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Analysis of nicotine-induced DNA damage in cells of the human respiratory tract

Christian Ginzkey[a], Thomas Stueber[a], Gudrun Friehs[a], Christian Koehler[a], Stephan Hackenberg[a], Elmar Richter[b], Rudolf Hagen[a], Norbert H. Kleinsasser[a,*]

[a] Department of Oto-Rhino-Laryngology, Plastic, Aesthetic and Reconstructive Head and Neck Surgery, Julius-Maximilian University Wuerzburg, Josef-Schneider-Str. 11, D-97080 Wuerzburg, Germany
[b] Walther Straub Institute, Department of Toxicology, Ludwig-Maximilian University Munich, Nussbaumstrasse 26, D-80336 Munich, Germany

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ABSTRACT

Epithelium of the upper and lower airways is a common origin of tobacco-related cancer. The main tobacco alkaloid nicotine may be associated with tumor progression. The potential of nicotine in inducing DNA mutations as a step towards cancer initiation is still controversially discussed. Different subtypes of nicotinic acetylcholine receptors (nAChR) are expressed in human nasal mucosa and a human bronchial cell line representing respiratory mucosa as a possible target for receptor-mediated pathways. In the present study, both cell systems were investigated with respect to DNA damage induced by nicotine and its mechanisms.

Specimens of human nasal mucosa were harvested during surgery of the nasal air passage. After enzymatic digestion over night, single cells were exposed to an increasing nicotine concentration between 0.001 mM and 4.0 mM. In a second step co-incubation was performed using the antioxidant *N*-acetylcysteine (NAC) and the nAChR antagonist mecamylamine. DNA damage was assessed using the alkali version of the comet assay. Dose finding experiments for mecamylamine to evaluate the maximal inhibitory effect were performed in the human bronchial cell line BEAS-2B with an increasing mecamylamine concentration and a constant nicotine concentration. The influence of nicotine in the apoptotic pathway was evaluated in BEAS-2B cells with the TUNEL assay combined with flow cytometry.

After 1 h of nicotine exposure with 0.001, 0.01, 0.1, 1.0 and 4.0 mM, significant DNA damage was determined at 1.0 mM. Further co-incubation experiments with mecamylamine and NAC were performed using 1.0 mM of nicotine. The strongest inhibitory effect was measured at 1.0 mM mecamylamine and this concentration was used for co-incubation. Both, the antioxidant NAC at a concentration of 1.0 mM, based on the literature, as well as the receptor antagonist were capable of complete inhibition of the nicotine-induced DNA migration in the comet assay. A nicotine-induced increase or decrease in apoptosis as assessed by the TUNEL assay in BEAS-2B could not be detected.

These results support the hypothesis that oxidative stress is responsible for nicotine-induced DNA damage. Similar results exist for other antioxidants in different cell systems. The decrease in DNA damage after co-incubation with a nAChR antagonist indicates a receptor-dependent pathway of induction for oxidative stress. Further investigations concerning pathways of receptor-mediated DNA damage via nAChR, the role of reactive oxygen species and apoptosis in this cell system will elucidate underlying mechanisms.

1. Introduction

Human tobacco smoke-related cancer predominantly originates from epithelia of the aerodigestive tract. Many constituents of tobacco smoke have been proven to play a crucial role in cancerogenesis (IARC, 2004). However, the role of the main tobacco alkaloid nicotine in cancerogenesis is still discussed controversially (Murray et al., 2009; Schuller, 2009). Most physiological effects of nicotine are mediated by nAChR. These receptors are ligand gated ion channels not only expressed in the human neuronal system but also on the surface of many non-neuronal cells such as pancreas, colon, bladder and airway epithelia where they are thought to be involved in tumor progression mediated via nAChR (Egleton et al., 2008; Keiger et al., 2003). Growing evidence for a tumor-promoting effect of nicotine has been supported by several reports, e.g., pro-angiogenetic effects of nicotine mediated by the

* Corresponding author. Tel.: +49 931 201 21323; fax: +49 931 201 21321.
E-mail address: kleinsasser_n@klinik.uni-wuerzburg.de (N.H. Kleinsasser).

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α7 subtype of nicotinic acetylcholine receptors (nAChR) (Heeschen et al., 2001). Besides such pro-mitogenic effects, nicotine promoted tumor growth of implanted gastric cancer cells (Shin et al., 2004) and breast cancer cells (Lee et al., 2010) in immunocompromised mice.

In human cell systems like lung cancer cells (Maneckjee and Minna, 1994), gingival fibroblasts (Argentin and Cicchetti, 2004), and oral cancer cells (Xu et al., 2007) nicotine was demonstrated to inhibit apoptosis and thus support tumor progression.

In addition to enhanced tumor promotion and progression, several investigations could demonstrate induction of DNA damage by nicotine in different human epithelial and non-epithelial cells (Arabi, 2004; Argentin and Cicchetti, 2004; Ginzkey et al., 2009; Kleinsasser et al., 2005). DNA damage such as single- or double-strand breaks are accepted to represent a possible step in causing DNA mutations if repair mechanisms are insufficient or apoptotic pathways are blocked. While the exact mechanisms of nicotine-related DNA damage are still unknown, oxidative stress seems to play an important role. Nicotine has been reported to cause oxidative stress to rat esophageal mucosa *in vitro* involving superoxide anions and hydrogen peroxide (Wetscher et al., 1995). This is in line with the observation that antioxidative substances are capable of inhibiting nicotine-induced genotoxic effects in rat peripheral blood lymphocytes (Sudheer et al., 2008).

Although no correlation of nicotine itself with malignancies of the nasal cavity is shown, nicotine was able to induce DNA damage in miniorgan cultures of human nasal mucosa as reported previously (Sassen et al., 2005). Epidemiologically, a relationship between smoking and sinonasal and nasopharyngeal cancer could be shown and nasal mucosa is exposed to active and passive smoking (Zhu et al., 2002). In South Africa the inhalation of local snuffs has also been suggested as risk factor for tumors of the paranasal sinuses (Keen et al., 1955).

The present study using freshly isolated single cells of human nasal epithelia and a permanent human bronchial cell line focuses on mechanisms leading to nicotine-induced DNA damage as assessed by the alkali version of the comet assay. Involvement of oxidative stress is investigated by the ability of the known antioxidant *N*-acetylcysteine to reduce DNA damage by nicotine. The role of nAChR is analyzed by co-incubation of nicotine with the non-competitive nAChR antagonist mecamylamine (Papke et al., 2001). The influence of nicotine with respect to apoptosis of exposed cells was evaluated with the TUNEL assay.

2. Materials and methods

2.1. Single-cell suspension of human nasal mucosa

Nasal mucosa specimens were obtained during surgery of the human nasal passage. The study was approved by the Ethics Commission of the Medical Faculty, Julius-Maximilian-University Würzburg, and all participants gave written informed consent.

Nasal mucosa was separated from bones and connective tissue. For enzymatic digestion, 100 μl of enzyme mix containing 0.1 g protease (Sigma–Aldrich, Taufkirchen, Germany) and 1.0 mg DNAse (Sigma–Aldrich) dissolved in 10 ml phosphate buffered saline (PBS, Roche, Mannheim, Germany) were prepared with 9 ml Airway Epithelial Growth Medium (AEGM, PromoCell, Heidelberg, Germany). Specimens were incubated with enzymes for 24 h on a shaker at 4 °C. After stopping the enzymatic reaction with fetal calf serum (FCS, Linaris, Wertheim-Bettingen, Germany), the cell suspension was filtered through sterile gauze and washed twice with PBS. Cell number and viability were assessed by the trypan blue exclusion test.

2.2. Human bronchial epithelial cell line BEAS-2B

Dose finding experiments for further treatment with mecamylamine were performed with the human bronchial epithelial cell line BEAS-2B (Sigma–Aldrich). Cells were cultured as monolayer at 37 °C and 4% CO_2 in coated T25 flasks in Bronchial Epithelial Growth Medium (BEGM, PromoCell, Heidelberg, Germany). 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) dissolved in BEGM was used and flasks were stored at 4 °C. For nicotine and mecamylamine exposure, cells were trypsinized with 0.25% trypsin (Biochrom, Berlin, Germany) for 3–5 min and washed twice with PBS.

2.3. Exposure to nicotine and co-incubation with mecamylamine and N-acetylcysteine

Single-cell suspensions of human nasal mucosa adjusted to 100,000 cells/ml in BEGM medium were exposed to nicotine (Sigma–Aldrich; purity >99%) at a concentration of 0.001, 0.01, 0.1, 1.0 and 4.0 mM. Mecamylamine and *N*-acetylcysteine (NAC, both from Sigma–Aldrich), were incubated with nasal mucosal cells at a concentration of 1.0 mM with or without 1 mM nicotine.

BEAS-2B cells were exposed to 1.0 mM nicotine and increasing concentrations of mecamylamine (0.001, 0.01, 0.1 and 1.0 mM) in BEGM medium.

In all experiments BEGM medium served as the negative control and the direct alkylating substance methyl methane sulfonate (MMS, Sigma–Aldrich) as the positive control. For co-incubation, substances were mixed directly prior to cell exposure. Exposure to all substances was carried out at 37 °C in a shaking water bath for 1 h, and after washing twice in PBS the trypan blue test was performed. Only samples with a viability of >80% were used for the subsequent comet assay.

2.4. Alkaline single cell microgel electrophoresis (comet) assay

The alkali version of the comet assay was used to determine single-strand breaks (SSB), alkali labile sites, and incomplete excision repair sites (Tice et al., 2000). In brief, cells were resuspended in 0.5% low melting agarose (Biozym, Hameln, Germany) and applied to slides (Langenbrinck) coated with 1.5% normal melting agarose (Biozym) (Kleinsasser et al., 2001). After cell lysis for 1.5 h in alkaline lysis buffer (10% DMSO, 1% Triton-X, 2.5 M NaCl, 10 mM Tris, 100 mM Na_2EDTA, pH 10), slides were placed in a horizontal gel electrophoresis chamber (Renner GmbH, Dannstadt, Germany) and covered with alkaline buffer (5 mM NaOH and 200 mM Na_2EDTA) at pH > 13. After a 20 min DNA "unwinding" period, the electrophoresis was performed under standard conditions (25 V, 300 mA, distance between electrodes 30 cm) for 20 min. Following neutralization at pH 7.5 (Trisma Base, Merck, Darmstadt, Germany), cells were stained with ethidium bromide (20 μg/ml; Sigma–Aldrich) and stored at 4 °C until analysis. All preparation steps were performed under red or yellow light to avoid DNA damage by UV light.

2.5. Analysis and statistics

For analysis of slides a DMLB fluorescence microscope (Leica Microsystems, Wetzlar, Germany) with an adapted CCD camera (Model KP-M1AP, Hitachi Ltd., Tokyo, Japan) was used. After coding and blinding of the slides the comet was determined by an image analysis system (Komet 5.5, Kinetic Imaging, Liverpool, UK). Two slides 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), product of the median migration distance, and the percentage of DNA in tail (Olive et al., 1993). Data of DT, TL and OTM are given in Tables 1 and 2. However, according to expert recommendations, statistical evaluation was based on OTM. Results are given as mean ± standard deviation (S.D.) Dose-dependent effects within treatment groups were analyzed by the Friedman test. To demonstrate differences between treatment groups and respective controls, the Wilcoxon signed-rank test was applied (SPSS for Windows 9.0, SPSS Inc., Chicago, IL, USA).

2.6. Detection of apoptosis in BEAS-2B with terminal deoxynucleotidyltransferase dUTP nick end labeling (TUNEL) assay

During later steps in apoptosis DNA is fragmented by endonucleases into small DNA pieces of about 50–300 kb. After addition of bromolated deoxyuridine triphosphate nucleotides (Br-dUTP) the terminal deoxynucleotidyltransferase (TdT) catalyzes the binding of Br-dUTP to 3′-hydroxyl termini of double- and single-stranded DNA. Exposure to nicotine (1.0 mM) was performed for 24 h in cell culture flasks under cell culture conditions. The cytostatic agent mitomycin C (MMC), a known inductor of apoptosis, served as positive control (50 mg/ml; Medac GmbH, Hamburg, Germany). Cells were fixed in 70% ethanol for 30 min and further steps, including staining with a fluorescein isothiocyanate (FITC)-labeled anti-BrdU monoclonal antibody and analysis with flow cytometry (FACS Canto, BD Biosciences, Heidelberg, Germany), were performed with the APO-BRDU™ kit according to the corresponding protocol (BD-Biosciences). For determination of apoptotic cells the quadrant with FITC positive cells was measured in percent related to the whole cell population.

3. Results

3.1. Genotoxic effects of nicotine in nasal mucosa

Single-cell suspensions of human nasal mucosa obtained by enzymatic digestion from samples of 8 donors were exposed to

Table 1
DNA damage by nicotine and MMS as measured by the comet assay in isolated single cells of human nasal mucosa.

Patient	Control			Nicotine 1 μM			Nicotine 10 μM			Nicotine 100 μM			Nicotine 1 mM			Nicotine 4 mM			MMS 100 μM		
	TL	OTM	DT	TL	OTM	DT	TL	OTM	DT	TL	OTM	DT	TL	OTM	DT	TL	OTM	DT	TL	OTM	DT
1	46.46	0.37	1.25	46.35	0.45	1.47	50.32	0.51	1.65	50.65	0.69	2.21	58.91	1.52	4.42	64.87	1.86	5.07	87.58	6.33	15.82
2	49.83	0.76	2.13	38.18	0.51	1.74	48.91	1.25	3.50	42.67	0.66	2.20	40.03	0.60	1.89	55.39	1.79	4.79	95.83	9.95	22.21
3	40.49	0.37	1.46	35.70	0.26	1.04	39.11	0.36	1.39	45.25	0.58	1.95	49.02	0.95	2.67	50.60	0.80	2.68	72.63	3.71	8.81
4	47.13	0.48	1.47	45.80	0.32	1.10	47.62	0.37	1.22	52.05	0.39	1.31	53.64	1.26	3.56	59.83	0.94	2.40	100.72	6.81	15.98
5	40.68	0.34	1.32	49.12	0.84	2.39	52.42	0.59	1.93	49.05	0.61	2.09	69.26	1.31	3.52	70.30	1.86	4.48	84.55	7.55	18.41
6	30.27	0.35	1.76	25.75	0.26	1.39	29.53	0.36	1.70	30.24	0.42	2.01	35.62	0.67	2.72	49.30	1.33	4.33	60.14	3.71	11.09
7	26.12	0.23	1.17	24.15	0.18	0.98	25.21	0.27	1.08	33.10	0.45	1.84	27.09	0.51	1.94	30.99	0.55	2.11	51.74	3.52	10.93
8	29.77	0.32	1.51	31.84	0.41	1.94	30.09	0.40	1.92	31.65	0.47	2.13	43.82	0.93	3.64	41.86	0.91	3.45	97.71	5.84	30.10
Mean	38.84	0.4	1.51	37.11	0.4	1.51	40.4	0.51	1.8	41.83	0.52	1.97	47.17	0.97	3.04	52.89	1.25	3.66	81.36	5.93	16.67
S.D.	9.03	0.16	0.31	9.51	0.21	0.50	10.85	0.31	0.75	8.95	0.12	0.29	13.47	0.37	0.89	12.65	0.53	1.16	18.14	2.25	6.98

MMS, methyl methane sulfonate; OTM, Olive tail moment; DT, % of DNA in tail; TL, tail length.

Table 2
DNA damage by nicotine, *N*-acetylcysteine, mecamylamine and MMS as measured by the comet assay in human nasal mucosa single cells.

Patient	Control			NAC 1 mM			MEC 1 mM			Nicotine 1 mM			Nicotine + MEC			Nicotine + NAC			MMS 100 μM		
	TL	OTM	DT	TL	OTM	DT	TL	OTM	DT	TL	OTM	DT	TL	OTM	DT	TL	OTM	DT	TL	OTM	DT
9	39.82	0.73	2.80	45.37	0.97	3.47	41.30	0.65	2.63	42.85	1.06	3.71	43.07	0.86	3.49	43.79	0.97	3.62	75.24	6.28	16.07
10	23.47	0.27	1.16	23.76	0.15	0.80	24.83	0.24	1.13	32.23	0.51	1.94	23.46	0.19	1.00	23.59	0.26	1.02	59.33	4.43	13.15
11	42.72	0.66	2.31	38.19	0.45	1.64	41.22	0.71	2.38	43.95	0.74	2.63	34.31	0.31	1.35	33.05	0.25	1.04	67.36	3.20	9.08
12	44.65	0.53	1.94	37.68	0.51	1.97	37.38	0.53	2.12	52.23	0.90	3.12	37.69	0.45	1.87	39.45	0.45	1.78	67.04	3.61	9.61
13	37.34	0.27	1.07	34.71	0.40	1.60	41.42	0.43	1.48	37.76	0.58	2.32	36.64	0.36	1.48	53.97	0.52	1.63	67.34	4.14	11.84
14	40.23	0.51	2.02	38.34	0.50	2.11	42.05	0.56	2.12	53.77	1.03	3.35	40.65	0.68	2.36	41.78	0.53	2.11	123.64	14.91	30.65
15	41.80	0.46	1.73	40.50	0.43	1.62	36.65	0.31	1.29	53.83	0.85	2.68	37.89	0.39	1.49	40.61	0.37	1.43	88.17	5.29	12.85
16	43.25	0.50	1.80	39.44	0.42	1.69	42.67	0.47	1.81	50.75	0.80	2.79	41.42	0.45	1.80	42.14	0.47	1.88	78.67	5.70	13.49
17	41.73	0.35	1.32	42.95	0.39	1.44	43.45	0.39	1.48	55.66	1.13	3.65	49.29	0.58	2.09	46.26	0.56	2.06	82.52	3.68	9.46
18	40.25	0.49	1.78	39.55	0.39	1.53	37.36	0.38	1.53	64.73	1.16	3.21	47.39	0.68	2.07	53.88	0.70	2.18	99.31	6.61	15.04
Mean	39.53	0.48	1.79	38.05	0.46	1.79	38.83	0.47	1.8	48.78	0.88	2.94	39.18	0.49	1.9	41.85	0.51	1.87	80.86	5.79	14.12
S.D.	6.00	0.15	0.53	5.81	0.20	0.68	5.48	0.15	0.50	9.56	0.22	0.57	7.25	0.20	0.69	9.03	0.21	0.74	19.10	3.41	6.27

NAC, *N*-acetylcysteine; MEC, mecamylamine; MMS, methyl methane sulfonate; OTM, Olive tail moment; DT, % of DNA in tail; TL, tail length.

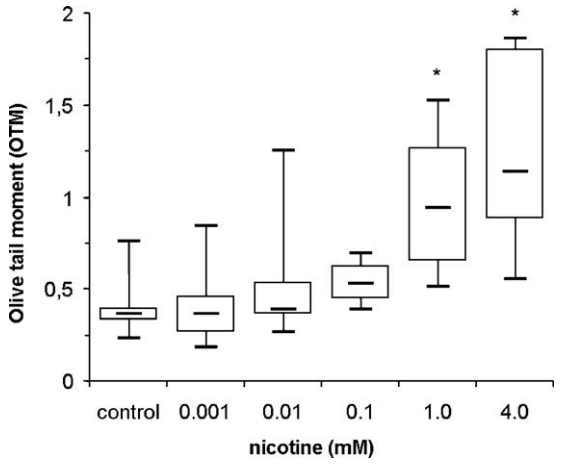

Fig. 1. Exposure of isolated single cells of human nasal mucosa ($n = 8$) to increasing nicotine concentrations for 1 h. Medium alone was used as negative control. DNA damage was determined using the comet assay. Olive tail moment (OTM) represents the product of the median migration distance and the percentage of DNA in tail. Box plots show median, 1st quartile, 3rd quartile, minimal and maximal values of OTM. The increase in DNA migration was dose-dependent ($p < 0.001$). Compared to the negative control, significant DNA damage started at 1.0 mM nicotine (*$p = 0.01$).

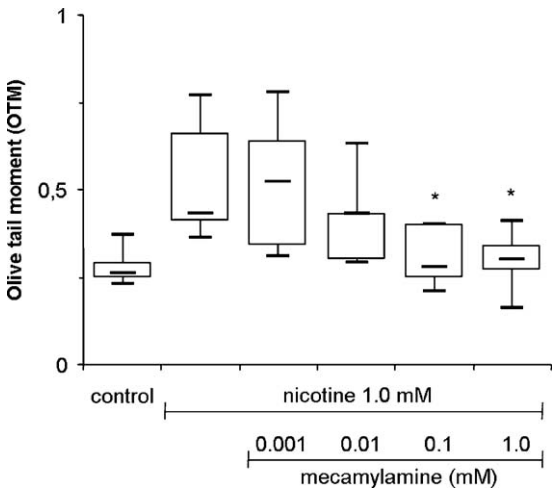

Fig. 2. Human bronchial epithelial cell line BEAS-2B was exposed to 1.0 mM nicotine and an increasing concentration of the nAChR blocker mecamylamine for 1 h. DNA damage was determined using the comet assay. Olive tail moment (OTM) represents the product of the median migration distance and the percentage of DNA in tail. Box plots show median, 1st quartile, 3rd quartile, minimal and maximal values of OTM. Reduction in nicotine-induced DNA damage reached significance at 0.1 and 1.0 mM mecamylamine (*$p < 0.05$).

increasing concentrations of nicotine for 1 h. Genotoxic effects of nicotine were assessed by the comet assay (see Table 1 for all individual values of OTM, DT and TL). Looking at the OTM, nicotine demonstrated a significant dose-dependent increase in DNA damage ($p < 0.001$; Fig. 1). Cytotoxic effects responsible for DNA migration was minimized by using trypan blue exclusion test, which show cell viability >80% in all tested samples. Compared to the control value (0.40 ± 0.16) a significant increase of OTM values was observed at 1.0 mM (0.97 ± 0.37; $p = 0.01$) and 4.0 mM (1.25 ± 0.53; $p < 0.01$). The positive control methyl methane sulfonate (MMS) caused distinct DNA damage (5.93 ± 2.25; Table 1).

3.2. Dose-dependent inhibition of nicotine-induced DNA damage by mecamylamine in BEAS-2B

The human bronchial epithelial cell line BEAS-2B was exposed to 1.0 mM nicotine for 1 h together with increasing concentrations of mecamylamine, with 5 replications being performed. Nicotine (0.53 ± 0.18) caused a distinct increase in DNA migration compared to the negative control (0.28 ± 0.05; $p < 0.05$) as assessed by OTM values in the comet assay. Addition of mecamylamine dose-dependently decreased nicotine-induced DNA damage (Fig. 2) reaching significance at 0.1 mM (0.31 ± 0.09; $p < 0.05$) and 1.0 mM (0.30 ± 0.09; $p < 0.05$) of mecamylamine. For further experiments mecamylamine was used at a concentration of 1.0 mM.

3.3. Inhibition of nicotine-induced DNA damage by N-acetylcysteine and mecamylamine in human nasal mucosa

Based on the initial data, further experiments were performed with a nicotine concentration of 1.0 mM. Nasal epithelial single-cell suspensions from samples of 10 patients were exposed to 1.0 mM nicotine for 1 h. Co-incubations were performed with the non-competitive antagonist of the nAChR mecamylamine (1.0 mM) and the antioxidant *N*-acetylcysteine (NAC, 1.0 mM) for the same incubation period and the comet assay was applied (see Table 2 for all individual values of OTM, DT and TL). None of the substances affected viability of the cells as determined by the trypan blue exclusion test.

No DNA damage could be measured when comparing OTM of the negative control (0.48 ± 0.15) after incubation with mecamylamine (0.47 ± 0.15) and NAC (0.46 ± 0.2) only (Fig. 3). After exposure to 1 mM nicotine, a significant DNA damage (0.88 ± 0.22; $p < 0.01$) could be confirmed. Co-incubation of nicotine with mecamylamine (0.49 ± 0.20; $p < 0.01$) as well as NAC (0.51 ± 0.21; $p < 0.01$) abolished DNA damage by nicotine.

3.4. Detection of apoptosis in BEAS-2B with the TUNEL assay

After exposure of BEAS-2B to 1.0 mM nicotine for 24 h apoptotic activity was measured by detection of 3′-hydroxyl termini of double- and single-stranded DNA in the TUNEL assay. Cell medium served as a negative control, mitomycin C (MMC) as a positive control, with 5 replications being performed. As shown in Fig. 4, compared to the negative control ($1.86 \pm 1.35\%$) exposure of the cells to nicotine ($2.04 \pm 1.11\%$; n.s.), did not affect apoptosis, whereas the positive control MMC significantly increased the percentage of apoptotic cells ($4.58 \pm 3.32\%$; $p < 0.05$).

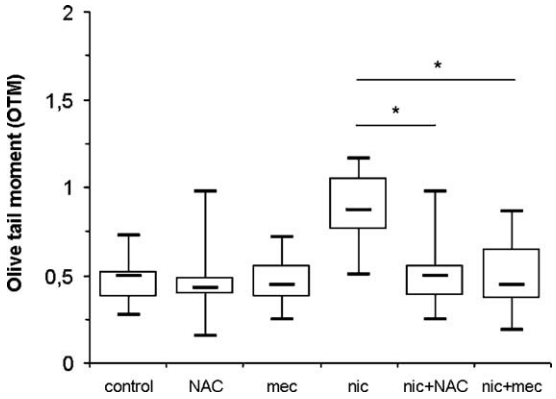

Fig. 3. DNA damage in isolated single cells of human nasal mucosa ($n = 10$) after 1 h exposure to nicotine (Nic, 1.0 mM), *N*-acetylcysteine (NAC, 1.0 mM) and mecamylamine (Mec, 1.0 mM) and co-incubation of nicotine with NAC or mecamylamine. DNA damage was determined using the comet assay. Olive tail moment (OTM) represents the product of the median migration distance and the percentage of DNA in tail. Box plots show median, 1st quartile, 3rd quartile, minimal and maximal values of OTM. NAC and mecamylamine completely inhibited nicotine-induced DNA damage at 1.0 mM (*$p < 0.01$).

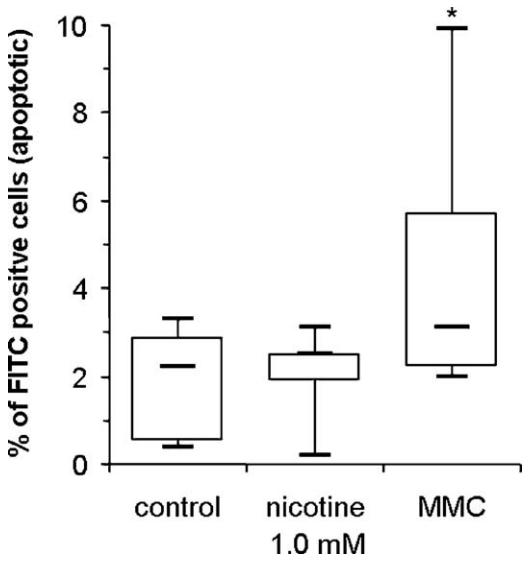

Fig. 4. The influence of nicotine on apoptosis was measured by the TUNEL assay after exposure of BEAS-2B cells to 1.0 mM nicotine for 24 h. DNA breaks were visualized using flow cytometry by staining with FITC-labeled antibodies of 3′-hydroxyl termini after Br-dUTP binding. Mitomycin C (MMC) served as a positive control. Box plots show median, 1st quartile, 3rd quartile, minimal and maximal values of the percentage of FITC positive cells. MMC but not nicotine induced a significant increase in apoptotic activity compared to the control ($^*p < 0.05$).

4. Discussion

4.1. Nicotine-induced DNA damage in nasal mucosa is dose dependent

A common origin of tobacco-related cancer is the epithelium of the upper and lower aerodigestive tract (IARC, 2004). The role of the tobacco alkaloid nicotine in the initial phase of tumor induction is still controversially discussed. To investigate the mechanisms leading to DNA damage due to exposure to nicotine, freshly isolated human nasal mucosa cells and a stable human bronchial epithelium cell line were used.

In the present study, single cells of human nasal epithelia were exposed to nicotine and genotoxic effects were assessed by the alkali version of the comet assay. This assay is an established test for measurement of genotoxic effects and DNA migration was shown to correlate with DNA damage (Tice et al., 2000). Significant reliable DNA damage was determined in this setting at a nicotine concentration of 1.0 mM. Similar results were obtained previously with miniorgan cultures of human nasal mucosa after repetitive exposure to nicotine (Sassen et al., 2005). In human lymphocytes and single cells isolated from human tissues like tonsils (Kleinsasser et al., 2005), and the parotid gland (Ginzkey et al., 2009), a significant increase in DNA migration in the comet assay was measured at 0.25 and 0.5 mM.

The content of nicotine in one cigarette varies between 0.3 and 2.0 mg and plasma nicotine levels in heavy smokers reach up to 100 ng/ml (=0.6 μM) and in saliva up to 25 μM with a much longer time of exposure (Hukkanen et al., 2005; Teneggi et al., 2002). The highest plasma nicotine concentration, 0.8 μM was recorded in a nasal snuffer (Russell et al., 1981). However, one pinch of nasal snuff (0.1–0.2 g) may contain 0.5–2.8 mg of nicotine (SmokeLess New Zealand, 2007) and even millimolar concentrations could be achieved within the nasal cavity. Nasal spray for nicotine replacement therapy (NRT) contains about 0.5 mg nicotine per application (Rubinstein et al., 2008). Trying a pharmacokinetic calculation, it is essential to know the volume of the fluid in the nose, in which nicotine is solved after usage. For example, 0.5 mg nicotine in 1 ml is equivalent to 3.0 mM nicotine. This could only be a theoretical approach and the realistic dispersion of nicotine after usage, e.g. nose spray, is difficult to measure. To our knowledge, there is not much published data to this term. Therefore, the nicotine concentration of 1.0 mM chosen for our further experiments is not unrealistically high.

4.2. Nicotinic acetylcholine receptors contribute to nicotine-induced genotoxicity

Is DNA damage by nicotine related to a receptor-mediated pathway? To evaluate this hypothesis blocking experiments with mecamylamine, a potent non-competitive, non-selective antagonist of the nicotinic acetylcholine receptor (nAChR), were performed. First, dose finding experiments were carried out in the human bronchial cell line BEAS-2B with a constant nicotine concentration of 1.0 mM and an increasing concentration of mecamylamine between 0.001 and 1.0 mM for 1 h followed by the comet assay. We could demonstrate significant DNA damage by nicotine and a maximal reduction in induced DNA damage at 0.1 and 1.0 mM of mecamylamine without affecting the viability of cells (Fig. 2). This effect may be explained by non-competitive ion-channel blocking of nAChR.

Although mecamylamine has been reported to be a preferential blocker of the ganglionic nAChR subtypes, such as α3- or α4-made nAChRs (Arredondo et al., 2006a), at a concentration of 0.1 mM mecamylamine completely inhibited all important subtypes of nAChRs including the α7 receptor (Papke et al., 2001). In order to completely inhibit nAChRs mecamylamine was added at 1.0 mM during exposure of nasal mucosa to nicotine at 1.0 mM. With respect to DNA migration in the comet assay, co-incubation with mecamylamine led to a complete and significant reduction in nicotine-induced DNA damage (Fig. 3 and Table 2). Both cell types, BEAS-2B and human nasal mucosa, are known to express nAChR as a possible target for nicotine and these results underscore the fact that nAChR plays an important role in nicotine-induced genotoxicity (Egleton et al., 2008; Keiger et al., 2003).

Since mecamylamine is a partial-agonist in nAChR it has a potential to up- and down-regulate nAChR expression. However, these effects depend on cell type and the chosen duration of exposure, which should always be taken into account analyzing specific results (Lukas, 1991; Mousavi and Hellström-Lindahl, 2009; Robinson and McGee, 1985). Considering agonistic and antagonistic properties, the reduction of the nicotine effect by mecamylamine in our cell system supports the hypothesis that DNA damage is caused by a receptor mediated pathway.

4.3. Nicotine-induced DNA damage is related to oxidative stress

One crucial step in the induction of DNA damage by nicotine seems to be the generation of oxidative stress. Reactive oxygen species (ROS) as mediators of oxidative stress are proven to induce DNA damage via direct interaction with cellular DNA, like induction of base modifications, cross links, single- and double-strand breaks as well as indirect interactions by lipid peroxidation.

To investigate the role of oxidative stress in DNA damage by nicotine as assessed by the comet assay a co-incubation experiment was performed with the known antioxidant *N*-acetylcysteine (NAC) and nicotine. At a concentration of 1.0 mM NAC completely prevented genotoxic effects induced by 1.0 mM nicotine. One explanation of this effect may be the induction of ROS by nicotine treatment and a protection of the cells by NAC. This is in line with results from experiments with rat *in vitro* as well as *in vivo* demonstrating protection against oxidative stress by NAC and other antioxidants in lymphocytes (Sudheer et al., 2007, 2008; Muthukumaran et al., 2008). Inhibition of nicotine-induced

genotoxicity by NAC was also reported for human fibroblasts (Argentin and Cicchetti, 2004) and in an oral cancer cell line (Wu et al., 2005). In employees of tobacco industries in India nicotine was made responsible for disturbance of the balance between oxidants, e.g., serum lipid peroxides and nitric oxides, and antioxidants like superoxide dismutase, thus causing oxidative stress (Swami et al., 2006).

ROS could be the product of CYP2A6 (Yamazaki et al., 1999), mitochondrial chain induction by nicotine (Gvozdjákova et al., 1992) and increased leucocyte activity (Gillespie et al., 1987). CYP2A subtypes, e.g., CYP2A6, are expressed in human and mammalian nasal mucosa (Chen et al., 2003; Getchell et al., 1993) and Kleinsasser et al. (2009) reported on CYP2A6 expression in human miniorgan cultures during 11 days of culture period.

Our results suggest an involvement of nAChRs in the induction of ROS. These receptors were hypothesized as being responsible for the induction of oxidative stress in fetal pancreatic cells after nicotine exposure of rat maternal animals (Bruin et al., 2008). A further possible link is shown by an investigation of human lymphocytes (Panayiotidis et al., 1999). After exposure of lymphocytes with H_2O_2, the increasing DNA damage was reduced by application of a Ca^{2+} chelator. The authors explained their results by an activation of an endonuclease responsible for DNA strand breaks. This is in line with the findings of activation of the Ras/Raf-1/MEK1/ERK pathway by Ca^{2+} influx after activation of nAChR in human oral keratinocytes (Arredondo et al., 2006b). Chronic exposure to nicotine itself was reported to increase production of ROS via activation of the Ras pathway with the potential of DNA damage and deregulation of cell cycle restriction in rat lung epithelial cells (Guo et al., 2005).

Beside ROS, an increase in nitric oxides (NO) caused by nicotine-mediated upregulation of the inducible nitric oxide synthetase (iNOS) is considered to play a role in nicotine-associated genotoxicity (Argentin and Cicchetti, 2006). iNOS was shown to be expressed in human nasal epithelia (Kawamoto et al., 1998). In neuronal epithelia a connection between nAChR stimulation, calcium influx, and iNOS induction has been shown (Garcia-Ratés et al., 2010; Haberberger et al., 2003).

4.4. Nicotine does not influence apoptotic activity in this cell system

By binding to nAChRs nicotine is able to initiate various pathways, e.g., activation of the serine/threonine kinase Akt, which controls cellular processes like cell cycle progression and apoptosis (Thunnissen, 2009; West et al., 2003). The influence of apoptosis by nicotine in this cell system was evaluated by the TUNEL assay after exposure of human bronchial cell line BEAS-2B to nicotine for 24 h. No measurable increase or decrease in the number apoptotic cells was seen after exposure to 1.0 mM of nicotine. This is in line with results from Zheng et al. (2007) showing no effect of nicotine on apoptosis of non-small cell lung carcinoma cells. After nicotine administration to rats induction of apoptosis was observed in pulmonary epithelial cells (Demiralay et al., 2006). However, in many other studies nicotine has been shown to support progression of cancer cells by inhibition of apoptotic pathways which may be mediated by nAChRs as well as ß-adrenoceptors (Tsurutani et al., 2005; Argentin and Cicchetti, 2006; Dasgupta et al., 2006; Jin et al., 2008; Chen et al., 2010). BEAS-2B cells are known to express different subtypes of nAChRs, e.g., α7, as a possible target for nicotine to activate anti-apoptotic pathways (Egleton et al., 2008). Though our results could not demonstrate pro- or anti-apoptotic effects by nicotine, these effects seem to be dependent on the target cells investigated, the concentration of nicotine, and the length of exposure (Zeidler et al., 2007). Therefore, further analyses in this cell system are required, especially at distinctly lower concentrations of nicotine and with varying periods of exposure time.

4.5. Conclusion

The results of the present study point to an involvement of nAChR in nicotine-associated DNA damage via induction of oxidative stress. Several intracellular pathways could be taken into consideration. Further research is essential to clarify the role of nAChR and oxidative stress in this cell system as a possible inductor of the observed DNA damage.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Assessment of nicotine-induced DNA damage in a genotoxicological test battery

Christian Ginzkey*, Gudrun Friehs, Christian Koehler, Stephan Hackenberg, Rudolf Hagen, Norbert H. Kleinsasser

Department of Oto-Rhino-Laryngology, Plastic, Aesthetic and Reconstructive Head and Neck Surgery, University Hospital of Wuerzburg, Josef-Schneider-Str. 11, D-97080 Wuerzburg, Germany

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ABSTRACT

The role of the tobacco-alkaloid nicotine in tumour biology is widely discussed in the literature. Due to a strong capacity to induce angiogenesis, a pro-mutagenic potential in non-tumour and cancer cells, and a pro- and anti-apoptotic influence, nicotine seems to promote the growth of established tumours. However, results indicating DNA damage and genetic instability associated with nicotine have been contradictory thus far. A variety of markers and endpoints of genotoxicity are required to characterize the genotoxic potential of nicotine. Induction of DNA single- and double-strand breaks, the formation of micronuclei, and the induction of sister chromatid exchange and chromosome aberrations represent possible genotoxicological endpoints at different cellular levels.

Human lymphocytes were exposed to nicotine concentrations between 1 μM and 1 mM for 24 h *in vitro*. The comet assay, the cytokinesis-block micronucleus test, the chromosome aberration (CA) test, and the sister chromatid exchange (SCE) test were then applied. Viability and apoptosis were measured by flow cytometry in combination with the annexin V-propidium iodide staining test.

In this test setting, no enhanced DNA migration was measured by the comet assay. An increase in the micronucleus frequency was detected at a concentration of 100 μM nicotine without affecting the frequency of apoptotic cells. A distinct genotoxic effect was determined by the CA test and the SCE test, with a significant increase in CA and SCE at a concentration of 1 μM. In the annexin V test, nicotine did not influence the proportion of apoptotic or necrotic cells.

The current data indicating the induction of CA by nicotine underscore the necessity of ongoing investigations on the potential of nicotine to initiate mutagenesis and tumour promotion. Taking into account the physiological nicotine plasma levels in smokers or in nicotine-replacement therapy, particularly the long-term use of nicotine should be critically discussed.

1. Introduction

The major tobacco-alkaloid nicotine is discussed in the literature as a factor in tumour progression. It has the potential to strongly stimulate angiogenesis via induction of endothelial cell growth and up-regulation of vascular endothelial growth factor (VEGF) [1,2]. Other investigators described pro-mitogenic effects of nicotine in normal and carcinoma cells [3,4]. As reviewed by Cardinale et al. in 2008 and by Catassi et al. in 2011, effects combine apoptotic pathways and nicotine seems to play a crucial role in tumour progression [5,6].

In previous studies, nicotine was shown to induce genotoxicity, as determined by means of the comet assay. Besides in human lymphocytes, these effects were described in human nasal mucosa [7,8], tonsillar tissue [9], cells of the parotid gland [10], and spermatozoa [11]. The comet assay is known to detect DNA single-strand breaks (SSB), alkali-labile sites, and incomplete excision-repair sites in proliferating and non-proliferating cells, representing transient promutagenic lesions. In principle, these lesions are assumed to represent a crucial step in causing DNA mutations if repair mechanisms are insufficient or apoptotic pathways are blocked. Compared with other genotoxicological tests, the comet assay does not require cell division in the cells investigated. However, DNA migration measured in this assay does not necessarily lead to the induction of DNA mutation [12]. Therefore, the comet assay is commonly used as a highly sensitive screening test. However, to investigate and evaluate the potential of xenobiotics to induce decisive DNA damage that may be followed by DNA mutations, it is essential to use genotoxicological tests with different endpoints.

An established genotoxicological test that measures a variety of endpoints such as chromosome instability, mitotic dysfunction, cell proliferation, and necrosis, is the cytokinesis-block micronucleus assay (CBMN). The mechanisms leading to the formation

* Corresponding author. Tel.: +49 931 201 21323; fax: +49 931 201 21321.
E-mail address: ginzkey_c@klinik.uni-wuerzburg.de (C. Ginzkey).

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of micronuclei (MN) are chromosome breakage and disturbance of the chromosome-segregation system. This mechanism requires mitotic or meiotic cell divisions. The chromosome breakage with formation of MN represents irreversible DNA damage with the risk of genetic instability, which may result in cancer [13].

At the chromosomal and the chromatid level, mis-repaired DNA lesions lead to the formation of chromosome aberrations (CA), which represent the test endpoints in the CA test. This assay is widely used, e.g., in toxicological and genotoxicological investigations, and detects both chromatid-type and chromosome-type aberrations [14]. Another test endpoint at the chromatid level is the sister chromatid exchange (SCE) as detected in the SCE test. By use of the thymidine analogue 5′-bromodeoxyuridine (BrdU), which is incorporated into the DNA strand during replication, the SCE can be determined after staining. The increase in SCE is very sensitive in indicating genotoxic potential of a tested xenobiotic, although the increase does not seem to be correlated with cancer [15].

This study investigated the potential of nicotine to induce reversible and irreversible DNA damage in human lymphocytes with a cluster of genotoxicological tests with different nicotine concentrations. A variety of test endpoints, as described above, were taken into account: DNA migration (comet assay), and frequency of MN, CA, and SCE. Additionally, cell viability and the induction of apoptosis were measured by the annexin V-propidium iodide staining apoptosis assay.

2. Material and methods

2.1. Preparation of human lymphocytes

Heparinized blood samples of 50 ml were obtained by venous puncture from ten healthy volunteers and transferred to the laboratory. Lymphocytes were separated by density-gradient centrifugation (20 min, 800 g on equal amounts of Ficoll (Biochrom, Berlin, Germany), washed in phosphate-buffered saline (PBS, Gibco, BRL Lifetechnologies, Eggenstein, Germany) and re-suspended in RPMI (Biochrom, Berlin, Germany) containing the supplement of bovine serum albumin (Linaris, Wertheim-Bettingen, Germany), 1% sodium-pyruvate, 1% non-essential amino acids, and 1% penicillin–streptomycin (all Biochrom). Lymphocytes were counted and their viability was determined by means of trypan-blue staining. The study was approved by the Ethics Commission of the Medical Faculty, Julius-Maximilian-University Wuerzburg, and all participants gave written informed consent.

2.2. Exposure of lymphocytes to nicotine

Due to different preparation protocols, lymphocyte cultures were divided in different samples for each assay. The comet assay and the annexin V assay were carried out in un-stimulated lymphocytes. Tests for chromosomal aberrations (CA) and sister chromatid exchange (SCE), as well as the cytokinesis-block micronucleus test (CBMN) were performed after stimulation with 2.4 μg/ml phytohaemagglutinin (PHA, Biochrom) for 42–44 h in RPMI with supplement (see Section 2.1) at 37 °C and 5% CO_2 to induce mitosis. Exposure to nicotine at concentrations of 1, 10, 100 μM as well as 1 mM for 24 h was done once per concentration tested in RPMI with supplement and under the same cell-culture conditions. Stimulated cells were washed twice with PBS before the subsequent exposure. The direct-alkylating substance methylmethane sulfonate (MMS) at a concentration of 100 μM served as a positive control.

2.3. Alkaline single-cell gel electrophoresis (comet) assay

The alkaline version of the comet assay was used to determine single-strand breaks (SSB), alkali-labile sites, and incomplete excision-repair sites [16]. The test procedure was as previously described [10]. RPMI medium with supplement was used for the negative control and 100 μM MMS for the positive control. Because of extremely extensive DNA migration, exposure to MMS was only for 1 h prior to comet assay. Slides were analysed with a DMLB fluorescence microscope (Leica Microsystems, Wetzlar, Germany) with an adapted CCD camera (model KP-M1AP, Hitachi Ltd., Tokyo, Japan) and comets were determined by means of an image-analysis system (Komet 5.5, Kinetic Imaging, Liverpool, UK). Two slides with 50 cells (total of 100 cells) for every test sample were counted and analysed for the following parameters to quantify the induced DNA damage: % DNA in tail (DT), tail length (TL) and Olive tail moment (OTM), product of the median migration distance and the percentage of DNA in tail [17]. Figures and statistical evaluation were based on the OTM.

2.4. Cytokinesis-block micronucleus test

The assay was performed as described previously [18]. After stimulation of 800,000 lymphocytes/ml with PHA (see Section 2.2), cells were exposed to nicotine for 24 h. To avoid cell division after mitosis, cells were incubated with cytochalasin B (Sigma–Aldrich, Taufkirchen, Germany) at a concentration of 5 μg/ml for 48 h. Thus, the cells that had undergone mitosis could be detected by their doubled nuclei. After staining with trypan blue, 30,000 cells were centrifuged on glass slides and fixed in methanol at −20 °C for 2 h. Staining was performed by incubation in an acridine-orange staining solution (15 mM $Na_2HPO4{\cdot}2H_2O$, 15 mM $KH_2PO_4{\cdot}H_2O$, 6.25 mg/ml acridine orange; Serva, Heidelberg, Germany) for 5 min, followed by two washings in Soerensen buffer (15 mM $Na_2HPO_4{\cdot}2H_2O$, 15 mM $KH_2PO_4{\cdot}H_2O$; Serva).

Analysis of cells was performed with a fluorescence microscope (Zeiss, Jena, Germany) according to the recommendations [19]. During analysis, 1000 cells were counted and the number of bi-nucleated cells, multi-nucleated cells, apoptotic cells, cells during mitosis, and necrotic cells were determined according to the criteria described by the HUman MicronNucleus (HUMN) project [20]. In a second count, the number of micronuclei in 500 bi-nucleated cells was determined.

2.5. Chromosome aberration assay and sister chromatid exchange test

The chromosome aberration (CA) assay and the sister chromatid exchange (SCE) test were applied in 800,000 lymphocytes/ml after stimulation with PHA and exposure to nicotine or MMS for 24 h. In addition, for the SCE test cells were incubated with BrdU for staining of sister chromatids. For metaphase arrest 0.1 μg/ml colcemid (Gibco, Eggenstein, Germany) was added to the cell culture medium for 1.5 h, followed by treatment with 0.4% KCl in PBS for 20 min at 37 °C and fixation in a 3:1 mixture of methanol/acetic acid (Carnoy's solution). Cells were fixed three times at −20 °C prior to seeding on glass slides. The slides were air-dried, placed on a 90 °C heating plate for 30 min and stored at 60 °C overnight. Cells were stained with a 3% Giemsa solution (pH 6.4; Merck Biosciences, Schwalbach, Germany) for 2 min, followed by rinsing in water and air-drying.

Analysis of structural chromosome aberrations and SCE was performed by a competent analyst, who was trained in the standard operating procedures at the test laboratory. Slides were evaluated with an inversed light microscope in Giemsa-stained metaphase cells. Fifty metaphase cells per culture were observed and CA (gaps and breaks) and reunions were recorded according to the International System for Human Cytogenetic Nomenclature (ISCN) system [21]. The ISCN defines a gap as a clear, non-stained region on a chromosome, and a break as a discontinuity of a chromosome showing a clear distal fragment-misalignment of a broken chromosome.

2.6. Annexin V-propidium iodide staining apoptosis assay

Flow cytometry was used to determine apoptosis and necrosis with an annexin V-propidium iodide (PI) kit (Becton-Dickinson Bioscience, Heidelberg, Germany) according to the manufacturer's protocol. Twenty-four hours after exposure to nicotine or MMS, cells were washed twice with PBS and the cell pellets were re-suspended in 4 °C binding buffer (0.1 M HEPES, pH 7.4, 1.4 M NaCl, 25 mM $CaCl_2$). The staining procedure for annexin V and PI was followed, with addition of 5 μl annexin V and 5 μl PI to each sample and incubation for 15 min in the dark, at room temperature. Flow-cytometry was used to determine fluorescence (FACScanto, Becton-Dickinson) as described previously [22]. Cells positive for PI and annexin V were counted as necrotic, cells positive for annexin V alone as apoptotic, and those positive for neither were counted as viable cells.

2.7. Analysis and statistics

The mean values obtained from cells scored for each concentration in the different assays were used within treatment groups to determine concentration-dependent effects according to the Friedman test. For analysis of statistical differences between the treatment groups and the respective controls, the Wilcoxon signed-rank test was performed. Statistical significance was reached when the p-value was <0.05.

3. Results

3.1. Comet assay

After 24 h of exposure of cells to nicotine at concentrations between 1 μM and 1 mM, the comet assay was applied on the exposed cells. Medium served as a negative control and the direct alkylating substance methylmethane sulfonate (MMS) was used as a positive control. Lymphocytes were incubated with 100 μM MMS 1 h prior to the comet assay. Looking at DNA in the tail (DT), tail length (TL) and Olive tail moment (OTM), no significant concentration-dependent DNA migration could be determined

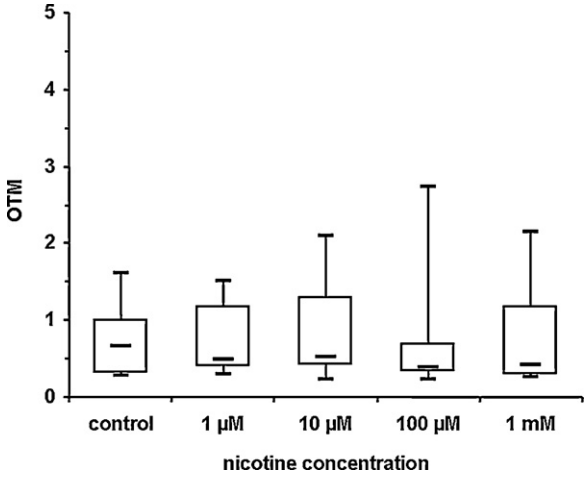

Fig. 1. Comet assay: OTM values after exposure of lymphocytes to nicotine for 24 h. Box plots represent median, 1st quartile, 3rd quartile, minimal and maximal values of OTM. Culture medium served as a negative control. No significant DNA damage was determined at any of the concentrations tested.

after exposure to nicotine in the Friedman-test. Values of OTM with respect to the nicotine concentration are presented in Fig. 1, mean and standard deviation were calculated as follows: control (0.76 ± 0.46), 0.001 mM (0.8 ± 0.48), 0.01 mM (0.84 ± 0.62), 0.1 mM (0.74 ± 0.8), 1.0 mM (0.81 ± 0.65). Significant DNA migration was caused by MMS (3.03 ± 1.85; $p < 0.05$). With the trypan-blue exclusion test, no cytotoxic effects could be measured for nicotine and MMS.

3.2. Cytokinesis-block micronucleus test

Exposure to nicotine for 24 h was followed by the CBMN test. Medium served as negative control and 100 μM MMS as positive control. The baseline frequency of MN without exposure to nicotine or MMS was 11.5 (mean ± 8.55) or 1.15% of 1000 bi-nuclear cells counted. A concentration-dependent increase of the MN frequency was detected in the Friedman-test ($p = 0.03$). Compared with the negative control, no increase could be detected at nicotine concentrations of 0.001 mM (13.92 ± 8.11; $p = 0.213$) and 0.01 mM (13.46 ± 7.49; $p = 0.125$), whereas a significant increase was measured at 0.1 mM (14.83 ± 8.8; $p = 0.028$) and 1.0 mM (16.59 ± 7.44; $p = 0.016$; Wilcoxon signed-rank test). MMS as the positive control was capable of inducing a strong increase in MN (23.15 ± 11.65; $p < 0.05$) (data presented in Fig. 2A).

In the same setting, the number of apoptotic cells per 1000 cells was counted. The percentage of apoptotic cells for the negative control was 1.27 ± 1.01 (mean ± SD). No significant concentration-dependent increase ($p = 0.23$; Friedman-test) or an increase compared with the negative control was measured for any of the nicotine concentrations (0.001 mM: $1.33\% \pm 0.72$, $p = 0.374$; 0.01 mM: $1.25\% \pm 0.7$, $p = 0.95$; 0.1 mM: $1.83\% \pm 1.17$, $p = 0.2$; 1.0 mM: $1.8\% \pm 1.27$, $p = 0.262$), whereas MMS was able to induce apoptosis ($3.65\% \pm 3.21$; $p = 0.008$) (Fig. 2B).

3.3. Chromosome aberrations

To evaluate genotoxic effects of nicotine at the chromosomal level, the CA test was performed. The structural aberrations mainly consisted of chromatid breaks. For statistical calculations all structural aberrations of chromatid- and chromosome-type (gaps, breaks, and reunions) were taken into account. A concentration-dependent increase of CA was detected in the Friedman-test ($p < 0.001$). Compared with the negative control, an increase in CA

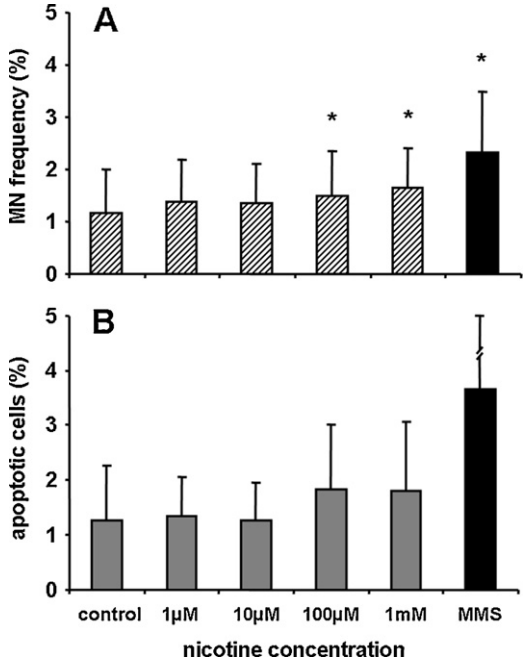

Fig. 2. Cytokinesis-block micronucleus test (CBMN): exposure to nicotine for 24 h was followed by the CBMN test. Medium served as a negative control, methylmethane sulfonate (MMS) as a positive control. (A) Presented are the percentages of 1000 binuclear cells containing MN and (B) the percentage of apoptotic cells among all 1000 counted cells. A significant increase of MN frequency compared with the negative control was measured at concentrations of 0.1 and 1.0 mM (*$p < 0.05$, Wilcoxon signed-rank test).

was assessed starting at 1 μM of nicotine and an increase for higher nicotine concentrations could be determined as well ($p = 0.005$ for 10, 100 and 1000 μM; Wilcoxon signed-rank test), see Fig. 3. The positive control MMS was able to induce a distinct increase of CA (data not shown). Detailed values are given in Table 1.

3.4. Sister chromatid exchange

SCE (Sister chromatid exchange) were counted manually after exposure to nicotine. Baseline SCE in untreated cells was 56.56

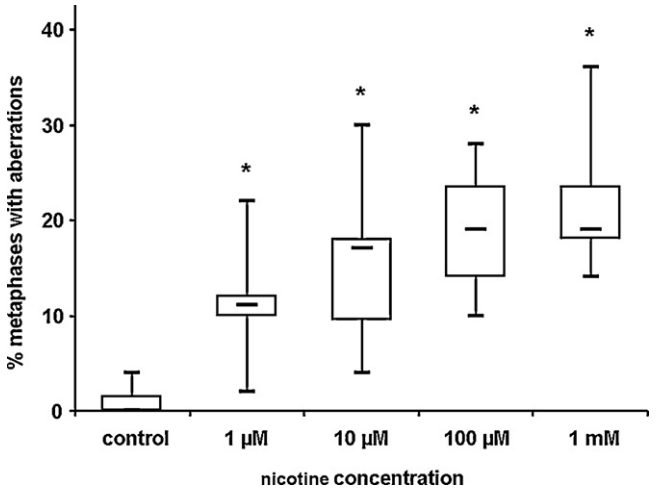

Fig. 3. Chromosome aberration test: structural aberrations mainly consisted of chromatid breaks. A significant increase in chromosome aberrations compared with the negative control was determined at concentrations of 1.0 μM up to 1.0 mM nicotine (*$p < 0.01$, Wilcoxon signed-rank test). Given is the percentage of metaphase chromosomes with aberrations. Box plots represent median, 1st quartile, 3rd quartile, minimal and maximal values of chromosome aberrations.

Table 1
Frequency and type of chromosome aberration in human lymphocyte cultures exposed to nicotine and MMS. Given are the mean values ± the standard deviation, $n = 10$.

Concentration (μM)	Chromatid type		Chromosome type		
	Breaks	Reunions	Breaks	Reunions	Gaps
Nicotine					
0 (control)	0.2 (±0.4)	0 (±0)	0.1 (±0.3)	0 (±0)	0.1 (±0.3)
1	3.5 (±2.1)	0 (±0)	1.6 (±1.4)	0 (±0)	0.5 (±1.0)
10	3.8 (±2.8)	0 (±0)	3.4 (±2.5)	0.1 (±0.3)	0.4 (±1.0)
100	5.0 (±2.1)	0 (±0)	3.7 (±3.2)	0.1 (±0.3)	0.5 (±0.7)
1000	5.7 (±1.8)	0.1 (±0.3)	4.3 (±3.7)	0 (±0)	0.4 (±0.7)
MMS					
100	26.9 (±29.3)	0.6 (±1.1)	8.0 (±5.2)	0.7 (±0.9)	2.0 (±3.4)

(mean) ± 41.26 (SD). A concentration-dependent increase of CA was detected in the Friedman-test ($p < 0.001$). A significant increase in SCE compared with the negative control was measured at a nicotine concentration of 1 μM (89.8 ± 54.78, $p = 0.007$, Wilcoxon signed-rank test), and a further rise in the nicotine concentration led to even more significant increases in SCE. At 10 μM 108 ± 60.04 ($p = 0.005$), at 100 μM 119.3 ± 73.88 ($p = 0.008$) and at 1 mM 190.75 ± 91.13 ($p = 0.005$) SCE were determined. MMS as the positive control was also able to induce a strong increase in SCE (271.2 ± 227.49). Data are presented in Fig. 4.

3.5. Annexin V-propidium iodide staining apoptosis assay

Viable, apoptotic and necrotic cells were determined with the annexin V-propidium iodide staining apoptosis assay after exposure to nicotine or MMS for 24 h prior to cytometric analysis. Baseline parameters were determined after incubation with medium alone (negative control). Percentage of viable cells was 82.6%, apoptotic cells 12.3%, and necrotic cells 4.7%. No significant change was measured after incubation to nicotine at 1 μM (83.7/12.2/3.9%), 10 μM (84.2/10.8/3.9%), 100 μM (84.0/11.4/4.2%), and 1 mM (82.8/13.0/3.9%). MMS was able to reduce the percentage of viable cells to 73.2% ($p = 0.018$) and to increase the ratio of necrotic cells to 12.2% ($p = 0.028$). No significant increase was determined in the part of apoptotic cells (13.4%; $p = 0.18$). Data are shown in Fig. 5.

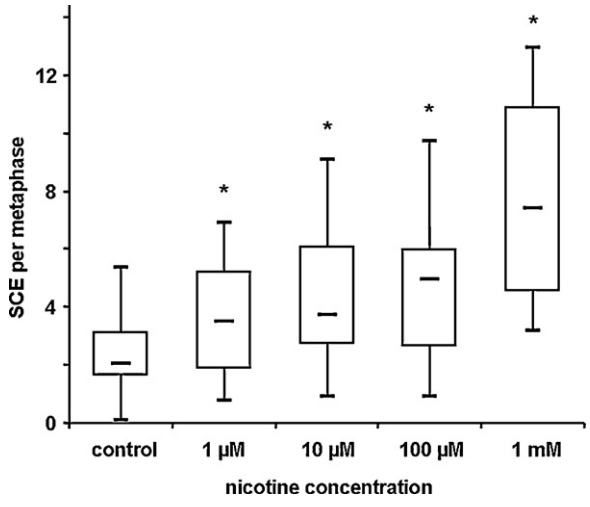

Fig. 4. Sister chromatid exchange (SCE) test: determination of SCE was performed after 24 h exposure to nicotine at concentrations between 1 μM and 1 mM. Given are SCEs per metaphase chromosome. All test concentrations demonstrated a significant increase in SCE compared with the baseline level of SCE (*$p < 0.01$, Wilcoxon signed-rank test). Box plots represent median, 1st quartile, 3rd quartile, minimal and maximal values of SCE.

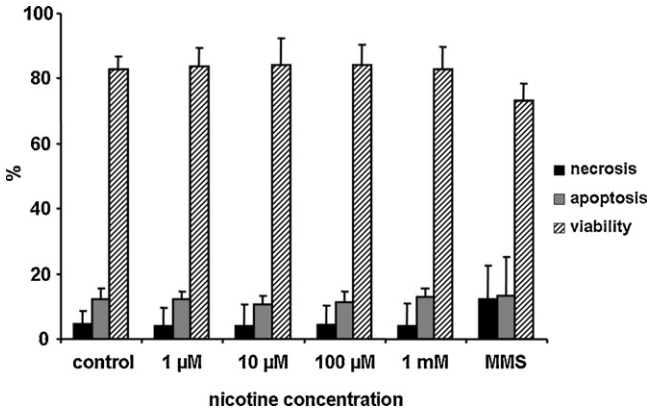

Fig. 5. Annexin V apoptosis assay: viable, apoptotic and necrotic cells were measured by flow cytometry after staining with annexin V and propidium iodide. Medium served as the negative control. At all nicotine concentrations, the percentage of viable cells was >80%. No significant change compared with medium alone was measured with respect to apoptotic and necrotic cells after nicotine exposure. MMS showed a slight decrease of viable cells (73.16%, $p < 0.05$) but no significant increase of apoptotic cells.

4. Discussion

The potential of the tobacco-alkaloid nicotine to induce genotoxic damage was investigated in different cell systems and with varyious genotoxicological tests. Initial results reported the induction of chromosome aberrations (CA) and sister chromatid exchange (SCE) in CHO cells after different exposure times and concentrations of nicotine [23]. Contradictory results showed no increase in SCE, or in the frequency of mutations in the Ames test after exposure to nicotine and its major metabolites [24]. In 2004, direct genotoxic effects induced by nicotine could be demonstrated via the micronucleus test in human gingival fibroblasts [25] and via the comet assay in human spermatozoa [11]. In experiments with the comet assay in human lymphocytes, tonsillar tissue, nasal mucosa, acinar cells of salivary glands, and embryonic cells of *Caenorhabditis elegans*, nicotine induced distinct DNA damage after 1 h of exposure [8–10,26,27]. In a mini-organ culture system of parotid-gland tissue, significant DNA damage was observed in the comet assay after 1 and 3 h of exposure [28], and in an animal model significant genotoxic effects were measured in the comet assay after exposure to nicotine between 2 and 24 h [29].

In the present study, human lymphocytes were exposed to increasing concentrations of nicotine for 24 h. A series of tests was performed to evaluate the potential of nicotine to induce DNA damage with a variety of genotoxicological endpoints.

The data collected from the comet assay did not show an increase in DNA migration compared with the negative control, indicating no significant DNA damage after 24 h at the DNA-strand level. Because genotoxic effects have been described after shorter periods of exposure, potentially induced DNA breaks may

be repaired before detection. The reversibility of effects detected in the comet assay was described previously [30,31].

The induction of micronuclei (MN) represents irreversible DNA damage caused by chromosome loss and/or breakage during mitosis. Chromosomes or chromosome fragments are able to form micronuclei when normal mitosis is interrupted. In 2004, nicotine was shown to increase the MN frequency in human gingival fibroblasts after an exposure time of at least 24 h and at a concentration of 1 and 10 μM [25]. In our study, MN frequencies were determined in stimulated human lymphocytes after exposure to different concentrations of nicotine. However, we only found an increase in MN frequency at a higher nicotine concentration (100 μM) compared with those used in the SCE and CA tests.

Another well-known test system looking into markers of cancer risk is the chromosome aberration (CA) test. High levels of CA in lymphocytes of peripheral blood were shown to correlate with an increased risk for cancer [32]. DNA double-strand breaks (DSB) with missing repair are considered to be the most important lesion leading to observed CA followed by biological effects [33]. In our study we could demonstrate a significant increase in chromatid-type and chromosome-type CA after 24 h exposure to nicotine at concentrations between 1 μM and 1 mM. There is evidence that both types of CA predict cancer risk. Chromosome-type CA are induced by S-phase-independent clastogens, whereas chromatid-type CA are dependent on the S-phase during mitosis. Depending on the subjects and clastogens studied, chromatid- or chromosome-type CA seemed to have a stronger association with cancer risk, although sometimes chromosome-type CA might have a stronger association [15].

The results of the SCE test in the current study showed a clear increase in the SCE frequency starting at the lowest concentration of nicotine tested (1 μM). This is in line with the results of Trivedi in 1990, who investigated the effects of nicotine in CHO cells [23]. This test is a well-established method for investigating the genotoxic potential of different substances at the chromatid level. Although the frequency of SCE does not seem to be associated with the risk for cancer, the test has a high sensitivity and is used as a screening test [15,34]. Compared with the comet assay, both tests have a high sensitivity, but the SCE test is not influenced by necrotic or apoptotic cells in the same cell culture.

The potential of nicotine to influence the apoptotic pathway seems to be dependent on the cell system investigated. Different authors have reported pro- and anti-apoptotic effects *in vitro* and *in vivo*, as reviewed in 2007 [35]. One major problem in most studies was a short exposure period and high nicotine concentrations compared with the physiological situation of repetitive exposures over years at distinctly lower concentrations. In a previous investigation, the influence of nicotine on apoptosis could not be demonstrated at a concentration of 1 mM for 1 h by means of the TUNEL assay [8]. In the current study, distinctly lower concentrations of nicotine were used with an exposure time of 24 h. With the annexin V-propidium iodide staining apoptosis assay, no increase or decrease in apoptotic cells was determined. Similar results were shown in the CBMN test. Other groups reported similar results regarding the influence on apoptosis in a caspase assay after repetitive treatment of lung-carcinoma cells with nicotine at concentrations up to 1 mM [36]. Nevertheless, it is important to consider the possibility of nicotine-induced inhibition of apoptotic pathways. Especially in cell systems in which genotoxic effects of nicotine at the same concentrations are shown, anti-apoptotic effects play a crucial role in the function of nicotine as a part of tumour promotion [6,37].

Comparing the present results from the different genotoxicological tests in the current study, nicotine demonstrated the potential to induce irreversible DNA damage in human lymphocytes. This investigation underscores the necessity of using different genotoxicological tests in parallel, as generally recommended [38]. For example, in the current study the comet assay and the MN test, both standardized tests to evaluate genotoxicity of different xenobiotics, were not able to detect relevant DNA damage at physiological nicotine concentrations, while the CA test and the SCE test clearly demonstrated significant genotoxic damage. One explanation for this difference is the chosen duration of exposure. Long-term incubation is needed to represent physiological test conditions compared with nicotine plasma levels during smoking, chewing tobacco, or in nicotine replacement therapy (NRT). *In vivo*, the plasma half-life of nicotine after intravenous infusion or cigarette smoking is about 2 h [39]. *In vitro* studies on the metabolism of nicotine are mostly done in cell systems containing cytochrome P450 enzymes [40,41] and exposure times of 1 h. Although monitoring of the nicotine concentration during the 24 h exposure period was not performed in this study, it is probable that the nicotine concentration in the cell culture decreases over time, e.g., due to metabolic or chemical processes. In test systems measuring reversible genotoxic effects, such as the comet assay, cells are able to repair the induced damage, whereas irreversible damage is accumulating [33]. Although the detailed mechanisms leading to DNA damage in lymphocytes were not investigated in the present study, the observed effects are probably mediated by binding to the nicotinic acetylcholine receptor (nAChR), which is physiologically expressed on the surface of lymphocytes [42]. Via this receptor, nicotine is able to activate different intracellular pathways, among others with the induction of reactive oxygen species [5,43].

Considering the pro-angiogenetic and pro-mitogenic effect of nicotine, a relationship with tumour progression due to nicotine should be anticipated. Taking into account that the nicotine plasma level in smokers is about 1 μM [3], the current data with the induction of CA at physiological nicotine concentrations underscores the necessity for further investigations of the potential of nicotine to initiate mutagenesis and tumour promotion after long-term exposure. A problem in investigating freshly isolated cells, e.g., of the parotid gland or the nasal mucosa, is the lack of cell proliferation. This excludes these cell systems from further genotoxicological tests like the CBMN test, CA, or SCE test since all tests require cells that are able to perform mitosis. Future studies will focus on genotoxicological test batteries in different human cell systems after the establishment of primary cell lines.

Conflict of interest statement

The authors declare that there are no conflicts of interests.

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Nicotine induces DNA damage in human salivary glands

Christian Ginzkey[a], Katja Kampfinger[a], Gudrun Friehs[a], Christian Köhler[a], Rudolf Hagen[a], Elmar Richter[b], Norbert H. Kleinsasser[a,*]

[a] Department of Oto-Rhino-Laryngology, Plastic, Aesthetic and Reconstructive Head and Neck Surgery, Julius-Maximilian-University Würzburg, Josef-Schneider-Str. 11, D-97080 Würzburg, Germany
[b] Walther Straub Institute, Department of Toxicology, Ludwig-Maximilians University Munich, Nussbaumstrasse 26, D-80336 Munich, Germany

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ABSTRACT

The tobacco alkaloid nicotine is responsible for addiction to tobacco and supposed to contribute to tobacco carcinogensis, too. Recently, genotoxic effects of nicotine have been reported in human cells from blood and upper aerodigestive tract. Because of nicotine accumulation in saliva, the study of possible *in vitro* genotoxic effects of nicotine have been extended to human salivary gland cells. Specimens of parotid glands of 10 tumor patients were obtained from tumor-free tissue. Single cells were prepared by enzymatic digestion immediately after surgery and exposed for 1 h to 0.125–4.0 mM of nicotine. Possible genotoxic effects were determined by the Comet assay using the % DNA in tail (DT) as a reliable indicator of DNA damage. Nicotine induced a significant dose-dependent increase of DNA migration in parotid gland single-cells. The mean DT was 1.12-fold (0.125 mM) to 2.24-fold (4.0 mM) higher compared to control. The lowest concentration eliciting significant DNA damage within 1 h, 0.25 mM nicotine, is only 10-fold higher than maximal concentrations of nicotine reported in saliva after unrestricted smoking. Although conclusive evidence for a carcinogenic potential of nicotine is still lacking, the safety of long-term nicotine replacement therapy should be carefully monitored.

1. Introduction

Recent findings suggest an active role of nicotine, the major tobacco alkaloid, in tobacco carcinogenesis. Mechanisms involved in tumor progression are inhibition of apoptosis and stimulation of cell proliferation and angiogenesis. Cooke and Bitterman (2004) and Heeschen et al. (2001) demonstrated a strong angiogenic potential via induction of endothelial cell growth and up-regulation of vascular endothelial growths factor (VEGF), whereas Dasgupta et al. (2006) and Waggoner and Wang (1994) described pro-mitogenic effects of nicotine. Effects on apoptosis are equivocal since both anti- as well as pro-apoptotic effects of nicotine have been reported (Zeidler et al., 2007). In normal and neoplastic cell lines inhibition of apoptosis by the tobacco alkaloid has been shown consistently (Heeschen et al., 2001; Heusch and Maneckjee, 1998; West et al., 2003; Arredondo et al., 2006).

Beside the tumor promoting potential there is accumulating evidence that nicotine causes DNA damage in different human epithelial and non-epithelial cells (Argentin and Cicchetti, 2004; Arabi, 2004; Kleinsasser et al., 2005; Sassen et al., 2005). Genotoxic effects in cells are supposed to be a crucial step in causing DNA mutations if repair mechanisms are insufficient or apoptotic pathways are blocked.

During unrestricted smoking blood or plasma nicotine concentrations in smokers generally range from 10 to 50 ng/ml (Hukkanen et al., 2005). Similar levels can be reached by snuffing or in nicotine replacement therapy (NRT). However, more than 100-fold higher concentrations of nicotine may be present in saliva and mouth washings after smoking, snuff dipping and nicotine replacement therapy (Hoffmann and Adams, 1981; Lindell et al., 1993, 1996; Teneggi et al., 2002). In addition to exposure from saliva and blood, small salivary glands found in the submucosa of the lips and palatine could be exposed locally by pH-dependent passive diffusion via the mucosa in the oral cavity (Adrian et al., 2006). Therefore, the genotoxic effect of nicotine in salivary gland cells was investigated in the present study in single-cells isolated from tumor-free tissue by evaluation of DNA damage with the Comet assay.

2. Materials and methods

2.1. Patient groups

Ten patients intended for partial or total parotidectomy were informed and asked to sign a consent form and a questionnaire concerning job, smoking and alcohol consumption prior to surgery (Table 1). Parotid specimens were removed

* Corresponding author. Tel.: +49 931 201 21323; fax: +49 931 201 21321.
E-mail addresses: kleinsasser_n@klinik.uni-wuerzburg.de, norbert.kleinsasser@web.de (N.H. Kleinsasser).

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Table 1
Patient characteristics.

Patient	Gender	Age (year)	Profession	Smoking (py)	Alcohol (g/day)[a]
1	F	71	Tailor	–	–
2	M	41	Tank clerk	25	120
3	M	63	Mechanic	35	100
4	M	65	Motorist	–	–
5	M	44	Driver	50	–
6	F	49	Tailor	–	–
7	M	71	Welder	6	30
8	M	69	Tax advisor	3	–
9	M	83	Pensioner	–	–
10	M	55	Mechanic	30	–

M: male; F: female.

[a] Alcohol consumption is given in grams per day (g/day) and smoking habit 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.).

distantly from tumors of the resected gland and transferred to the laboratory immediately for further preparation. The study was approved by the Ethic Commission of the Medical Faculty, Julius-Maximilian University Würzburg, according to the note from February 2006, no. 16/06.

2.2. Single-cell separation

Healthy pieces from parotid glands were separated from coagulation artefacts and minced with a scalpel in a common Petri dish. For enzymatic digestion protease type XIV from *Streptomyces griseus* (Sigma–Aldrich, Taufkirchen, Germany, 6.25 mg/ml), hyaluronidase from bovine testis (Sigma–Aldrich, 1.25 mg/dl) and collagenase P (Roche, Mannheim, Germany, 1.25 mg/dl) were dissolved in Airway Epithelial Cell Growth Medium (AEGM, PromoCell, Heidelberg, Germany) and samples were incubated for 1 h in a shaking water bath at 37 °C (Harréus et al., 1999). After stopping the enzymatic reaction with fetal calf serum (FCS, Linaris, Wertheim-Bettingen, Germany) and filtration through sterile gaze the cell suspension was washed once and resuspended in AEGM with SupplementMix (PromoCell) and 1% penicillin/streptomycin (Biochrom, Berlin, Germany). To reduce cell clotting the cells were incubated under standard culture conditions (37 °C, 5% CO_2) for additional 20 h before exposure. For determination of cell viability the Trypan blue exclusion test was performed directly prior to nicotine exposure.

2.3. Exposure to nicotine

Cells were adjusted to a concentration of 80,000 cells/ml in AEGM with SupplementMix. Nicotine with a purity of more than 99% (Sigma–Aldrich) was freshly dissolved in AEGM for 1 h incubation of single cells with 0.125–4.0 mM of nicotine under cell culture conditions (37 °C, 5% CO_2). Medium alone served as negative control. The direct alkylating methyl methane sulfonate (MMS, purity >99%; Sigma–Aldrich) was used at 100 μM as a reliable positive control not causing cytotoxic effects (data not shown). After exposure cells were washed in phosphate buffered saline (Roche) at 4 °C once and preparations were done on ice to avoid enzymatic repair of DNA damage. Viability was determined using the Trypan blue exclusion test prior to the single-cell microgel electrophoresis. Only cell samples with a viability of more than 80% were used to reduce cytotoxic artefacts as postulated by the expert panel (Tice et al., 2000).

2.4. Alkaline single-cell microgel electrophoresis (Comet assay)

The alkali version of the single-cell microgel electrophoresis to determine single strand breaks (SSB), alkali labile sites and excision repair sites was used as described by Tice et al. (2000). In brief, slides (Langenbrinck, Emmendingen, Germany) were coated with 1.5% normal melting agarose (Biozym, Hameln, Germany) and dried at room temperature. Cell samples with a viability of more than 80% were resuspended in 0.5% low melting agarose (Biozym) and applied to the prepared slides. After a lysis period for 1.5 h in an alkaline lysis buffer (10% DMSO, 1% Triton-X, 2.5 M NaCl, 10 mM Tris, 100 mM Na_2EDTA, pH 10) slides were placed in a horizontal gel electrophoresis chamber (Renner, Dannstadt, Germany) and covered with alkaline buffer (5 mM NaOH and 200 mM Na_2EDTA) at pH >13. After a 20 min DNA "unwinding" period, the electrophoresis was performed under standard conditions (25 V, 300 mA) for 20 min. Followed by neutralization at pH 7.5 (Trisma base, Merck, Darmstadt, Germany) cells were stained with ethidium bromide (20 μg/ml; Sigma–Aldrich) and stored at 4 °C until analysis. All preparation steps were performed at red or yellow light to avoid DNA damage by UV light.

2.5. Analysis and statistics

For analysis of slides a DMLB fluorescence microscope (Leica Microsystems, Wetzlar, Germany) with an adapted CCD camera (model KP-M1AP, Hitachi Ltd., Tokyo, Japan) was used. The comet was determined by an image analysis system (Komet 4.0, 1998, Kinetic Imaging, Liverpool, UK). Two slides 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, product of the median migration distance and the percentage of DNA in tail; Olive et al., 1993). After confirming normal distribution with the Kolmogorov–Smirnov test, further statistical analysis was performed for the DT values with the repeated measures ANOVA for paired data with post-test for linear trend and Dunnett's multiple comparison test to detect concentration-dependent differences in DNA damage by nicotine (Prism 4.01, GraphPad Software Inc., San Diego).

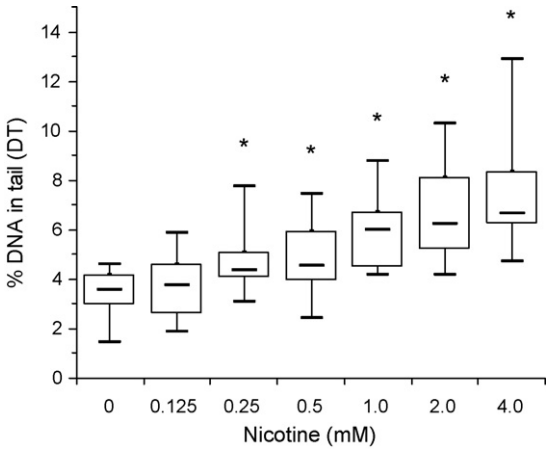

Fig. 1. Concentration-dependent DNA damage in single cells of tumor-free human parotid glands from 10 patients after 1 h incubation with nicotine as determined by the Comet assay. Lines in the boxes represent the median values of the % DNA in tail (DT). Box plots show the lowest and highest values of DT, as well as the first and third quartile. The increase of DNA migration with rising concentrations of nicotine was significant according to Repeated Measures ANOVA with post-test for linearity ($p < 0.0001$). Significant difference compared to control in Dunnett's multiple comparison test: $^{*}p < 0.01$.

3. Results

Single-cell suspensions of the tumor-free parts of human parotid glands from 10 patients were exposed to nicotine for 1 h *in vitro* to assess genotoxic effects by the Comet assay. Looking at the DT, nicotine demonstrated a highly significant dose-dependent increase of DNA damage ($p < 0.0001$; Fig. 1) without affecting the viability of the cells which was always more than 80% by the Trypan blue exclusion test. The differences in DT values started to become significant at a concentration of 0.25 mM nicotine (mean ± S.D., 4.7 ± 1.33) corresponding to a 1.4-fold increase compared to control values (3.37 ± 1.09; $p < 0.01$). The highest DT value obtained after incubation with 4.0 mM nicotine (7.56 ± 2.64; $p < 0.01$) was still considerably less than the DNA damage by the positive control MMS (14.26 ± 4.28; data not shown). All individual values of the DT, together with TL and OTM are summarized in Table 2. Whereas DT was slightly more sensitive than OTM giving a significant 1.4-fold increase by nicotine at 0.250 mM (4.70 ± 1.33) versus control (3.37 ± 1.09; $p < 0.01$), TL was somewhat less sensitive requiring 1.0 mM of nicotine (117 ± 22) to reach a significant 1.11-fold increase versus control (105 ± 19; $p < 0.05$). Neither age nor smoking and drinking status had any effect on basal DT, TL or OTM values and the effect of nicotine.

4. Discussion

The present study using parotid gland single cells confirms the genotoxic effect of nicotine in freshly isolated tonsillar cells and in lymphocytes as determined by the Comet assay (Kleinsasser et al., 2005). The lowest concentration eliciting a significant effect,

Table 2
DNA damage by nicotine in single cells isolated from tumor-free parotid glands.

Patient	Control			Nicotine (mM)																
	0			0.125			0.25			0.5			1.0			2.0			4.0		
	OTM	DT	TL	OTM	DT	TL	OTM	DT	TL	OTM	DT	TL	OTM	DT	TL	OTM	DT	TL	OTM	DT	TL
1	2.44	3.4	97.9	3.66	4	113	3.08	4.04	108	5.83	6.15	122	6.02	6.33	143	8.29	8.19	142	7.31	8.45	133
2	0.82	1.44	71.4	1.5	1.87	90.5	2.68	3.48	93.4	2.36	3.1	95.2	3.53	4.35	99.1	4.28	5.19	102	4.75	6.31	113
3	1.98	3.16	105	2.18	3.55	99.3	2.6	4.12	104	2.28	3.84	105	2.47	4.18	102	3.96	5.81	104	4.18	6.22	115
4	3.18	4.62	130	4.1	5.57	124	3.56	5.12	102	4.6	6.47	131	6.4	8.8	145	7.15	10.3	138	7.48	11	123
5	3.6	4.57	130	4.8	5.87	138	7.2	7.73	151	6.17	7.46	153	6.75	7.81	151	9.46	9.78	156	12.7	12.9	170
6	3.04	4.31	117	3.21	4.65	122	3.3	4.86	110	3.01	4.56	108	4.13	6.18	113	4.66	6.71	123	5.88	7.96	119
7	2.04	2.92	91.9	1.38	2.35	85.2	2.45	4.33	83.4	2.63	4.56	88.5	2.51	4.46	89	2.89	4.46	91.1	4.02	6.76	107
8	1.1	1.8	82.6	1.33	2.24	80.6	2.1	3.07	82.6	1.62	2.44	81.6	3.31	4.51	103	2.77	4.16	97	2.87	4.7	91.9
9	2.79	3.76	111	3.22	4.41	110	4.1	5.86	108	3.92	5.35	100	5.16	6.87	110	5.73	7.92	112	4.67	6.58	108
10	2.68	3.76	114	2.25	3.35	109	2.98	4.34	108	2.84	4.19	104	3.98	5.74	110	3.54	5.18	108	3.06	4.69	102
Mean	2.37	3.37	105	2.76	3.79	107	3.41	4.7	105	3.53	4.81	109	4.43	5.92	117	5.27	6.77	117	5.69	7.56	118
S.D.	0.89	1.09	19.3	1.22	1.38	18.2	1.45	1.33	19.0	1.55	1.56	21.3	1.57	1.59	21.6	2.32	2.18	21.5	2.92	2.65	21.5

OTM, Olive tail moment; DT, % of DNA in tail; TL, tail length.

0.25 mM of nicotine using DT and 0.5 mM using OTM, was identical and the maximal DT and OTM values achieved after 1 h incubation with 4.0 mM nicotine (DT 7.56 ± 2.64; OTM 5.69 ± 2.92) were similar to the results obtained with human tonsillar cells (DT 5.98 ± 2.86; OTM 4.78 ± 2.81). Genotoxic *in vitro* effects of nicotine assessed by the Comet assay have also been reported in miniorgan cultures of human nasal mucosa (Sassen et al., 2005), human spermatozoa (Arabi, 2004), human oral cancer cells (Wu et al., 2005), rat lymphocytes (Sudheer et al., 2007a,b) and hamster cheek pouch cells (Barley et al., 2004).

Maximal nicotine concentrations found in saliva of heavy smokers, 4000 ng/ml (Teneggi et al., 2002) are only 10-fold lower than the lowest concentration giving a significant increase in DNA damage after 1 h *in vitro* incubation of isolated salivary gland cells. Therefore, salivary glands may be exposed to high concentrations of genotoxic nicotine for extended time. Additionally, oral mucosal cells including small salivary glands found in the submucosa of the lips and palatine could be exposed locally by pH-dependent passive diffusion via the mucosa in the oral cavity (Adrian et al., 2006). Alkaline saliva occurring in betel quid chewers will increase nicotine exposure from oral tobacco or smoking and this may contribute to the high incidence of oral cancer in south Asia (Warnakulasuriya and Ralhan, 2007) which contrasts to the low risk in recent epidemiological studies from the US and Sweden showing no correlation with the use of oral smokeless tobacco (Henley et al., 2005; Rosenquist et al., 2005).

In a large Finnish epidemiological study a considerable increase of age-adjusted incidence of cancer of the salivary glands (ICD10 C07-08) was noted at the end of the last century (Tarvainen et al., 2004). Nevertheless, the implication of the genotoxic effects of nicotine in salivary gland tissue is still controversial. Major and minor salivary glands are sites in which both benign and malignant tumors may develop. Salivary gland cancer is a rare disease with a largely unknown origin. According to Muscat and Wynder (1998) neither cigarette smoking nor use of chewing tobacco and snuff increase the risk of salivary gland cancer. In contrast, Horn-Ross et al. (1997) reported an increased risk for smoking men. Smoking has also been implicated as a risk factor in the etiology of Warthin's tumor, a benign parotid gland tumor (Pinkston and Cole, 1996; Klussmann et al., 2006).

According to Tice et al. (2000) and Faust et al. (2004) effects measured in the Comet assay are due to single-strand breaks, alkali labile and excision repair sites. Insufficient repair mechanisms for example could potentially lead to manifest DNA damage followed by DNA mutations. Argentin and Cicchetti (2004) demonstrated the induction of manifest DNA damages by nicotine using the micronucleus test in a human fibroblast cell line. Similar genotoxic potential were shown via induction of sister chromatid exchanges (SCE) and chromosome aberrations in Chinese hamster ovarial (CHO) cells (Trivedi et al., 1990). Although other authors like Doolittle et al. (1995) did not observe genotoxic effects of the tobacco alkaloid and its metabolites, e.g., cotinine, in the Ames test and the SCE in CHO cells, the data above indicate a DNA damaging potential of nicotine. Although there are no convincing data showing a carcinogenic effect of nicotine in humans, co-carcinogenic effects and/or promotion of tumors by nicotine in smokers cannot be excluded. Besides direct genotoxicity, a plethora of nicotine effects on apoptosis, oxidative damage, ras, Akt, NF-κB and JAK-2/STAT-3 and others have been reported (Wright et al., 1993; Rakowicz-Szulczynska et al., 1994; Tipton and Dabbous, 1995; West et al., 2003; Crowley-Weber et al., 2003; Ye et al., 2004a; Xin and Deng, 2005; Tsurutani et al., 2005; Guo et al., 2005; Arredondo et al., 2006). In experimental animals nicotine promotes lung tumor growth and angiogenesis not only *in vitro* but also *in vivo* (Chen et al., 1994; Schuller et al., 1995; Heeschen et al., 2001, 2002; Ye et al., 2004b).

Taken together, genotoxic effects could induce manifest DNA damage. In combination with suppression of apoptosis this damage could persist and might represent a first step in tumor initiation. In smoking cessation, nicotine replacement therapy leads to a high and constant exposure of salivary glands to nicotine for weeks and months. Although there is no conclusive evidence for a carcinogenic potential of nicotine, because of the effects described above, the safety of long-term nicotine replacement ought to be considered with some caution.

Conflict of interest

The authors declare to have no conflict of interests.

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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

Christian Ginzkey *, Gudrun Steussloff, Christian Koehler, Marc Burghartz, Agmal Scherzed, Stephan Hackenberg, Rudolf Hagen, Norbert H. Kleinsasser

Department of Oto-Rhino-Laryngology, Plastic, Aesthetic and Reconstructive Head and Neck Surgery, University Hospital of Wuerzburg, Josef-Schneider-Str. 11, D-97080 Wuerzburg, Germany

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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 $\geqslant 1$ 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.

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

Abbreviations: OTM, Olive tail moment; CBMN, cytokinesis-block micronucleus test; MN, micronucleus; CA, chromosome aberration; MMS, methyl-methanesulfonate.

* Corresponding author. Tel.: +49 931 201 21323; fax: +49 931 201 21321.
E-mail addresses: ginzkey_c@ukw.de (C. Ginzkey), friehs_g@ukw.de (G. Steussloff), koehler_c@ukw.de (C. Koehler), burghartz_m@ukw.de (M. Burghartz), scherzed_a@ukw.de (A. Scherzed), hackenberg_s@ukw.de (S. Hackenberg), hagen_r@ukw.de (R. Hagen), kleinsasser_n@ukw.de (N.H. Kleinsasser).

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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 (Doolittle 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. Maximun 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 palatine 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 Ralhan, 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 mm^3 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% CO_2 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% confluence.

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% CO_2 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 α-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% CO_2 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 resuspended in BEG medium. The alkali version of the Comet assay was used as described previously (Ginzkey et al., 2009). Slides were analyzed with a DMLB fluorescence microscope (Leica Microsystems, Wetzlar, Germany) with an adapted CCD camera (model KP-M1AP, Hitachi Ltd., Tokyo, Japan) and comets were determined 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 $Na_2HPO_4 \times 2H_2O$, 15 mmol/l $KH_2PO_4 \times H_2O$, 6.25 mg/ml acridine orange; Serva, Heidelberg, Germany) for 5 min, followed by washing twice in Soerensen buffer (15 mmol/l $Na_2HPO_4 \times 2H_2O$, 15 mmol/l $KH_2PO_4 \times H_2O$; 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 air dried, placed on a 90 °C heating plate for 30 min and stored at 60 °C overnight. Cells were stained with a 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 inversed 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% CO_2. 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 ± 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 ± 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 μM 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 ± 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 μM (mean OTM 0.43 ± 0.22) and 10 μM (0.38 ± 0.14), whereas a significant increase in DNA migration could be measured at nicotine concentrations of 100 μM (0.47 ± 0.18; $p = 0.01$) and 1000 μM (0.62 ± 0.33; $p < 0.01$). MMS as positive control induced strong DNA damage (mean OTM 8.39 ± 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

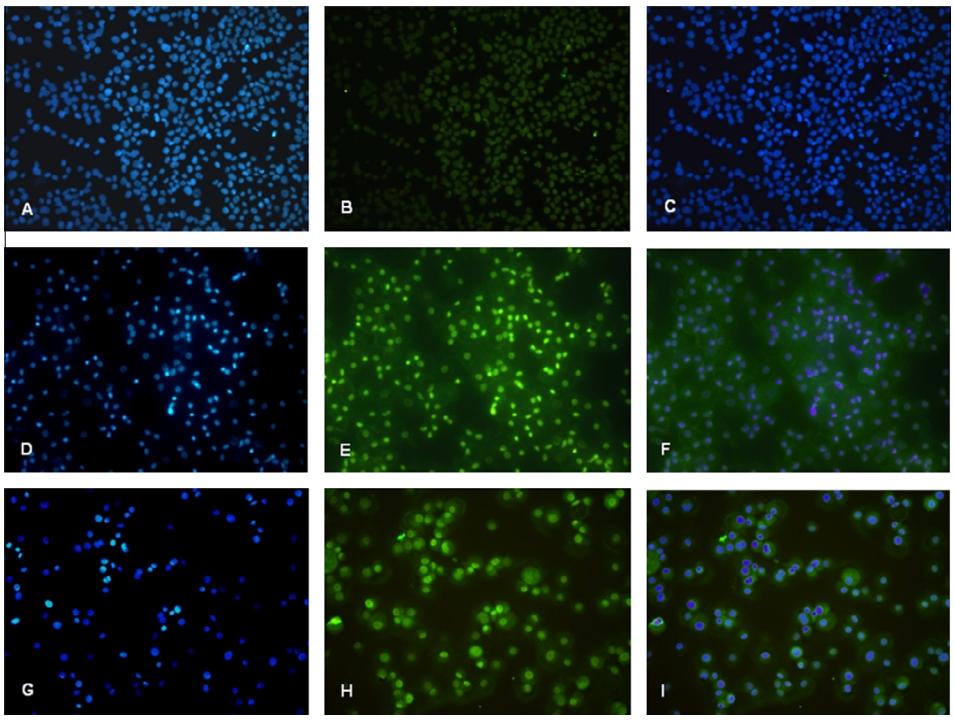

Fig. 1. Immunofluorescent staining of alpha-amylase. Human bronchial epithelial cell line BEAS-2b was used for negative control (A–C) and freshly isolated cells of human parotid gland for positive control (D–F). Staining of the cell nucleus was performed with DAPI (A, D, G). No expression of alpha-amylase was determined in BEAS 2b (B), whereas a strong expression pattern could be shown for freshly isolated (E) and for cultured parotid gland cells (H). C, F and I show an overlay of DAPI and alpha-amylase expression (magnification 200×).

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).

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

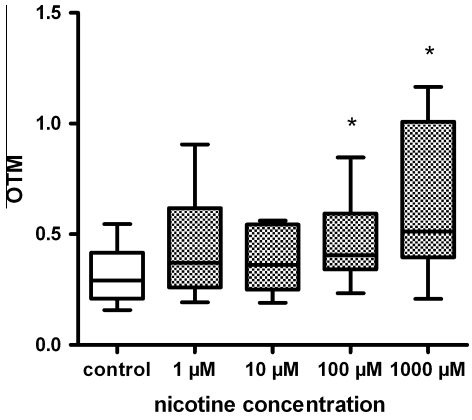

Fig. 2. Comet assay. Olive tail moment (OTM) values after exposure of human salivary gland epithelial cells to nicotine for 1 h. Box plots represent median, 1st quartile, 3rd quartile, minimal and maximal values of OTM, $n = 10$. Medium (white box) served as negative control. Significant DNA migration was determined at 100 µM and 1000 µM of nicotine (*, Wilcoxon signed-rank test), whereas no increase was measured at 1 µM and 10 µM.

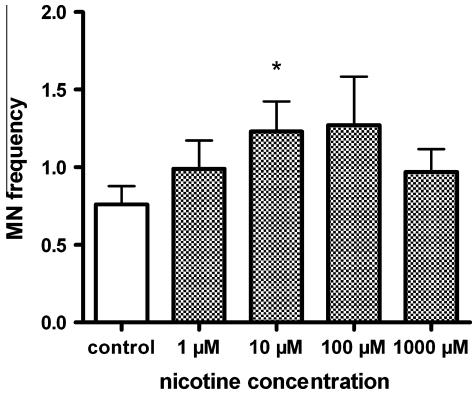

Fig. 3. Cytokinesis-block micronucleus test (CBMN). Exposure to nicotine in human salivary gland epithelial cells for 1 h was followed by the CBMN test ($n = 10$), medium (white box) served as negative control. MN frequency is shown as percentage of 1000 binuclear cells. A significant increase in MN frequency compared to the negative control was measured at a concentration of 10 µM (*, Wilcoxon signed-rank test). No further significant rise was determined at higher nicotine concentrations (100 and 1000 µM).

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 µM (0.99% ± 0.6; $p > 0.0125$), 100 µM (1.27% ± 1.0; $p > 0.0125$) and 1000 (0.97% ± 0.5; $p > 0.0125$), whereas a significant increase was measured at 10 µM (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 µM ($p < 0.0125$). Further significant increases for the concentrations 10 µM, 100 µM and 1000 µM 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-

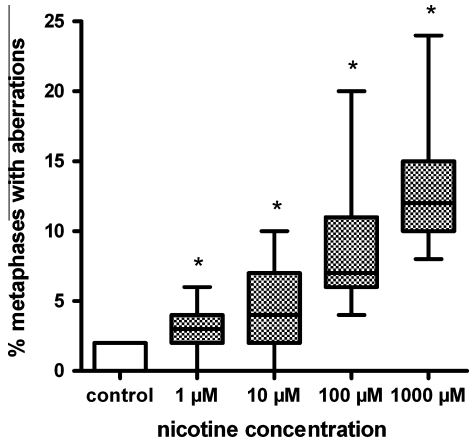

Fig. 4. Chromosome aberration test. Structural aberrations after exposure to nicotine in human salivary gland epithelial cells consisted of chromatid-type and chromosome-type aberrations. A significant increase in chromosome aberrations compared to negative control (white box) was determined at all tested concentrations, starting at 1 µM (*, Wilcoxon signed-rank test). The percentage of metaphase chromosomes with aberrations are given. Box plots represent median, 1st quartile, 3rd quartile, minimal and maximal values of chromosome aberrations, $n = 10$.

Table 2
Frequency and type of chromosome aberrations in human salivary gland epithelial cells exposed to nicotine at concentrations between 1 μM and 1000 μM for 1 h. The mean values ± the standard deviation from 50 metaphase cells are given from all 10 patients. No polyploidy or cells with pulverized chromosomes or >10 aberrations per cells were detected. Medium served as negative control, methyl-methane-sulfonate (MMS) at a concentration of 100 μM as positive control.

Nicotine concentration (μM)	Structural aberrations					% Aberrant cells for structural defects	% Aberrant cells for numerical defects
	Chromatid type		Chromosome type				
	Breaks/fragments	Exchanges	Breaks/fragments	Dicentric	Ring		
0 (control)	0.1 (± 0.3)	0 (± 0)	0.2 (± 0.4)	0 (± 0)	0 (± 0)	0.5 (± 0.9)	4.9 (± 2.3)
1	0.6 (± 0.5)	0.1 (± 0.3)	0.6 (± 0.9)	0 (± 0)	0 (± 0)	2.7 (± 1.8)	8.5 (± 4.3)
10	0.7 (± 0.8)	0 (± 0)	0.9 (± 1.0)	0.1 (± 0.3)	0.2 (± 0.4)	3.8 (± 3.2)	12.0 (± 6.0)
100	1.5 (± 1.1)	0.1 (± 0.3)	2.5 (± 1.9)	0 (± 0)	0 (± 0)	7.8 (± 5.1)	12.0 (± 4.6)
1000	2.5 (± 1.4)	0.2 (± 0.4)	2.5 (± 1.9)	0.7 (± 0.9)	0 (± 0)	11.8 (± 5.8)	10.9 (± 5.2)
MMS	4.5 (± 3.6)	0.4 (± 0.5)	4.2 (± 2.5)	0.5 (± 0.7)	0.2 (± 0.6)	19.3 (± 11.6)	13.6 (± 6.3)

cal aberrations per individual consisted of missing chromosomes not exceeding one per metaphase. One individual presented a trisomy 13 in a sample of nicotine treatment with 10 μM. A significant dose-dependent increase of numerical aberrations was detected, starting at 1 μM of nicotine ($p < 0.0125$). Percentage of cells with numerical and structural aberrations is presented in Table 2.

3.6. 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).

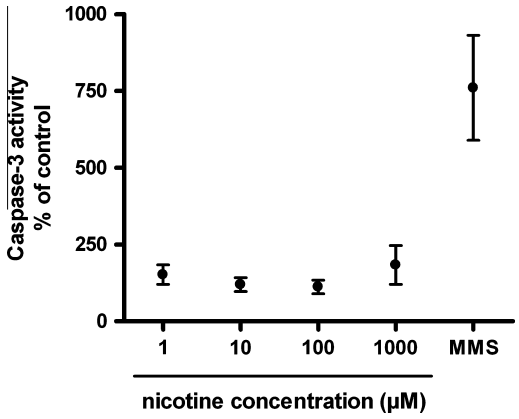

Fig. 5. Caspase-3 activity. The ELISA assay was used to determine the caspase-3 activity in human salivary gland epithelial cells after exposure to nicotine for 1 h, $n = 10$. Mean values with standard deviation in percentage of control are given. No significant increase or decrease in apoptosis could be determined in nicotine-treated cells, whereas the positiv control MMS showed a strong increase in caspase-3 activity.

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 difficulties 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 *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 μM 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 chromosome-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′-nitrosonornicotine (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; Stepanov 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 ⩾80 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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