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Nicotine causes genotoxic damage but is not metabolized during long-term exposure of human nasal miniorgan cultures

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HIGHLIGHTS

- Human nasal miniorgan cultures were exposed to nicotine for 1–3 weeks.
- Genotoxic effects could be shown after 1 week of exposure using the comet assay.
- Nicotine and cotinine levels were detected by GC/MS.
- No metabolism of nicotine could be detected after 1, 3 and 48 h.

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ABSTRACT

Human nasal miniorgan cultures (MOC) are a useful tool in ecogenotoxicology. Repetitive exposure to nicotine showed reversible DNA damage, and stable CYP2A6 expression was demonstrated in nasal MOC in previous investigations. The aim of the present study was to evaluate the genotoxic effect of nicotine in nasal MOC after chronic nicotine exposure, and to monitor possible metabolism capacities. MOC were dissected from human nasal mucosa and cultured under standard cell culture conditions. MOC were exposed to nicotine for 3 weeks at concentrations of 1 μM and 1 mM. The concentrations were chosen based on nicotine plasma levels in heavy smokers, and possible concentrations used in topical application of nicotine nasal spray. DNA damage was assessed by the comet assay at days 7, 14 and 21. Concentrations of nicotine and cotinine were analyzed in cell culture medium by gas chromatography/mass spectrometry to determine a possible metabolism of nicotine by MOC. Distinct DNA damage in MOC could be demonstrated after 1 week of exposure to 1 μM and 1 mM nicotine. This effect decreased after 2 and 3 weeks with no statistically relevant DNA migration. No nicotine metabolism could be detected by changes in nicotine and cotinine concentrations in the supernatants. This is the first time genotoxic effects have been evaluated in nasal MOC after chronic nicotine exposure for up to 3 weeks. Genotoxic effects were present after 1 week of culture with a decrease over time. Down-regulation of nicotinic acetylcholine receptors, which are expressed in nasal mucosa, may be a possible explanation. The lack of nicotine metabolism in this model could be explained by the functional loss of CYP2A6 during chronic nicotine exposure. Further investigations are necessary to provide a more detailed description of the underlying mechanisms involved in DNA damage by nicotine.

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1. Introduction

Nicotine is the major tobacco alkaloid and is responsible for tobacco addiction. In humans, 70–80% of nicotine is metabolized to cotinine primarily by the liver. The transformation from nicotine to the major metabolite cotinine is catalyzed by a cytochrome P450 system with the generation of the nicotine-$\Delta^{1'(5)}$-iminium ion, which is in equilibrium with 5′-hydroxynicotine. In a second oxidation step by a cytosolic aldehyde oxidase, 5′-hydroxynicotine is transformed to cotinine. CYP2A6 is the enzyme that is primarily responsible for the oxidation of nicotine and cotinine in the liver (Hukkanen et al., 2005). Depending on the subtypes, CYP2A

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proteins are expressed in extrahepatic tissues as well. Different members of the cytochrome P450 family are present in the nasal cavity (Thornton-Manning and Dahl, 1997), including the CYP2A proteins. Expression of CYP2A6 has been described in nasal mucosa, but also in other extrahepatic tissues, such as bronchial epithelial cells (Crawford et al., 1998) and esophageal mucosa (Godoy et al., 2002). Another subtype of CYP2A proteins is CYP2A13, which is expressed at much higher levels in extrahepatic tissues, especially in nasal mucosa, but also in the lung and the esophagus. The amino acid sequence of both proteins (CYP2A6 and CYP2A13) is 95.4% identical. Therefore, antibodies, e.g., in flow cytometry, and different chemical probes are able to interact with both (Su et al., 2000). CYP2A6 and CYP2A13 also have a number of similar metabolic substrates but there are differences in the product distribution and activities. For example, CYP2A13 was demonstrated to have the highest efficiency for metabolic activation of the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (Su et al., 2000) whereas its role in nicotine metabolism is not yet well understood (Bao et al., 2005; DeVore and Scott, 2012). The metabolic activation of NNK in the nasal mucosa was initially described three decades ago (Brittebo et al., 1983) and has been shown to play a key role in the initiation of tumors in the nose (Jalas et al., 2005).

Besides causing addiction, nicotine and its metabolites are also involved in many intracellular processes. Via an endogenous nicotinic cholinergic pathway it has demonstrated strong angiogenic potential in endothelial cells of vascular lesions and tumors (Cooke and Bitterman, 2004; Heeschen et al., 2001). Pro-mitogenic effects were described in different cell systems, e.g., in normal and neoplastic lung cells (Schuller, 1994), gastric tumors (Shin et al., 2004), pancreatic cells (Chowdhury et al., 2007), and via the nicotinic acetylcholine receptor (nAChR) in non-small cell lung cancer cells (Dasgupta et al., 2006). In addition to the stimulatory effects on proliferation, the influence of apoptotic pathways has also been investigated. Depending on the cell system, inhibition of apoptosis was described in normal and neoplastic cells (Arredondo et al., 2006; Cardinale et al., 2012). These findings have led to the hypothesis that nicotine contributes to tumor progression (Catassi et al., 2008).

Even though nicotine itself is not classified as a carcinogen, there is growing evidence that it may have genotoxic potential. Most investigations were performed in single cell cultures in vitro. In 1990, nicotine was reported to induce chromosome aberrations and sister chromatid exchanges (SCE) in CHO cells (Trivedi et al., 1990), a result which could not be reproduced by others (Doolittle et al., 1995). In 2004, a distinct rise in the formation of micronuclei in human gingival fibroblast was reported (Argentin and Cicchetti, 2004). Using the comet assay, dose-dependent DNA damage was shown in human lymphocytes, tonsillar tissue, salivary gland cells, embryonic cells, amniotic cells and spermatozoa after short-term exposure to nicotine (Arabi, 2004; Demirhan et al., 2011; Ginzkey et al., 2009; Kleinsasser et al., 2005; Sobkowiak and Lesicki, 2009).

Miniorgan cultures (MOC) of adenoid tissue (Steinsvag et al., 1991), bronchial epithelium (Fjellbirkeland et al., 1996), nasal epithelium (Buehrlen et al., 2007; Kleinsasser et al., 2004) and human salivary glands (Ginzkey et al., 2010) have been established, and are widely used as a model for long-term and repetitive exposure protocols in ecogenotoxicology. For studying the metabolism and toxicity of xenobiotics, precision-cut organ slices in vitro models were developed for liver, lung, heart, spleen and kidney (De Kanter et al., 1999). Due to the maintenance of tissue structure with epithelium, basal membrane and connective tissue, organ cultures are believed to imitate a more in vivo-like test condition. In MOC of both nasal and salivary gland tissue, nicotine was shown to induce reproducible DNA damage after repetitive exposure for a culture period between 1 and 11 days (Ginzkey et al., 2010; Sassen et al., 2005). However, exposure was performed for only 1 h and the used concentrations were quite high compared to plasma levels (Hukkanen et al., 2005).

The present study in human nasal MOC is the first time to evaluate the genotoxic effect of nicotine after chronic exposure. Therefore, (i) DNA damage was determined after long-term exposure to nicotine over a period of 3 weeks using the comet assay, and (ii) a possible nicotine metabolism was measured by using highly sensitive gas chromatography/mass spectrometry (GC/MS) to determine nicotine and cotinine concentrations at different time points.

2. Material and methods

2.1. Preparation of miniorgan cultures (MOC)

During endoscopic surgery specimens of inferior nasal turbinates from 10 patients were resected and transferred to the laboratory, followed by separation from coagulation artefacts and connective tissue. The tissues were cut into small cubes of about 1 mm^3 and washed three times in bronchial epithelium growth medium (BEGM, PromoCell, Heidelberg, Germany). The MOC were subsequently cultivated at 37 °C and 5% CO_2 atmosphere in 24-well plates (BD Biosciences, Heidelberg, Germany) in 250 μl BEGM (with supplement according to the manufacturer's instructions) with or without nicotine. Nicotine (purity >99%; Sigma–Aldrich) was diluted from stock solution in BEG medium prior to treatment.

To avoid adhesion to the surface, wells were coated with >0.75% Agar Noble (BD Biosciences) dissolved in Dulbecco's modified eagle medium (DMEM, Invitrogen, Karlsruhe, Germany) containing 10% fetal calf serum (FCS; Linaris, Wertheim-Bettingen, Germany), non-essential amino acids, penicillin and streptomycin (Biochrom, Berlin, Germany) and stored at 4 °C.

Prior to surgery participants provided written informed consent and were asked to answer a questionnaire regarding smoking habits and occupation. The study was approved by the Ethics Commission of the Medical Faculty, Julius-Maximilian University Wuerzburg, according to their approval notification dated February 2006, no. 16/06.

2.2. Exposure protocol

Starting from the day of preparation (day 0), MOC were assigned to three different groups: a control group without nicotine and two groups with MOC containing nicotine at concentrations of 1 μM and 1 mM, respectively. In all incubations the medium (with or without nicotine) was changed every 2nd day.

At days 7, 14 and 21 a single cell microgel electrophoresis assay (comet assay) was performed for all 10 patients of each group. To have enough cells for the comet assay, 4 MOC per sample were treated in the same way and cells were pooled after enzymatic digestion. Serving as a positive control, additional MOCs of the control group were incubated for 1 h prior to the comet assay with the alkylating methyl methanesulfonate (MMS, purity >99%; Sigma–Aldrich) at a concentration of 100 μM.

2.3. Single cell separation and alkaline single cell microgel electrophoresis (comet) assay

After exposure, MOC were digested enzymatically for 45 min at 37 °C with a combination of protease type XIV from *Streptomyces griseus* (Sigma–Aldrich, Taufkirchen, Germany, 5.0 mg/ml), hyaluronidase from bovine testis (Sigma–Aldrich, 1.0 mg/dl) and collagenase P (Roche, Mannheim, Germany, 1.0 mg/dl). The enzymatic reaction was stopped with FCS and the digestion was put on ice to avoid any further repair of DNA damage. Following digestion, reaction tubes were vortexed and residual connective

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