Effects of cigarette smoke residues from textiles on fibroblasts, neurocytes and zebrafish embryos and nicotine permeation through human skin
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ABSTRACT

Toxic substances from cigarette smoke can attach to carpets, curtains, clothes or other surfaces and thus may pose risks to affected persons. The phenomenon itself and the potential hazards are discussed controversially, but scientific data are rare. The objective of this study was to examine the potential of textile-bound nicotine for permeation through human skin and to assess the effects of cigarette smoke extracts from clothes on fibroblasts, neurocytes and zebrafish embryos. Tritiated nicotine from contaminated cotton textiles penetrated through adult human full-thickness skin as well as through a 3D in vitro skin model in diffusion chambers. We also observed a significant concentration-dependent cytotoxicity of textile smoke extracts on fibroblast viability and structure as well as on neurocytes. Early larval tests with zebrafish embryos were used as a valid assay for testing acute vertebrate toxicity. Zebrafish development was delayed and most of the embryos died when exposed to smoke extracts from textiles. Our data show that textiles contaminated with cigarette smoke represent a potential source of nicotine uptake and can provoke adverse health effects.

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Introduction

Many studies show the negative health effects of tobacco smoke on the smokers themselves and their environment with tobacco smoke containing more than 4000 chemicals including numerous known and suspected carcinogens (Rodgman and Perfetti, 2008). While the smokers inhale mainstream smoke, people around them are exposed to second-hand or sidestream smoke, which contains even higher concentrations of many tobacco components than mainstream smoke (Löfroth, 1989; Wong et al., 2004). Environmental Tobacco Smoke (ETS) is a combination of both which becomes rapidly diluted in ambient air. The effects of passive smoking on human health were studied extensively (Argacha et al., 2008; Bakirtas, 2009; Flouris, 2009; Gül et al., 2010; Kraemer et al., 2004; Lefcoe, 1986; Magnussen et al., 1993; Otsuka et al., 2001; Raupach et al., 2006; Simonet et al., 2007; Wiedemann et al., 1986; Yarlioglues et al., 2010) and a recent global study found that 600,000 people die because of passive smoking each year (Öberg et al., 2010).

During the last few years, another part of cigarette smoke – third-hand smoke (THS) – has moved to the focus of attention and is discussed controversially (Dreyfuss, 2010; Tuma, 2010; Winickoff et al., 2009). The phenomenon of third-hand smoke (THS) is not inhaled but adheres to curtains, carpets, hair or clothing and other surfaces in the environment of the smoker. Also the smoker’s breath is a source of tobacco-related volatile organic compounds (Buszewski et al., 2009). Sleiman et al. (2010) and Petrick et al. (2011) showed the formation of toxicants by surface-mediated reactions of nicotine with other substances and thus the potential hazard of residual tobacco smoke contamination, which could especially be dangerous for infants and children (Matt et al., 1999, 2004; Winickoff et al., 2010). While particle size and amount (Beccquemin et al., 2010) as well as volatile organic compounds (Ueta et al., 2010) of third-hand smoke were determined, to our knowledge, a direct effect of smoke residues from textiles on living tissues or cells has not been proven so far. These data could be very important, however, as sidestream smoke was shown to have effects on structure and function of fibroblasts with impacts on wound healing and tissue repair (Wong et al., 2004). Furthermore, the enzyme activity of human lung fibroblasts is affected by cigarette smoke (Ning et al., 2007).

The nicotine levels of tobacco products vary (Gritz et al., 1981; Hecht et al., 1975; Squier, 1986). For instance, the smoke of one cigarette usually contains between 0.4 and 3.0 mg nicotine. Since 2004 the maximum nicotine content per cigarette in the European Union is restricted to 1.0 mg. Various acute effects of nicotine via nicotinic acetylcholine receptors could be shown for example on skin (Grando, 2001), oral epithelial cells or keratinocytes (Arredondo et al., 2001, 2005, 2006, 2007, 2008; Lee et al., 2005) as well as direct effects on dermal fibroblasts (Cho et al., 2010) and epithelial cells (Conklin et al., 2002). Other studies indicate...
direct impacts of nicotine on nervous system development by the affection of axonal outgrowth of spinal motoneurons in zebrafish (Svoboda et al., 2002; Welsh et al., 2009), cholinceptive neurons in developing chick tectum (Torrao et al., 2003) or hippocampal H19-7 cells (Shin-young et al., 2007).

Although numerous adverse effects of tobacco smoke on the skin are known (Krug et al., 2004), the effects of pure nicotine on skin and wound healing are even more controversial (Forrest et al., 1987; Misery, 2004). For example, the alkaloid was shown to promote wound healing at low concentrations (Jacobi et al., 2002; Morimoto et al., 2008), to induce angiogenesis (Heeschen et al., 2001; Jacobi et al., 2002) or to increase cutaneous blood flow (Sorenson et al., 2009; Usuki et al., 1998) when applied in its pure form, not as a tobacco ingredient. On the other hand, Inaloz et al. (2000) found considerable teratogenic effects of nicotine on new born rat skin. The ability of nicotine to penetrate skin (Farahmand and Maibach, 2009) and mucosa (Squier, 1986) is used for smoking replacement therapies with nicotine-containing patches or chewing gums.

In our study we intended to examine the effects of cigarette smoke residues from textiles on skin and living cells. As the adhesion and dehiscence of smoke components to and from textiles strongly depend on the material the fabric is made of (Ueta et al., 2010), we decided to use standardized cotton cloth since cotton is the predominantly used cloth worldwide. Artificial human sweat, a common solvent for testing textile-associated toxicity, was used for extracting cigarette smoke from the treated cotton. Radioactively labelled nicotine was used for tracer experiments to evaluate the risk potential of nicotine transfer and penetration capacity from contaminated textile patches to adult human full-thickness skin and to 3D in vitro skin model. Subsequently, the effects of textile sweat extracts (TSE) on fibroblasts and neurocytes were documented. To evaluate vertebrate toxicity, experiments on the impact of TSE on zebrafish development were conducted.

Materials and methods

Preparation of textile sweat extracts (TSE)

Swatches of standard cotton cloth (10 g) were fixed inside a 5 l glass flask and slightly moistened with deionized water. The flask was arranged upside-down on 1 cm thick plastic strips to obtain a window for inserting the cigarettes. 1, 5 or 10 commercially available cigarettes (smoke of each containing 10 mg tar, 0.8 mg nicotine and 10 mg CO) were lit up and put immediately under the glass flask. As the whole experimental set-up was placed in a room free of air turbulence, the smoke remained within the glass bell. After 60 min, the contaminated textiles were taken out, dried and subsequently conducted to extraction with artificial human sweat (0.5 g/l histidine, 5 g/l NaCl, 2.2 g/l NaH₂PO₄, pH 5.5; 10 ml/g textile) for 24 h at 37 °C with gentle agitation. Artificial sweat is the standard solvent used in textile toxicology testing procedures. Finally, the textile sweat extracts from textiles exposed to 1 cigarette (TSE-1), 5 cigarettes (TSE-5) and 10 cigarettes (TSE-10) were obtained, adjusted to pH 7.4 and frozen at −26 °C until use. In addition, a non-treated textile was extracted likewise with artificial sweat as a negative control for subsequent experiments (TSE-0).

Full-thickness skin penetration assay

Fresh adult full-thickness donor skin removed from the breast of a female patient during plastic surgery was kept in PBS at 4 °C until use. For the penetration assays, circular pieces of the skin (diameter: 3 cm) were fixed in Franz-type diffusion cells with the epidermis upwards. The lower chamber was completely filled with 12 ml of phosphate buffered saline (PBS, pH 7.4) without leaving any residual air underneath the skin. A circular swatch of cotton textile (diameter: 1 cm) endowed with ²H-labelled nicotine (85 Ci/mmol, 25 pmol/textile patch, dried) was placed in the middle of each skin sample. 50 µl of PBS (pH 7.4) or artificial sweat (pH 5.5) were added to the textile swatches to dissolve the dried nicotine. The Franz-type diffusion cell was closed with parafilm to prevent drying-out of the system. After incubation of the whole diffusion cell for 24 h at 37 °C, the textile swatches were removed and the residual textile-bound nicotine was eluted for 30 min at 37 °C with gentle agitation in 6.5 ml of PBS. This solution, as well as the PBS from the lower chamber of the Franz-type diffusion cell, were transferred directly to liquid scintillation counting (LSC) to determine the percentage of residual textile-bound nicotine and of the nicotine which completely permeated through the skin. The skin sample was homogenized, diluted in PBS and also transferred to LSC. In an additional assay, 1 ml of TSE-10 was added onto the skin sample instead of the textile swatch and the PBS in the lower chamber was replaced by 12 ml of DMEM cell culture medium. After incubation for 24 h at 37 °C, the medium from the lower chamber was used for the fibroblast cytotoxicity assay. To repeat the experiments with a more susceptible skin equivalent, a 3D in vitro skin model was produced as described elsewhere (Mertsching et al., 2008) and skin penetration assays were performed as described above.

Liquid scintillation counting (LSC)

Samples (eluted residual textile-bound nicotine, PBS from lower chamber in Franz-type diffusion cell and homogenized skin in PBS) were diluted with deionized water to a final volume of 6 ml in a 20 ml plastic scintillation vial. 8 ml of scintillation cocktail (Aquasol-Light, Hidex) were added to each sample and the mixture was gently inverted to obtain a homogenous suspension. Subsequently, the samples were placed in a liquid scintillation counter (Hidex 300 SL) and measured with 120 s detection time for ²H-detection. The obtained data were processed using MikroWin software (Hidex).

Fibroblast cytotoxicity assay

Murine fibroblasts (ATCC#CCL1 clone L929) were grown in cell culture flasks with DMEM culture medium at standard culture conditions (37 °C with 5% CO₂ in a ‘Cytoperm 2’ CO₂-incubator). The cytotoxicity assay was performed according to DIN EN ISO 10993-5. In brief, confluent cells were detached from the cell culture flask, adjusted with fresh DMEM to a final concentration of 1 x 10⁵ cells/ml and transferred to a 96-well culture plate with 50 µl per well. After 30 min of incubation at 37 °C and 5% CO₂, 100 µl of the sample solutions (TSE-1, TSE-5, TSE-10, 12 mM nicotine in artificial sweat, medium from skin penetration assay with TSE-10) were added to each well. Sample concentration was varied to obtain final sample concentrations of 33.3%, 22.2%, 14.8% and 9.9%. 5% DMSO was used as a negative control. Each sample concentration was tested at least in 3 independent experiments. After addition of the sample solutions, the cells were incubated for 72 h as described above. To measure cell viability, a BCA-assay was conducted. The medium was removed from the cells and the cells were washed 3 times with PBS (pH 7.4). Subsequently, the cells were frozen at −28 °C for 30 min and thawed at 37 °C with gentle agitation. The freezing/thawing procedure was repeated twice. BCA-A and BCA-B reagent were mixed 1:50 and 200 µl of the mixture were added to each well. The cells were mechanically detached and mixed with the reagent using a pipette tip. After a 30 min incubation step at 60 °C and subsequent chilling at room temperature, absorption at 540 nm was determined with a multiwell plate.
Permeation of nicotine through the skin

Textile sweat extracts (TSE) from cotton textiles exposed to smoke from 1, 5 or 10 cigarettes in a practical approach were used for BCA cytotoxicity assays with L929 fibroblasts. In addition, we also tested the medium which was underneath the TSE-treated skin model in a Franz-type diffusion cell for cytotoxicity according to DIN EN ISO 10993-5. A significant dose-dependent cytotoxic effect on L929 fibroblasts could be observed. The results are summarized in Fig. 1. According to the mentioned standard, an extract is cytotoxic if cell survival is below 70% at the 33.3% sweat extract concentration. Thus, all textile extracts tested whereas nicotine solution was only cytotoxic in the 33.3% concentration.

Fibroblast viability

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Fibroblast structure

TSE-10 (33.3%) was applied on human fibroblasts for 6 h to assess putative effects on cell morphology and structure prior to cell death. Representative pictures are shown in Fig. 2. Fibroblasts exposed to TSE-10 were significantly smaller in size and more separated from each other compared to the control with 33.3% of uncontaminated artificial sweat (TSE-0). In addition, fibroblasts in the TSE-10-assays were less elongated than the control cells. In about 1% of the TSE-10-exposed human fibroblasts we found a nicotine-driven formation of vacuoles. All fibroblasts grown in medium with TSE-10 showed an accumulation of black particles inside the cells, which were likely to be particulate matter from the cigarette smoke. As cell shape was clearly affected by TSE-10, we looked for changes in the actin-cytoskeleton in detail. No apparent alterations in the stress fibre pattern could be observed in the TSE-treated cells compared to the control.

Neurotoxicity

A neurite growth assay was run with avian tectum neurons exposed to TSE-0 as a control, nicotine in artificial sweat and TSE-10. Cells died off completely when exposed to 33.3% and 22.2% artificial sweat/nicotine or 33.3%, 22.2% and 14.8% TSE-10, respectively. Applying lower concentrations, neurite length could be measured in 100 individual cells per sample. Medium neurite length and statistical length distribution were determined. The results are shown in Fig. 3. Our data show that neurocytes are significantly affected by nicotine and especially by TSE-10. Medium neurite length of the AS-nicotine-exposed cells was reduced to 62.7% (with 14.8% of nicotine-solution) and 36.9% (with 9.9% of nicotine solution) compared to the control (AS without nicotine). The effect of TSE-10 was stronger with a neurite length reduction of 23.0% in the lowest concentration tested (9.9% TSE-10) and a significantly increased in the presence of TSE-5 and TSE-10 during the following 20 h with the heart beat of the treated fish being significantly lower than in the control group. In the late phase, prior to hatching, most of the smoke extract- and nicotine-treated embryos died off. Throughout the assays, TSE-1 had no apparent effect on zebrafish development. Fig. 4 gives an overview of the zebrafish embryo experiments.

Discussion

Several studies show the toxicity of cigarette smoke inhaled directly by the smoker or passively. The phenomenon and the hazards of smoke attached to surfaces like curtains, carpets or clothes are discussed controversially and scientific data are rare. Here we show for the first time the toxic effects of cigarette smoke residues dissolved from cotton textiles on fibroblasts, neurocytes and zebrafish embryos and the permeation of textile-bound nicotine through human skin.

Our results imply that about half of the nicotine bound to clothing and dissolved by skin sweat penetrates into and permeates through the adult skin. In this respect, our data are consistent with the nicotine permeation values of the full-thickness skin model published by Ackermann et al. (2010) who found permeation of 46.3% ± 6.5% of total nicotine dissolved in PBS after 6 h and 70.4% ± 4.3% after 26 h in a Franz-type diffusion cell. It is known that penetration rates of substances depend on the solvent used which is also true for our data. Although it is known that most in vitro-based permeability models underestimate the in vivo data up to 10,000-fold, especially for nicotine (Farahmand and Maibach, 2009), we used a low nicotine concentration (about 32 pmol nicotine/cm² cotton textile) for the permeation experiments. In comparison, common nicotine swatches in smoking replacement therapies contain up to 25 mg (~154 nmol) nicotine. In our study, the most realistic scenario for cigarette smoke residuals exposure to skin from clothes is with human donor skin and artificial sweat as a nicotine solvent. As about 50% of the nicotine entered or permeated the skin, we assume that tobacco smoke residue on clothes is a potential source of nicotine uptake. We obtained significantly higher permeation rates with a 3D in vitro skin model (data not shown) which may be explained by differences in structure of the stratum corneum, which is known to form a rate-controlling barrier for diffusion of most compounds.

For the toxicity experiments, we generated smoke-contaminated cotton textiles in a realistic practical approach rather than producing defined third-hand smoke with a constant source of oxygen. The extracted smoke residues from the prepared textiles were toxic to fibroblasts and neurocytes. Fibroblast viability and structure was affected significantly, a finding which is consistent with Wong et al. (2004). On the other hand, the fibroblasts in our TSE-10-assays were less elongated than the control cells, which is contradictory to the above mentioned data from 2004. Vacuolization was already shown in human keratinocytes exposed to nicotine by Theilig et al. (1994) and one decade later, the same effect was revealed in chicken primary fibroblasts with second-hand smoke as a toxicant (Wong et al., 2004). We were able to confirm the nicotine-driven formation of vacuoles in human fibroblasts and found the same effect stimulated by TSE-10 in about 1% of the exposed fibroblasts. We did not observe alterations

Table 1

<table>
<thead>
<tr>
<th>Artificial sweat</th>
<th>PBS</th>
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<tbody>
<tr>
<td><strong>Textile</strong></td>
<td></td>
</tr>
<tr>
<td>1H-signal [Bq]</td>
<td>504 ± 124.3</td>
</tr>
<tr>
<td>Nicotine [pmol]</td>
<td>13.6 ± 3.4</td>
</tr>
<tr>
<td>% of total nicotine</td>
<td>543 ± 13.4</td>
</tr>
<tr>
<td><strong>Skin</strong></td>
<td>372 ± 84.5</td>
</tr>
<tr>
<td>1H-signal [Bq]</td>
<td>10.0 ± 2.3</td>
</tr>
<tr>
<td>Nicotine [pmol]</td>
<td>40.0 ± 9.5</td>
</tr>
<tr>
<td>% of total nicotine</td>
<td>5.7 ± 4.5</td>
</tr>
<tr>
<td><strong>Diffusion chamber</strong></td>
<td>54 ± 44.1</td>
</tr>
<tr>
<td>1H-signal [Bq]</td>
<td>1.5 ± 1.2</td>
</tr>
<tr>
<td>Nicotine [pmol]</td>
<td>5.7 ± 4.5</td>
</tr>
<tr>
<td>% of total nicotine</td>
<td>5.7 ± 4.5</td>
</tr>
</tbody>
</table>
in the stress fibre pattern and the migration potential of the cells
exposed to the textile smoke extracts, although second-hand
smoke has been shown to increase stress-fibre formation in fibrob-
lasts (Wong et al., 2004). Fibroblast propagation and migration are
crucial for wound healing. Several studies on the effect of active
and passive smoking on wound healing have been published so
far, displaying contradictory results. In cutaneous injuries of rats
less fibroblasts were found when the animals were exposed to
nicotine (Biondo-Simoes et al., 2009) and in another study wound
closure in mice exposed to sidestream smoke was significantly
delayed compared to the control group (Wong et al., 2004). Jacobi
et al. (2002) however, report the nicotine-driven acceleration of
angiogenesis and wound healing in diabetic mice. In our study,
the extracted smoke residuals had significant negative effects on
the viability and cell structure of fibroblasts in a dose-dependent
manner. Although we concentrated on nicotine in high doses as
a positive control, one should be aware that nicotine is not the
only component of cigarette smoke which could explain cyto- and
neurotoxicity. Substantial affection of neural cells by nicotine alone
as well as by tobacco smoke could be shown in various studies
with implications for alterations in the nervous system (Berger
et al., 1998; Roy et al., 2002; Slotkin, 1998; Tomassini et al., 2007;
Touiki et al., 2007). With our findings we add the aspect of cigarette
smoke residue from textiles as a potential neurotoxic agent, which
might be critical for example for children sucking on clothes or
other textiles which were previously exposed to cigarette smoke.
Therefore, potential effects of such extracts on zebrafish embryo
development were used for testing acute vertebrate toxicity. We
were able to show the acute toxicity of extracted cigarette smoke
residues from textiles on zebrafish development. TSE- as well
as nicotine exposure resulted in an increased heart beat during
the first 35 h of incubation, followed by a decrease in heart beat
during the following 20 h and a high embryo mortality prior to
hatching. This phenomenon might be explained by the initial
nicotine-driven stimulation of heart rate and the associated higher
energy consumption and a subsequent lack of energy resulting in
embryo mortality.

To conclude, people should be aware of the negative effects of
cigarette smoke residues on various cell types, for example when
infants get into contact with contaminated textiles as a source of
Fig. 3. Neurite growth assay with isolated chicken tectum neurons. (A) Effect of artificial sweat (AS) and 12 mM nicotine in AS (Nic) in two different concentrations (9.9% and 14.8%) on medium neurite length and on statistical neurite length distribution. (B) Effect of TSE-0 and 9.9% TSE-10 on medium neurite length and on statistical neurite length distribution. Neurite length was measured in pixels on cell microscopy photographs of 100 neurons per sample. Nicotine and TSE-10 clearly inhibited neurite outgrowth in avian tectum neurocytes.

Fig. 4. Effects of TSE-0, 12 mM nicotine in AS (Nic), TSE-5 and TSE-10 on zebrafish embryo development compared to a negative control (Co). Nicotine, TSE-5 and TSE-10 significantly delayed embryo development in the period from 25 to 53 h. Scale bar = 300 μm.
smoke residues. While our study focuses on the acute toxicity, chronic effects of smoke residue exposure still have to be studied. In addition, the putative interactions of the tobacco components with textile finishes or the fibre surfaces should also be considered. In the future, further research has to be done to clarify open issues and to support the controversial discussion on the risks of third-hand smoke scientifically.

Acknowledgments

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