DOI:10.1111/j.1600-0625.2010.01068.x
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Original Article

Tattoo inks contain polycyclic aromatic hydrocarbons that additionally generate deleterious singlet oxygen

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Accepted for publication 18 December 2009

Abstract: In the past years, tattoos have become very popular worldwide, and millions of people have tattoos with mainly black colours. Black tattoo inks are usually based on soot, are not regulated and may contain hazardous polycyclic aromatic hydrocarbons (PAHs). Part of PAHs possibly stay lifelong in skin, absorb UV radiation and generate singlet oxygen, which may affect skin integrity. Therefore, we analysed 19 commercially available tattoo inks using HPLC and mass spectrometry. The total concentrations of PAHs in the different inks ranged from 0.14 to 201 $\mu g/g$ tattoo ink. Benz(a)pyrene was found in four ink samples at a mean concentration of 0.3 ± 0.2 $\mu g/g$. We also found high concentrations of phenol ranging from 0.2 to 385 $\mu g/g$ tattoo ink. PAHs partly show high quantum yields of singlet oxygen (Φ_Δ) in the range from 0.18 to 0.85. We incubated keratinocytes with extracts of different inks. Subsequent UVA irradiation decreased the mitochondrial activity of cells when the extracts contained PAHs, which sufficiently absorb UVA and show simultaneously high Φ_Δ value. Tattooing with black inks entails an injection of substantial amounts of phenol and PAHs into skin. Most of these PAHs are carcinogenic and may additionally generate deleterious singlet oxygen inside the dermis when skin is exposed to UVA (e.g. solar radiation).

Key words: black tattoo ink – PAHs – singlet oxygen – skin – UVA

Please cite this paper as: Tattoo inks contain polycyclic aromatic hydrocarbons that additionally generate deleterious singlet oxygen. Experimental Dermatology 2010; **19**: e275–e281.

Introduction

In recent years, the number of tattooed individuals has increased significantly (1,2). In the United States, up to 24% of the population are tattooed (2), in Germany approximately 9% and about 12% in the United Kingdom (3). Despite the high number of tattooed individuals, the composition of tattoo inks, its pharmacokinetics in the human body and the potential risks of the inks are unknown.

Most of the tattoos are either completely or partly black. Tattooists purchase black inks from tattoo suppliers or through the internet. The manufacturing of black inks usually involves the thermal combustion of feedstock oil and we expect in the black inks compounds such as carbon black and polycyclic aromatic hydrocarbons (PAHs). Carbon black is listed as a group 2B carcinogen: possibly carcinogenic to humans (4,5). Most of the PAHs are carcinogenic or mutagenic (6,7). Nevertheless, black tattoo inks are usually neither analysed nor controlled prior to use.

If black tattoo inks contain PAHs, the uptake of PAHs is no longer only a matter of cigarette smoke (8) and dietary intake (9). Moreover, transportation of black carbon inside the human body, probably together with PAHs, has been already observed for the lymph nodes (10,11). Beside the carcinogenicity or mutagenicity, PAHs may pose another risk to the skin. Some of the PAHs are known to generate singlet oxygen under UV-irradiation (12), which is of importance when the PAHs remain in the skin, e.g. adsorbed to black carbon particles. If black tattooed skin is exposed to solar light, PAHs can generate singlet oxygen inside the dermis, which might affect skin integrity as for other endogenous photosensitizers (13–15).

Therefore, we analysed commercially available tattoo inks using HPLC and mass spectrometry to quantify the

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concentrations of 20 well-known PAHs. We determined the efficiency of PAHs to generate singlet oxygen and we examined possible cytotoxic effects of black ink extracts in skin cells by measuring the mitochondrial activity.

Materials and methods

Materials

The 19 black tattoo colours were purchased from different tattoo ink providers. PAHs were obtained from Sigma Aldrich (Steinheim, Germany). Benzene and acetone were of reagent grade, obtained from Merck (Darmstadt, Germany). Acetonitrile as solvent B for LC–MS analysis was of gradient grade quality for liquid chromatography (LiChroSolv® Darmstadt, Germany). Millipore water as solvent A for LC–MS analysis was freshly produced by a novel Milli-Q Advantage A10 system (TOC 5 ppb, Millipore, Molsheim Cédex). All 20 USEPA PAHs were dissolved in 1 ml of acetonitrile and treated by ultrasonic (Bandelin Sonorex Super RK 103 H) for 10 min, respectively. For internal standard (ISTD), 9,10-diphenylanthracene was obtained from Riedel-de Haën and prepared as an 100 μg/ml stock solution in acetonitrile.

Extraction and chemical analysis

Extraction with ultrasonic assistance is a frequently applied method (16–18). 1 mL of each ink sample was extracted three times in 2 ml of benzene and 1 ml of acetone in a duran-glas (8 mm × 10 mm, NS 14; Neubert-Glas, Ilmenau, Germany) by treating with ultrasonic (Bandelin Sonorex Super RK 103 H) for 60 min and 60°C each.

After cooling down, the samples were centrifugated at 4°C with 2500 ***g*** r.c.f. (Eppendorf Centrifuge, 5702 RH), and the supernatant was concentrated by nitrogen flow. The residual compounds were resolved in 1 mL of acetonitrile, filtered using PTFE-filter (CHROMAFIL®, O-20/15, organic, pore size 0.2 μm, Machery-Nagel, Düren, Germany) and analysed with internal standard method by high-pressure liquid chromatography.

One hundred microlitres of the clear to light-yellow resolved solution was analysed using a model 1100 HPLC (Agilent Technologies, Waldbronn, Germany) fitted with a C18 analytical column for nanoscalic environmental analysis (Phenomenex Environsep PP, particle size 3 μm, 125 mm × 2.00 mm, Aschaffenburg, Germany), diode array detector (DAD). The data-files were analysed using a HPLC-3D-ChemStation Rev. B.03.01. The PAHs could be separated by gradient elution with water [0.0059 w % trifluoroacetic acid] (solvent A) and acetonitrile (solvent B) at a constant flow rate of 0.3 ml/min. A gradient profile with the following proportions of solvent B was applied [t (min), % B]: (0, 40), (2, 40), (27, 98), (35, 98). The chromatograms were monitored at 220 nm.

The concentration of phenol and PAHs in the solutions was determined by the method of internal standard. For each compound (i), the calibration factor (CF_i) was determined in a calibration run (single level calibration). The respective concentration of the internal standard was chosen to be in the range of the concentration of the PAHs.

$$CF_i = \frac{f_{Tr}}{f_i} = \frac{m_i^K \cdot a_{Tr}^K}{m_{Tr}^K \cdot a_i^K}$$

Where f_{Tr} is the response factor of the ISTD, m_i^K the mass of compound i in the solution k and m_{Tr}^K the mass of ISTD in solution k. a_{Tr}^K is the area of ISTD in solution k and a_i^K the area of compound i in solution k.

Statistical methods

The complete procedure of extraction and quantification of PAHs and phenol was repeated three times for three different subsamples. The values for the three different subsamples are given as mean ± standard deviation. To determine the mitochondrial activity of cells, $n = 3$ independent experiments were carried out with five values per each parameter. Pair comparison of selected data points was performed by analysis of variance (ANOVA), whereas differences were regarded as significant for $P \leq 0.05$.

Singlet oxygen quantification

To determine the singlet oxygen quantum yield, the PAHs were dissolved in benzene to show an absorbance of 0.5 at 355 nm. The solutions were transferred in a cuvette (101-QS; Hellma Optik, Jena, Germany) in which PAHs were excited with 355 nm of a frequency-tripled Nd:YAG laser. Singlet oxygen was detected by its luminescence, and the quantum yield was determined as reported previously (13,19).

Cell experiments

Primary normal human dermal keratinocytes (NHEK) were purchased from Cascade Biologics (Invitrogen GmbH, Karlsruhe, Germany). NHEK were propagated in complete Epilife media (Cascade Biologics) in a humidified atmosphere containing 5% CO_2 at 37°C. Sub-confluent cells were washed with PBS and harvested using a treatment with 0.05% trypsin/0.02% EDTA in PBS for 10 min. 1×10^4 cells/well of a 96-mircotitreplate were used for the phototoxicity experiments ($n = 3$). NHEK were used between passages 3–4. Extracts #10, #11 and #12 were diluted 1:1000 in Epilife media. Incubation time of the extracts was 300 min. Thereafter, the cells were washed; fresh media was added prior illumination with 4 or 8 J/cm^2 (UVA 700; Waldmann, Schwenningen, Germany). To measure mitochondrial activity (20), MTT assay was performed 24 h after irradiation. The mitochondrial

activity was normalised to 100%. That is, the OD values of cells without irradiation and without extract incubation (medium only) were normalised to 100%. Every experiment was performed in triplicate.

Results and discussion

Black tattoo inks in skin

Black tattoos are widespread in most of the countries worldwide. Recently, a patient with a large black tattoo showed up in our department Fig. 1a. Tattooists deposit the black inks in the dermis by piercing the skin with tiny solid needles moistened with the ink that is illustrated in a typical histology slice (Fig. 1b). Part of the ink is transported to other anatomical locations like the lymph nodes as shown in Fig. 1c (11,21,22). This sentinel lymph node was removed in the axilla of a tattooed patient with a melanoma in the tattoo. Prior to tattooing, the ink mainly consists of black carbon nanoparticles (diameter approximately 40 nm) as shown by transmission electron microscopy (Fig. 1d). When using coloured tattoo pigments, we have recently determined a value of 2.53 mg per 1 cm^2 skin (23). If this value is also valid for black inks, such high amounts of unregulated inks in the skin may pose a health risk on the tattooed individual, in particular when the inks contain hazardous PAHs.

There are various case reports about various adverse reactions after tattooing including malignancies (24–29). However, the cited papers are case reports, in which the authors frequently cannot identify a specific compound in the tattoo colourant that acted as a trigger for the adverse skin reactions. But the skin reactions are clearly associated with the tattooed skin. We recently performed a nationwide survey in German-speaking countries. Among the 3411 participants, 6% stated persisting health problems in the tattooed skin (30).

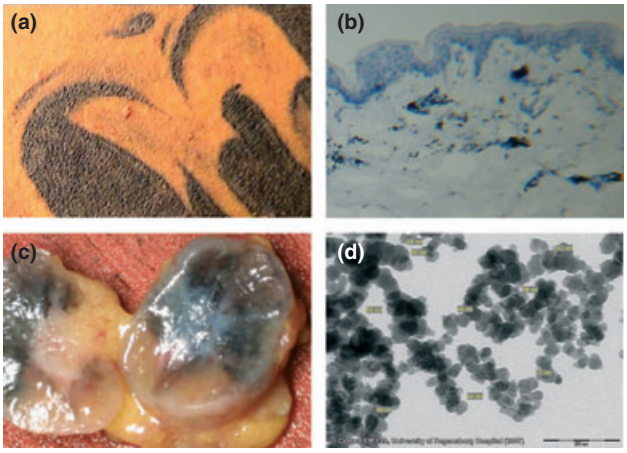

Figure 1. Localisation of black tattoo inks. A black tattoo at the back of a patient (a). The histology of tattooed skin shows black ink inside the dermis that obviously stays there for years (b). Part of the black ink is transported away via lymphatic system to the lymph node as shown in the same patient after lymph node dissection (c). Black tattoo ink consists usually of small globular particles with a typical cross section of 40 nm as shown by TEM analysis (d).

PAHs and phenol content in commercial black tattoo inks

We purchased 19 inks from different tattoo suppliers in Europe and US, and the inks were delivered as 'ready to use'. They appeared as black viscous suspensions. We received no list of ingredients or other product information together with the ink, which seems to be common for tattoo inks.

We established a procedure to extract PAHs from the inks. Several series of experiments with different solvents (n-hexane, acetone, acetonitrile, toluene and benzene) showed a maximum extraction of PAHs from all inks when using the mixture of benzene and acetone. To identify PAHs in black inks using HPLC, we selected 20 PAHs as reference, which are frequently found in environment, food (31) or cigarette smoke (32): naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a] pyrene, dibenzo[a,h] anthracene, benzo[ghi]perylene, indeno[1,2,3-cd]pyrene, dibenzo[a,e]pyrene, dibenzo[a,l]pyrene, 5-methylchrysene and benzo[j]fluoranthene. These PAHs are listed by the United States Environmental Protection Agency (USEPA) because of their toxicity and carcinogenicity or Scientific Committee on Food (SCF) of HEALTH and CONSUMER PROTECTION DIRECTORATE-GENERAL in Europe.

It is known that PAHs either can be dissolved in the liquid phase or can be adsorbed to the surface of the black carbon particles with different partition coefficients (33). We took samples from the respective inks and extracted the PAHs and phenol. The maximum total concentration of PAHs of 201.1 ± 19.5 $\mu g/g$ (mean value) was found in ink sample #11. In addition, we found high phenol concentrations of up to 385 $\mu g/g$ (Fig. 2). Phenol is toxic and can damage kidney and the central nervous system. It is classified as having a teratogenic and carcinogenic effect (7).

Out of the 20 reference PAHs, we found 16 PAHs at different concentrations whose mean values ranged from 0.1 $\mu g/g$ (dibenzo[a,h]anthracene) to 24.5 $\mu g/g$ (phenanthrene) (Table 1) (not found: dibenzo[a,e]pyrene, dibenzo[a,l]pyrene, 5-methylchrysene, benzo[j]fluoranthene). The deviation of the mean values reflects the different values of three independent extraction experiments of the subsamples. It indicates an inhomogeneous distribution of PAHs in the ink suspensions. We also detected the leading

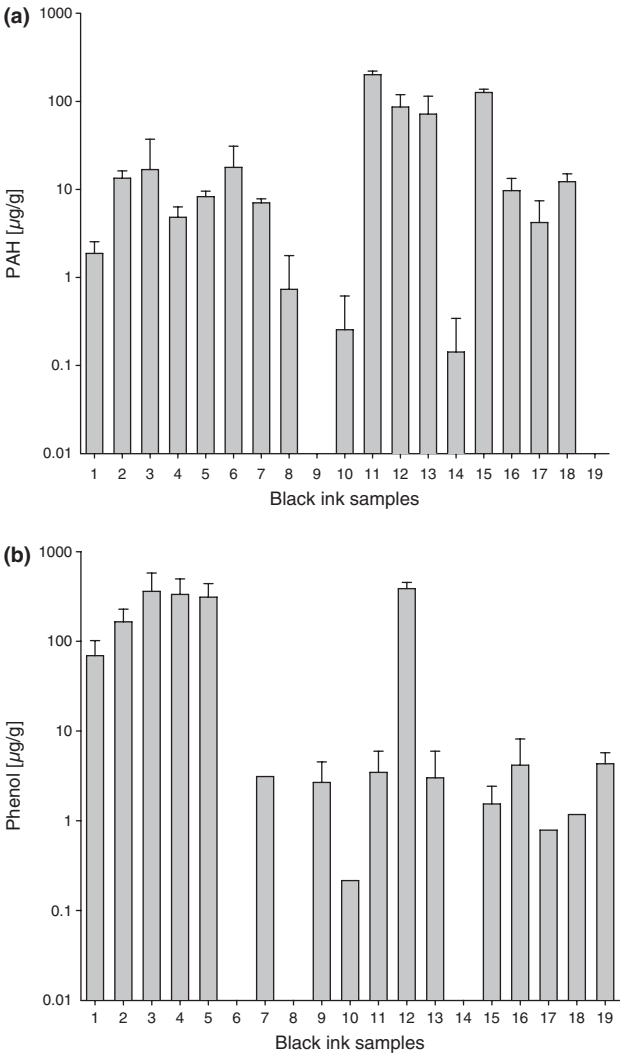

Figure 2. PAHs and phenol content of commercial tattoo inks. PAHs were extracted from the 19 black inks. The black inks contained different total amounts of PAHs, and the mean values ranged from 0.14 to 201 $\mu g/g$ (a). PAHs concentrations were below detection limit for inks #9 and #19. The 19 black inks additionally contained high amounts of phenol. The mean values ranged from 0.2 to 385 $\mu g/g$ phenol (b). Phenol concentrations were below detection limit for inks #6, #8 and #14. The extent of phenol concentration did not correlate to PAHs concentration.

PAH benz[a]pyrene in four ink samples at a mean concentration of 0.3 $\mu g/g$. Recently, IARC classified benzo[a] pyrene to group 1 (carcinogenic to humans) (34).

The USEPA classification and the Toxic Equivalent Factor (TEF) assess carcinogenicity and toxicity of substances like PAHs (7,35) (Table 1). Class D means not classifiable as to human carcinogenicity, C possible human carcinogen and B2 probable human carcinogen. This is based on sufficient evidence of carcinogenicity in animals. It should be emphasised that classification of PAHs in Group D do not necessarily imply non-carcinogenicity or overall safety. We found 7 PAHs in part of the inks that are classified B2. TEF is the toxicity potency factor that is used by the World Health Organisation (WHO) and by scientists and regulators globally as a consistent method to evaluate the toxicities of highly variable mixtures of PAHs. The highest value is attributed to benzo[a]pyrene (TEF = 1.0).

When assessing health risks of PAHs, three major pathways of incorporation to human are considered: ingestion of contaminated food or water, inhalation and skin contact. However, tattooing is a new modality of PAHs incorporation. PAHs are not applied topically on the skin but are injected into skin together with black carbon. In addition, tattooists inject a considerable amount of black tattoo inks into skin. We have recently shown that about 2.53 mg of Pigment Red 22 (dry chemical) is placed in the skin to tattoo 1 cm^2 (23).

As a first estimation, we assume this value of 2.53 per cm^2 also for black tattoos. Suspensions frequently contain a mixture of water and glycerol to a variable extent. After lyophilisation of inks, we obtained nearly dry powder of carbon black, which shows a mean value (19 inks) of 54 ± 22% weight per cent when compared to ink suspensions. Thus, tattooists should inject about 2 g black ink into skin for a typical tattoo of 400 cm^2. Therefore, the values in Fig. 2 should be approximately doubled to estimate the uptake of the individual or total amount of PAHs. In case of the most burdened ink, we estimate a mean skin uptake of about 402 μg (tattoo size; 400 cm^2). Based on the same assumption, a tattoo of 400 cm^2 implies an injection of up to 770 μg phenol.

For non-smoking humans, food is the main source of exposure to PAHs. Smoked meat products show total PAH concentrations in the range of 0.01–19 $\mu g/kg$ (36). Based on data from EU surveys, a maximum daily exposure of adults to the most important PAHs may be 5 μg per person (6). The maximum concentration limit in drinking water is 0.2 $\mu g/l$ for PAHs or 1 $\mu g/l$ for phenol. These values are substantially exceeded by such a tattoo.

It is unknown so far whether PAHs in black tattoo inks can contribute to any carcinogenic risk for tattooed humans. First, it is difficult to compare the risk of tattooing to other routes of administration such as inhalation, dietary intake or dermal contact (36–38). When tattooing skin, tattooists may inject the total amount of PAHs or phenol into tissue that is present in the respective amount of ink suspension. Secondly, black tattoos usually do not disappear and therefore carbon black together with PAHs stays in the skin lifelong. Thirdly, tattooing can be considered rather a bolus injection of high PAHs concentrations than a daily exposure to little PAHs concentration. Fourthly, skin is not the final destination of the injected black inks. Black carbon together with PAHs is transported

Table 1. PAHs found in black tattoo inks

PAHs[1]	Mean value extracted [μg/g]	Mean daily dietary intake (49) [μg/person]	Carcinogenicity USEPA (7)	Toxicity TEF (35)
Phenanthrene (12)	24.5 ± 6.0	1.54	D	0.001
Acenaphthylene (8)	14.5 ± 5.5	0.13	D	0.001
Benzo[b]fluoranthene (2)	4.5 ± 4.3	**0.04**	**B2**	0.1
Pyrene (12)	4.4 ± 0.8	0.35	D	0.001
Anthracene (8)	3.3 ± 0.8	0.07	D	0.01
Fluoranthene (14)	2.8 ± 1.0	0.35	D	0.001
Chrysene (4)	1.7 ± 0.8	**0.11**	**B2**	0.01
Benz[a]anthracene (6)	1.6 ± 0.2	**0.05**	**B2**	0.1
Benzo[ghi]perylene (3)	1.2 ± 1.5	0.05	D	0.01
Indeno[1,2,3-cd]pyrene (2)	1.1 ± 1.0	**0.03**	**B2**	0.1
Acenaphthene (8)	0.9 ± 0.3	0.98	-[2]	0.001
Fluorene (6)	0.9 ± 0.2	0.59	D	0.001
Benzo[k]fluoranthene (2)	0.4 ± 0.2	**0.01**	**B2**	0.1
Benzo[a]pyrene (4)	0.3 ± 0.2	**0.04**	**B2**	1.0
Naphthalene (7)	0.3 ± 0.1	-[2]	**C**	0.001
Dibenzo[a,h]anthracene (1)	0.1 ± 0.1	**0**	**B2**	1.0

[1]The number in brackets indicate the total number of inks in which the respective PAH was found.
[2]Data not available.
The bold characters highlight the probable or possible carcinogenic PAHs.

to other organs such as the lymph nodes (Fig. 1) (11). As recently shown in animal studies, about 30% of tattooed black ink is transported in the body during a time span of about 4 weeks (21). The number of organs involved and the extent of transportation are unknown so far.

After tattooing, a fraction of black carbon particles is transported in the human body. Another part stays in the dermis that is responsible for the black colour of the skin (Fig. 1). PAHs may be desorbed from black carbon in skin or stay adsorbed on black carbon particles for any length of time. The long-lasting sorption of PAHs on black carbon has been already shown in animal studies (37). To date, exact values of PAHs desorption rates are unknown. USEPA has suggested a maximum of 20% of PAHs that adsorbed onto soil could be desorbed (39).

Generation of singlet oxygen by PAHs and phototoxicity *in vitro*

A long-lasting presence of PAHs in skin may pose another risk on the tattooed skin. It is known that some of the PAHs can absorb UV radiation and generate reactive oxygen species such as singlet oxygen (40,41). In contrast to UVB, UVA shows a penetration depth in skin [up to 1.5 mm, (42)] that is sufficient to reach the black ink particles and the adsorbed PAHs in the dermis. In addition, many of the PAHs can absorb UVA radiation. Therefore, we excited the PAHs in the mid of the UVA spectrum at 355 nm.

To quantify the generation of singlet oxygen, we determined the quantum efficiency (Φ_Δ) of PAHs by exciting them in solution. We used the direct detection of singlet oxygen by its weak luminescence, which was measured time- and spectral resolved ('finger-printing' of singlet oxygen). We found partly high Φ_Δ for the most frequent PAHs detected in the black inks (Table 2). The values range from 0.18 to 0.85 and are comparable to or even more efficient than porphyrine photosensitizers, which are used in photodynamic therapy to kill cancer cells or bacteria (19).

Table 2. Quantum yields of singlet oxygen production

PAHs	Φ_Δ
Benz[a]anthracene	0.85
Anthracene	0.81
Benzo[ghi]perylene	0.81
Benzo[a]pyrene	0.66
Fluoranthene	0.57
Benzo[b]fluoranthene	0.45
Benzo[k]fluoranthene	0.27
Indeno[1,2,3-cd]pyrene	0.18

Any exposure of PAHs to UVA radiation generates singlet oxygen, which might affect cellular integrity. UVA radiation and PAHs generate singlet oxygen if the PAH molecules present in such PAH mixtures absorb UVA radiation and its Φ_Δ is sufficiently high. We exemplarily incubated human keratinocytes with the respective extract of three different inks, which contained less (#10), medium (#12) or maximum (#11) total concentrations of PAHs. As the extracts were diluted by a factor 1000, every well (10^4 cells) received a total PAH amount of 0.03 ng (#10), 9 ng

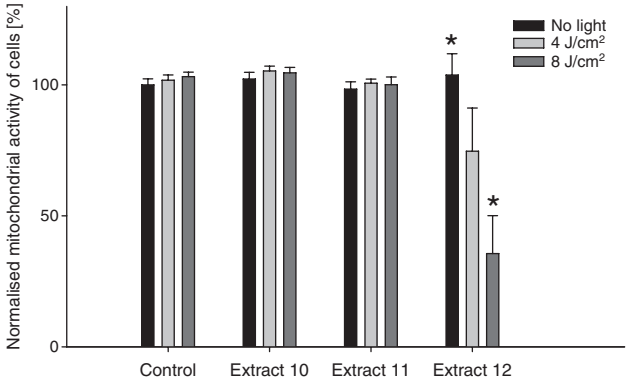

Figure 3. Phototoxicity of PAHs. When comparing to dark control (black bar: no light, no extract), mitochondrial activity of cells did not change after incubation with diluted ink extracts and UVA irradiation for inks 10 and 11. The mitochondrial activity of cells decreased with increasing light dose for black ink sample #12 (black: control, red: 4 J/cm^2, green: 8 J/cm^2). The decrease in mitochondrial activity (asterisk) was statistically significant ($P \leq 0.05$) for 8 J/cm^2 when compared to dark control (black bar: without light, with extract). This specific ink sample contained a mixture of PAHs that effectively absorb UVA radiation and exhibit high values of singlet oxygen quantum yield Φ_Δ at the same time.

(#12) or 20 ng (#11). The cells with ink extracts were subsequently irradiated with broadband UVA (330–400 nm) at a radiant exposure of 4 or 8 J/cm^2 (Fig. 3). The dose is below the MED for UVA irradiation for Caucasians subjects [about 21 J/cm^2, (43)]. The dose is well below the value that is caused by natural sunlight. The total annual UV dose (including the UVB) of the human skin is ranging from 27 J/cm^2 (United Kingdom) to 156 J/cm^2 (Australia) (44).

Although the total PAHs content for extract of ink #11 was maximal, the mitochondrial activity of cells was comparable to the dark control, the same for extract # 10. However, the medium PAHs content in extract #12 caused a significant decrease in mitochondrial activity with increasing radiant exposure when compared to the control (cells without UVA or extract). This is attributed to the composition of extract #12 that mostly contained UVA absorbing PAHs like anthracene, fluoranthene, benzo[b]fluoranthene and benzo[ghi]perylene with high Φ_Δ values in the range 0.45–0.81 (Table 2). Neither phenol nor any other solvent used in the setting did affect mitochondrial activity of cells, with or without UVA exposure.

The high quantum yield of singlet oxygen generation indicates that phototoxic reactions occurred in cells might be mediated by singlet oxygen. A black ink tattoo is repeatedly exposed to the ultraviolet spectrum of solar radiation, which repeatedly cause singlet oxygen and may pose an additional risk to the skin. Singlet oxygen is known to damage cellular structure by oxidation of lipids and proteins. Numerous papers on singlet oxygen generation by UVA irradiation of skin have provided evidence that singlet oxygen is responsible for many deleterious effects in tissue (45–48).

Acknowledgments

The work is supported by a grant of the 'Deutsche Forschungsgemeinschaft' (DFG), grant number BA1741/3-2. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. All authors declare no conflict of interest.

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