
<th>Profession</th>
<th>Nicotine (py)</th>
<th>Alcohol (g/d)</th>
<th>Tumor histology</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>f</td>
<td>47</td>
<td>Seller</td>
<td>0</td>
<td>0</td>
<td>Pleomorphic adenoma</td>
</tr>
<tr>
<td>2</td>
<td>f</td>
<td>40</td>
<td>Controller</td>
<td>10</td>
<td>0</td>
<td>Pleomorphic adenoma</td>
</tr>
<tr>
<td>3</td>
<td>f</td>
<td>39</td>
<td>Teacher</td>
<td>6</td>
<td>0</td>
<td>Pleomorphic adenoma</td>
</tr>
<tr>
<td>4</td>
<td>f</td>
<td>25</td>
<td>Housewife</td>
<td>0</td>
<td>0</td>
<td>Pleomorphic adenoma</td>
</tr>
<tr>
<td>5</td>
<td>m</td>
<td>59</td>
<td>Clerk</td>
<td>30</td>
<td>7</td>
<td>Warthin’s tumor</td>
</tr>
<tr>
<td>6</td>
<td>m</td>
<td>71</td>
<td>Salesman</td>
<td>0</td>
<td>6</td>
<td>Warthin’s tumor</td>
</tr>
<tr>
<td>7</td>
<td>f</td>
<td>38</td>
<td>Social worker</td>
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<td>0</td>
<td>Basal cell adenoma</td>
</tr>
<tr>
<td>8</td>
<td>f</td>
<td>44</td>
<td>Salesman</td>
<td>24</td>
<td>0</td>
<td>Pleomorphic adenoma</td>
</tr>
<tr>
<td>9</td>
<td>m</td>
<td>68</td>
<td>Driver</td>
<td>15</td>
<td>0</td>
<td>Warthin’s tumor</td>
</tr>
<tr>
<td>10</td>
<td>m</td>
<td>82</td>
<td>Pensioner</td>
<td>30</td>
<td>12</td>
<td>Basal cell adenoma</td>
</tr>
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</table>
Table 2

<table>
<thead>
<tr>
<th>Nicotine concentration (µM)</th>
<th>Structural aberrations</th>
<th>% Aberrant cells for structural defects</th>
<th>% Aberrant cells for numerical defects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chromatid type</td>
<td>Chromosome type</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Breaks/fragments</td>
<td>Exchanges</td>
<td>Breaks/fragments</td>
</tr>
<tr>
<td>0 (control)</td>
<td>0.1 (± 0.3)</td>
<td>0 (± 0)</td>
<td>0.2 (± 0.4)</td>
</tr>
<tr>
<td>1</td>
<td>0.6 (± 0.5)</td>
<td>0.1 (± 0.3)</td>
<td>0.6 (± 0.9)</td>
</tr>
<tr>
<td>10</td>
<td>0.7 (± 0.8)</td>
<td>0 (± 0)</td>
<td>0.9 (± 1.0)</td>
</tr>
<tr>
<td>100</td>
<td>1.5 (± 1.1)</td>
<td>0.1 (± 0.3)</td>
<td>2.5 (± 1.9)</td>
</tr>
<tr>
<td>1000</td>
<td>2.5 (± 1.4)</td>
<td>0.2 (± 0.4)</td>
<td>2.5 (± 1.9)</td>
</tr>
<tr>
<td>MMS</td>
<td>4.5 (± 3.6)</td>
<td>0.4 (± 0.5)</td>
<td>4.2 (± 2.5)</td>
</tr>
</tbody>
</table>

Viability of cells and caspase-3 activity

Viability of cells was determined by the trypan blue exclusion test before and after exposure to medium, nicotine and MMS. The range of viable cells was above 80% with no significant differences between groups. Overall, no significant cytotoxic damage could be determined.

To determine a possible influence on the induction of apoptosis, caspase-3 activity was measured by an ELISA assay in all 10 patients. After 1 h of incubation with medium or nicotine as described above, no significant increase or decrease in caspase-3, or differences between groups at any concentration level, could be determined, whereas MMS was able to strongly induce caspase-3 activity (Fig. 5).

4. Discussion

The potential of the tobacco alkaloid nicotine to induce genotoxic damage in salivary gland tissue was shown in freshly isolated parotid gland cells and in the so-called mini-organ cultures of parotid glands (Ginzkey et al., 2009, 2010). In both cell systems, the evaluation of DNA damage was done on isolated single cells using the Comet assay as described above. The Comet assay could be used as a simple screening test for repairable and non-repairable genotoxicity, and one of its advantages is the possibility to perform the test on all nucleated cells, irrespective of S-phase during proliferation (Tice et al., 2000).

Most cells have the ability to perform DNA repair. However, if DNA lesions are mis-repaired and the physiological pathway of apoptosis is interrupted, DNA lesions can cause chromosomal aberrations or other changes with the potential of inducing mutagenesis in a multistep mechanism (Natarajan and Palitti, 2008). Hence, it is recommended to measure genotoxicity at different levels, e.g., in a test battery including tests with the ability to detect different DNA lesions. S-phase-dependent mutagens like many chemical mutagens require the ability of proliferation including mitosis (S-phase), e.g., chromosome aberration or CBMN test to visualize the aberrations.

In this study, the first step was to develop a primary cell line of epithelial salivary gland cells with the potential of growing as described previously (Ping et al., 2005). The spontaneously growing monolayer cells developed from specimens of 10 human parotid gland tissues were investigated. We could demonstrate a strong intracellular content of alpha-amylase in the cell culture by immunofluorescence staining, proving the salivary gland phenotype of these cells.

The half-life of nicotine plasma levels after intravenous infusion of nicotine or cigarette smoking averages about 100–150 min (Hukkanen et al., 2005). Therefore, epithelial salivary gland cells were exposed to increasing concentrations of nicotine for 1 h. The genotoxic effects were detected by an in vitro test battery containing the Comet assay, CBMN test and chromosome aberration test. Significant DNA migration as detected in the Comet assay was measured at higher nicotine concentrations of 0.1 and 1.0 mM without affecting the cell viability. Previous investigations in freshly isolated parotid gland cells could demonstrate elicited DNA damage starting at a nicotine concentration of 0.25 mM (Ginzkey et al., 2009). The current results are in a similar range with a significant increase in DNA migration at 0.1 mM, suggestive of a reliable behavior of salivary gland cells regarding DNA damage induced by nicotine.

In contrast to the effects detected in the Comet assay, which are possibly repairable, micronuclei (MN) as determined in the CBMN test and chromosome aberrations (CA) reflect non-repairable DNA damage. Though the effect in the CBMN test was small, we could demonstrate a significant increase in MN frequency at the nicotine concentration of 10 µM, whereas no significant increase was detectable at 100 and 1000 µM. The potential of nicotine to induce
MN is consistent with published data. In human gingival fibroblasts, rising frequency of MN was shown at distinctly lower levels of nicotine (1 and 10 μM) after 24 h of exposure with decreasing frequency at higher nicotine levels (Argentin and Cicchetti, 2004).

The data from an investigation regarding the role of MN as a biomarker for elevated cancer risk was published in 2007. A large cohort study in lymphocytes of disease-free humans was presented, reporting a possible causal association between MN frequency and cancer risk in non-haematological malignancies (Bonassi et al., 2007). Most studies with the CBMN test were performed in lymphocytes or exfoliated cells (Bonassi et al., 2009; Knasmueller et al., 2011) and exposure to different substances was mostly performed in vivo. However, many studies and meta-analyses report differences in the performance and interpretation of MN (Cardinale et al., 2012), leading to the founding of the Human Micronucleus project (Fenech et al., 2003, 2007).

The origin of MN is a chromosome breakage and/or chromosome loss (aneuploidy), followed by complete nuclear division. In contrast, the CA test detects CA during the metaphase stage of the cell cycle. A possible explanation of an increase of MN frequency in lower nicotine concentrations with a decrease in higher ones could be the influence of nicotine to the cell cycle and proliferation. High DNA damage is known to influence apoptosis and cell cycle (Wang, 2001). A reduced proliferation rate or a loss of cells could be responsible for not achieving telophase, in which MN are scored (Argentin and Cicchetti, 2004). However, in our investigations, no reduction in cell viability or increase in apoptotic cells could be detected in the CBMN test.

Furthermore, no influence of intracellular caspase-3 activity by nicotine could be shown in the Caspase-3 ELISA, indicating no induction of caspase-dependent apoptosis in salivary gland cells after 1 h.

Pro- and anti-apoptotic effects of nicotine in vivo and in vitro models have been described. The different results were partly contributed to the varying exposure times and different nicotine concentrations. Effects in vitro were mostly reported as anti-apoptotic with a wide range of cell systems and different assays used (Zeidler et al., 2007). A physiological experimental setting would be represented by repetitive exposure over years with lower nicotine concentrations.

No effects regarding apoptosis were described after exposure of human lung carcinoma cells to nicotine at concentrations between $10^{-8}$ and $10^{-3}$ M up to 5 days (Zheng et al., 2007). In previous studies from our laboratory, no effects of nicotine concerning apoptotic activity could be determined using the TUNEL assay after exposure of nasal mucosa cells to 1 mM nicotine for 1 h, or the annexin-V-propidium iodide assay with exposure to 1 mM nicotine for 24 h in human lymphocytes (Ginzkey et al., 2012, 2013).

Compared to the Comet assay and CBMN test, the potential of nicotine to induce CA at low nicotine concentrations was even stronger. CA are well-validated biomarkers of genotoxic effects and are believed to develop after mis-repaired double-strand breaks (DSB) (Bender et al., 1974) or converted single-strand breaks to DSB (Natarajan and Palitti, 2008).

In our study, after 1 h of exposure in vitro, the DNA damage in the CA test was detectable at a concentration of 1 μM, inducing chromatid-type and slightly more chromosome-type aberrations, both mainly consisting of breaks. This nicotine concentration is comparable to measured nicotine levels in vivo: nicotine levels in saliva reach up to $>$10 μM in heavy smokers and 0.5–1.4 μM in healthy volunteers after transdermally administered nicotine (Lindell et al., 1996). Additionally, nicotine plasma levels during unrestricted smoking are measured up to 0.6 μM (Hukkanen et al., 2005; Teneggi et al., 2002). In a previous study on human lymphocytes, a similar pattern of CA could be shown after exposure to nicotine for 24 h beginning at a concentration of 1 μM (Ginzkey et al., 2013). In human fetal amniotic cells, nicotine at a concentration of 25 ng/ml (0.15 μM) was able to induce structural chromosome aberrations after exposure to 11 days in vitro (Demirhan et al., 2011). Clastogenic effects of nicotine were published in mouse lymphoma cells (DeMarini et al., 2008) and bovine oocytes (Liu et al., 2008), too.

CAs are an indicator for the risk of developing cancer, which was shown in cohort studies in human peripheral lymphocytes (Hagmar et al., 1998). The impact of chromatid-type and chromosome-type aberrations was discussed in the literature and there is evidence for both types being predictors for cancer risk, although some authors reported a stronger association for chromatoid-type aberrations (Norppa et al., 2006). Cells containing CAs are able to survive and especially chromatid-type aberrations, which can be converted to chromosome-type aberrations, are expected to survive for a long time with the risk of inducing mutagenesis (Bonassi and Au, 2002).

Overall, nicotine was able to induce genotoxic damage in epithelial salivary gland cells derived from human parotid glands. In the Comet assay significant damage could be detected at nicotine concentrations of 100 and 1000 μM and in the CBMN test at 10 μM. The induction of structural chromosome aberrations was measurable starting at a nicotine concentration of 1 μM in the CA test.

As reviewed in the introduction, salivary glands are highly exposed to nicotine, probably due to an ion-trapping mechanism. Saliva nicotine levels could reach concentrations $>$1 μM after oral or transdermal short-term exposure to nicotine. However, physiological situations involve even longer exposure periods to nicotine, e.g., in replacement therapy with lower nicotine concentrations (Hukkanen et al., 2005). Recent results could demonstrate the endogenous synthesis of N-nitrosornicotine (NNN) in human saliva by nitrosation from nicotine and nornicotine in users of nicotine replacement therapy (NRT) such as patch, gum and lozenge (Knezevich et al., 2013; Stepánov et al., 2009a,b). In further studies, results of the used test battery in our test system should be compared with values after metabolic activation, e.g., S9 mix, to detect a possible decrease of the effective nicotine concentrations.

Smoking is known to cause cancer, e.g., of the lung, oral cavity, pharynx, larynx, esophagus (squamous-cell carcinoma), pancreas and urinary bladder as reviewed by the International Agency for Research on Cancer (IARC, 1986; Sasco et al., 2004). Although smoking has proved to be a risk factor for developing benign Warthin’s tumor (Freedman et al., 2009; Pinkston and Cole, 1996), the role of tobacco use as a risk factor for developing malignant tumors in salivary glands is still discussed controversially. In a case-control study with 128 patients in each group, no increase in the risk of salivary gland cancer was found for smoking, chewing tobacco or snuff (Muscat and Wynder, 1998). A population-based, case-control study of 199 cases with salivary gland tumors reported an association between current smoking and a 2-fold increase in the risk of salivary gland cancer (Horn-Ross et al., 1997). Similar results were shown for heavy smokers with $>$ 50 pack years with an odds ratio of 1.8 (Swanson and Burns, 1997). A stronger association between tobacco use and salivary gland cancer was reported in 1999 with an odds ratio of 9 for men and 4.2 for women (Hayes et al., 1999). In an epidemiological study with more than 17,000 patients an increase in salivary gland cancer in males and females between 1953 and 1999 was found, parallel to a rising consumption in alcohol and smoking (Tarvainen et al., 2004). Finally, in 2008 a case-control study with 459 patients presenting with parotid gland tumors found a trend towards an increased risk of developing malignant tumors related to high smoking intensity and early ages at smoking initiation (Sadetzki et al., 2008).

Considering the still controversial epidemiological data regarding smoking and nicotine as a possible risk factor for tumor initia-
tion in salivary glands, our results suggest the potential of inducing genotoxic effects in human epithelial salivary gland cells. When considered together with the reported carcinogenic metabolites generated from nicotine, a careful clinical monitoring of the use of nicotine in humans is mandatory. Further studies will focus on the cellular mechanisms of DNA damage involved in this model and the role of the nicotinic acetylcholine receptors.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tiv.2014.03.012.

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