Journal of Hazardous Materials 296 (2015) 192–200

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Bioanalytical evidence that chemicals in tattoo ink can induce adaptive stress responses

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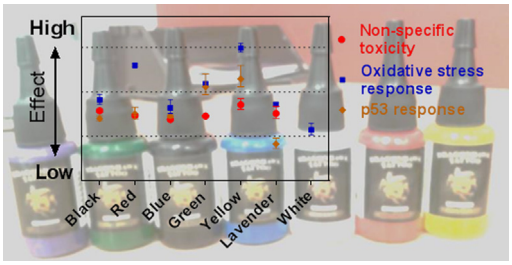
HIGHLIGHTS

- *In vitro* bioassays were applied to assess the toxicological hazard of tattoo inks.
- Studied assays were indicative of cytotoxicity, genotoxicity and oxidative stress.
- Greatest response for red and yellow tattoo inks in oxidative stress response assay.
- Detected PAHs in black tattoo ink could explain <0.1% of oxidative stress response.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 3 July 2014
Received in revised form 15 April 2015
Accepted 16 April 2015
Available online 20 April 2015

Keywords:
Tattoo ink
Bioanalytical tools
Polycyclic aromatic hydrocarbon
Genotoxicity
Oxidative stress
Cytotoxicity

ABSTRACT

Tattooing is becoming increasingly popular, particularly amongst young people. However, tattoo inks contain a complex mixture of chemical impurities that may pose a long-term risk for human health. As a first step towards the risk assessment of these complex mixtures we propose to assess the toxicological hazard potential of tattoo ink chemicals with cell-based bioassays. Targeted modes of toxic action and cellular endpoints included cytotoxicity, genotoxicity and adaptive stress response pathways. The studied tattoo inks, which were extracted with hexane as a proxy for the bioavailable fraction, caused effects in all bioassays, with the red and yellow tattoo inks having the greatest response, particularly inducing genotoxicity and oxidative stress response endpoints. Chemical analysis revealed the presence of polycyclic aromatic hydrocarbons in the tested black tattoo ink at concentrations twice the recommended level. The detected polycyclic aromatic hydrocarbons only explained 0.06% of the oxidative stress response of the black tattoo ink, thus the majority of the effect was caused by unidentified components. The study indicates that currently available tattoo inks contain components that induce adaptive stress response pathways, but to evaluate the risk to human health further work is required to understand the toxicokinetics of tattoo ink chemicals in the body.

1. Introduction

Tattooing is becoming increasingly popular in many countries. For example, the number of adult Australians with one or more tattoos increased from 10.1% to 14.5% over a seven-year period [1,2]. Further, young people tend to have a higher incidence of tattooing, with one recent study of US university students finding that 29.6% of participants were tattooed [3]. In addition to concerns regarding hygiene and disease transmission, there is growing interest in the

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http://dx.doi.org/10.1016/j.jhazmat.2015.04.051
0304-3894/

chemical composition of tattoo ink. Tattoo inks are typically composed of negligibly soluble or insoluble pigments, dispersants in which the pigments are suspended and other additives for preservation or to alter the viscosity of the ink [4]. While coloured tattoo inks traditionally contained metals, modern coloured inks contain organic pigments, such as azo dyes for red and yellows inks or phthalocyanines for blue and green inks [5]. Tattoo inks are typically manufactured in industrial processes, for applications including paints and printing, and can contain up to 10% impurities [6]. Black ink often contains carbon black, which is formed from the incomplete combustion of hydrocarbons, and unsurprisingly, recent studies have found polycyclic aromatic hydrocarbons (PAHs) in black tattoo ink [7,8]. Within the literature a number of adverse health effects from tattoos ranging from skin irritation to tumour formation have been reported [9], but the association between tattooing and cancer remains coincidental to date [10].

Given the complex mixture of chemicals that is likely to be present in tattoo ink, targeted chemical analysis alone is not sufficient to assess the potential health hazards associated with tattoo ink chemicals. *In vitro* bioanalytical tools can be applied complementary to chemical analysis. Bioanalytical tools or bioassays have the advantage that they can detect the mixture effects of all chemicals that act by the same mode of action and they are risk-scaled, so a more potent chemical will have a greater contribution to the effect than the same concentration of a less potent chemical [11]. These tools have been applied extensively for water quality assessment in recent years [12], but they can also be applied to other matrices of interest, such as extracts from animal tissue samples [13] or sediments [14]. They can also be used for the assessment of chemical products including tattoo ink. For example, Falconi et al. [15] found a significant reduction in cell viability of human fibroblast cells after exposure to red tattoo ink, but not black ink, while Regensburger et al. [8] assessed mitochondrial activity of human dermal keratinocytes in the presence of black tattoo ink extracts and UV light and found reduced activity for some black inks, but not others.

Knowledge of the mode of toxic action of tattoo ink chemicals is essential for understanding the potential impacts on human health. Modes of toxic action include non-specific (*e.g.* chemical partitioning to cell membrane), specific (*e.g.* chemical binding to an enzyme) and reactive (*e.g.* chemical reaction with biological molecules) and these can be targeted by applying different bioassays [11]. Also of relevance for human health are assays that focus on adaptive stress response pathways. These pathways, which are well conserved in all metazoan cells, are activated to restore the cell to homeostasis after damage to the cell structure [16].

The aim of this study was to evaluate the toxicity potential of chemicals present in commercially available tattoo inks with cell-based bioassays indicative of cytotoxicity and adaptive stress response pathways. The studied assays included two measures of cytotoxicity, the bioluminescence inhibition test with bacteria *Vibrio fischeri* and the resazurin cell viability test with human colon carcinoma cells (HCT-116). While not directly relevant to human health, the bioluminescence inhibition test was selected as it is highly sensitive to organic compounds [17] and typically more sensitive than cytotoxicity assays with mammalian cells. In addition the cellular response to genotoxicity, p53 response and oxidative stress response were assessed. The bacterial umuC assay quantifies the SOS response after DNA damage [18] and the mammalian p53 assay is a measure of adaptive stress response regulation that responses to DNA damage, either inducing DNA repair and/or apoptosis [19]. The activation of p53 in mammalian cells has also been used as an indicator for genotoxic carcinogens [20]. The oxidative stress response, which is induced by reactive oxygen species or electrophilic chemicals [21] and mediated by Nrf2 and the antioxidant response element, was quantified with the AREc32 assay [22]. Such a comprehensive bioanalytical assessment of tattoo inks has not been attempted previously. The SOS response and p53 protein induction are relevant for the tattoo ink cancer concerns as DNA damage can result in mutations, which can lead to cancer [11]. Further, Hutton Carlsen and Serup [23] hypothesised that skin complaints associated with tattooing may be related to the formation of reactive oxygen species (ROS), which is captured with the oxidative stress response endpoint [16]. Various coloured tattoo inks were tested in all bioassays. The bioanalytical assessment of black tattoo ink was complemented by chemical analysis to assess the contribution of known chemicals to the biological effect.

2. Materials and methods

2.1. Chemicals

All chemicals were of analytical grade. Naphthalene, acenaphthylene, fluoranthene, phenanthrene and pyrene were purchased from Sigma–Aldrich (Castle Hill, Australia). The liquid tattoo inks were purchased online from eBay and included Dragonhawk (tribal black, crimson red, country blue, true green, golden yellow, lavender, brite white) and Intenze (banana yellow) tattoo inks.

2.2. Sample treatment

1 g of liquid ink was extracted in 3 mL of hexane by sonicating for 60 min, then centrifuging for 10 min at 2500 × *g*. The supernatant was removed and the extraction process was repeated another two times. The combined solvent extract was blown down to 0.5 mL, enriching the sample 18 times and giving a final concentration of 2000 $g_{ink}/L_{extract}$.

2.3. Chemical analysis

The black ink hexane extract was blown to dryness and resuspended in 0.5 mL ethyl acetate. The extract was analysed for PAHs using GC–MS by a National Association of Testing Authorities (NATA) accredited analytical laboratory (Queensland Health Forensic and Scientific Service (QHFSS), Coopers Plains, QLD, Australia). The analysed PAHs included naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benz[*a*]anthracene, chrysene, benzo(*b* + *k*) fluoranthene, perylene, benzo(*a*) pyrene, benzo(*e*) pyrene, indeno(1,2,3-cd) pyrene, dibenz(a,h) anthracene, benzo(ghi) perylene, biphenyl, 2-ethyl naphthalene, 2,6-dimethyl naphthalene, 1,7-dimethyl naphthalene, 2,2-dimethyl biphenyl, 1,4-dimethyl naphthalene, 2-methoxy naphthalene, 1,2-dimethyl naphthalene, 1,8-dimethyl naphthalene, 3,3-dimethyl biphenyl, 4,4-dimethyl biphenyl, 1-methyl fluorene, 2-methyl anthracene and 9-methyl anthracene. Details of the analysis method can be found in Bi et al. [24]. The concentration was expressed as micrograms per gram of ink ($\mu g/g_{ink}$).

2.4. Bioanalytical tools

Prior to bioanalysis the hexane extracts were blown to dryness and resolubilized in the appropriate assay medium to prevent solvent effects in the assay, while the PAH stocks were prepared in methanol with low volumes added to the assay (not exceeding 1.04% methanol in final assay volume for the AREc32 and p53 assays and not exceeding 2.8% methanol in final assay volume for the bioluminescence inhibition assay). All methanol extracts were blown to dryness in the umuC assay due to the larger volume requirements. Solvent controls were included in all assays to ensure no effect from the methanol.

The bacterial bioluminescence inhibition assay is based on ISO11348-3 [25], but was performed in a 96-well format [26]. Lab-grown *V. fischeri* were prepared according to Tang et al. [17]. Briefly,