Original Article
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Determination of the phototoxicity potential of commercially available tattoo inks using the 3T3 neutral red uptake (NRU) phototoxicity test

Piyasada satılan dövme boyalarının 3T3 nötr kırmızı alım (NRU) fototoksisite testi ile fototoksisite potansiyelinin belirlenmesi

Short title: In vitro phototoxicity of tattoo inks

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ABSTRACT
INTRODUCTION: Tattooing is an ancient practice and its popularity has been increasing in the recent years. After tattooing, complications may occur related to composition of tattoo inks. In this study, the phototoxicity potential of the blue, red and black colors of the most commonly used three different commercially-available tattoo ink brands have been examined by performing in vitro 3T3 Neutral Red Uptake (NRU) phototoxicity test.
METHODS: In the study, the phototoxicity of serial diluted concentrations of tattoo inks were evaluated with in vitro 3T3 NRU phototoxicity test method according to OECD Guide 432. The data obtained from the NRU test result were uploaded to Phototox software (Version 2.0) and the phototoxicity potentials of tattoo inks were determined via calculation of the mean photo effect (MPE) and photo irritation factor (PIF) values.
RESULTS: The red, black and blue colors of three different commercially available tattoo inks did not cause a cytotoxic activity on BALB/c 3T3 cells with 3T3 NRU test. The IC50 values could not be determined +UV and –UV conditions. PIF values could not be calculated and MPE values were < 0.1, which predicts the absence of phototoxic effect for all of the tested tattoo inks.
DISCUSSION AND CONCLUSION: All tested inks were evaluated as non-phototoxic according to the results of MPE values calculated with Phototox software. However, test results should be verified by other phototoxicity test methods in order to obtain a comprehensive evaluation of phototoxic complications of different tattoo inks.
Keywords: Phototoxicity, in vitro phototoxicity, 3T3 NRU phototoxicity test, tattoo ink, Balb/c 3T3 cells

ÖZ
GİRİŞ ve AMAÇ: Dövme yapmak eski zamanlardan beri süregelen bir uygulamadır ve popülaritesi son yıllarda artmaktadır. Dövme yapılmasından sonra dövme boyalarının bileşimine bağlı olarak çeşitli komplikasyonlar oluşabilir. Bu çalışmada, piyasada en çok kullanılan üç farklı dövme boyası markasının mavi, kırmızı ve siyah renklerinin fototoksisite potansiyeli in vitro 3T3 Nötral Kırmızı Alım (NRU) fototoksisite testi yapılarak incelenmiştir.
YÖNTEM ve GEREÇLER: Çalışmada, dövme boyalarının seri olarak seyreltilmiş konsantrasyonlarının fototoksisitesi, OECD Kılavuz 432'ye göre in vitro 3T3 NRU fototoksisite test yöntemi ile değerlendirilmiştir. NRU test sonucundan elde edilen veriler Phototox yazılımıyla (Sürüm 2.0) değerlendirilmiştir. Dövme boyalarının fototoksisite potansiyelleri Medyan Foto Etki (MPE) ve Foto İrritan Faktör (PIF) değerlerinin hesaplanmasıyla belirlenmiştir.
BULGULAR: Ticari olarak temin edilebilen üç farklı dövme mürekkebinin kırmızı, siyah ve mavi renkleri, 3T3 NRU testi ile BALB/c 3T3 hücrelerinde sitotoksik aktiviteye neden olmamıştır. +UV ve –UV koşullarnda IC50 değerleri belirlenememiştir. PIF değerleri hesaplanamamıştır ve i MPE değerleri <0.1 olarak belirlenmiştir. Bu sonuçlar test edilen tüm dövme boyalarının fototoksik etkilerinin olmadığını göstermektedir.
TARTIŞMA ve SONUÇ: Phototox yazılımı ile hesaplanan MPE değerlerinin sonuçlarına göre test edilen tüm mürekkeplerin fototoksik olmadığı sonucuna ulaşılmıştır. Bununla birlikte, farklı dövme boyalarının fototoksik komplikasyonlarının kapsamlı bir değerlendirmesini yapabilmek için test sonuçlarının diğer fototoksisite test yöntemleriyle doğrulanması gerekmektedir.
Anahtar Kelimeler: Fototoksisite, in vitro fototoksisite, 3T3 NRU fototoksisite testi, dövme boyası, Balb/c 3T3 hücreleri

INTRODUCTION
Tattooing has existed worldwide for many centuries and increases its popularity, especially among young people today. Tattooing is applied by injecting tattoo ink into the dermis layer that is 1.5-2 mm below the skin with the help of a needle.[1] Despite the increasing popularity of tattooing, the toxicity profile of tattoo inks and the potential risks of these inks remain unknown. Tattoo inks have different formulations. Generally, tattoo inks are prepared by suspending pigments in a solvent. Apart from pigments and solvents, tattoo inks contain additives such as binding agents, preservatives, thickeners, and antioxidants.[2] One of the most significant problems in the toxicological evaluation of tattoo inks is the lack of information about the ink compositions. In addition, there is no regulation on their use in tattoo inks for organic/inorganic pigments, carbon black, and many different chemicals used as coloring agents in tattoo inks.[3] Dermatological severe complications may occur during tattooing and there is a serious increase in the number of patients who apply to the dermatology doctor due to skin conditions caused by a tattoo. Allergic skin reactions caused by tattoos are the most common skin problem. Especially red tattoo ink can cause allergic skin reactions.[4]
Phototoxicity describes the toxic response of the skin after exposure to light due to chemicals that are present on the skin following cutaneous or systemic application. To produce a phototoxic effect, a chromophore or a photosensitizing molecule must absorb photons. The absorbed photon causes reactive oxygen species (ROS) formation, and as a result of increased ROS level, symptoms such as edema, burning, and pain occur in the skin.[5,6] The European Union banned animal testing in cosmetic products in 2009 and their import and sale in 2013 to reduce the number of laboratory animals used and protect animals from unnecessary pain and injuries.[7,8] Subsequently, an increasing number of countries worldwide including Turkey have adopted the ban on animal testing for cosmetics.[9] Therefore, the need for validated relevant alternative *in vitro* test methods has increased in order to make the toxicologic

evaluation of cosmetic products.[10] *In vivo* experiments to determine acute phototoxicity are not allowed in Europe since 2000. Instead, the validated and regulatory accepted 3T3 Neutral Red Uptake Phototoxicity Test is primarily used as an *in vitro* alternative method.[11] The 3T3 Neutral Red Uptake Phototoxicity Test allows users to test many factors such as different test material concentrations, exposure times, and UV irradiation dose. Also, it has been determined to be reliable as results obtained with *in vivo* acute phototoxicity tests in animals and humans.[12,13]

Therefore, the aim of the study was to evaluate the phototoxic potential of the black, blue, and red colors of three different commercially available tattoo ink brands that are widely used by *in vitro* 3T3 Neutral Red Uptake Phototoxicity Test (NRU) according to the Organisation for Economic Cooperation and Development (OECD) 432 guideline.

MATERIALS AND METHODS

Cell culture

The BALB/c 3T3 fibroblast cells were purchased from ATCC. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1x antibiotic-antimycotic solution (all from Gibco) in a humidified atmosphere at 37 °C.

Test compounds

The Black, red and blue colors of three commercially available tattoo inks were used for the tests. Black-Triple Black, Red-Lipstick Red, Blue-Muter Earth (Eternal Tattoo Supply, Brighton, MI, USA), Black-True Black, Red-Bright Red, Blue-Mario's Light Blue (Intenze Products Inc. NJ, USA), Black, Red, Blue (Tang Dragon Tattoo, China) were used. Chlorpromazine was used as the positive control (Eczacibasi, Turkey).

The absorption spectrum of the tattoo inks

Before starting the phototoxicity test, the absorption spectra of the tattoo inks were measured in the 250-700 nm range (Epoc, Biotek). For this purpose, tattoo inks were dissolved in DMEM medium without phenol red at 1% final concentration, and this diluent was used as blank.

Photosensitivity of the BALB/c 3T3 fibroblast cells

To determine photosensitivity of the cells, the BALB/c 3T3 fibroblast cells were seeded on clear 96-well plates at a seeding density of 1 x 10^4 in DMEM without phenol red containing 10% FBS and 1x antibiotic antimycotic solution. The day after, cells were irradiated with 0-2.5-5-10-15 Joule/cm^2 of UVA radiation. The UVA sensitivity of the cells was evaluated with neutral red uptake (NRU) cell viability assay after 24 hours (h), as mentioned in detail below.

Neutral Red Uptake (NRU) Phototoxicity assay

The BALB/c 3T3 fibroblast NRU assay was carried out as described by the OECD 432 guideline with minor modifications.[13] Extra washing steps due to the high coloring of tattoo inks were added to the assay. Briefly, the BALB/c 3T3 cells were seeded on clear 96-well plates at a seeding density of 1 x 10^4 in DMEM without phenol red with 10% FBS and 1 x antibiotic antimycotic solution (Assay medium). After culturing the cells in the plate for 24 h, wells were washed with 1 x PBS. Briefly, eight logarithmic dilution series of tattoo inks starting from 200 µg/mL to 1 µg/mL with assay medium were prepared. The prepared concentrations of tattoo inks were added into wells and allowed to incubate for 1 h. 2 different sets of plates (*–UV* and *+UV*) were prepared for the assay. Then, *+UV* plates were irradiated with 5 Joule/cm^2 of UVA radiation from a Philips PL-L UVA lamp in a home-designed and constructed wooden box. UVA radiation calculation was made with a UVA light meter (Lutron UVA-365SD, Taiwan). While *+UV* plates were irradiating, the *–UV* plates were kept in the dark. After irradiation, the solutions were discarded, and wells were washed with 1xPBS. Then the cells were allowed to incubate overnight with the assay medium. To determine the phototoxic effects of the tattoo inks, the medium was replaced with 50 µg/mL

neutral red in assay medium and incubated for 3 h in a humidified atmosphere at 37 °C. Then, the medium was discarded, and the uptaken neutral red was dissolved with a mixture of acetic acid, water and ethanol (1:49:50). The absorbance was read at 540 nm with a plate reader. Then, the phototoxicity of the tattoo inks was evaluated with Phototox Version 2.0 software (ZEBET Germany) by calculating the photo irritation factor (PIF) and the mean photo effect (MPE). Since the tattoo ink dilutions were prepared with DMEM without phenol red with 10% FBS and 1x antibiotic antimycotic solution (Assay medium) and there was not any different solvent effect on the cells, only assay medium containing wells were used as the negative control. Therefore, negative control results used in the negative control sections in the Phototox program and IC50, PIF, MPE values were not calculated for negative controls. According to the OECD guideline, MPE < 0.1 predicts the absence of a phototoxic effect. MPE >0.1 and 0.15 predict a probable phototoxic effect and MPE > 0.15 predicts a phototoxic effect.[13]

Statistical analysis

Phototox Version 2.0 software (ZEBET Germany) was used for concentration-response analysis. Results are presented as mean standard deviation (±S.D.) of three independent experiments run in triplicate.

RESULTS

According to OECD Guideline 432, a substance must show absorption in the UV/Visible area in order to be photoreactive.[13] For this reason, absorption spectra of each ink were taken before starting the 3T3 NRU phototoxicity test. Absorption peaks of inks were found to be between 410-420 nm in black inks, 610-640 nm in blue inks and 560-570 nm in red inks. Photosensitivity study results showed that increasing doses of UVA caused a phototoxic effect on the BALB/c 3T3 fibroblast cells. The viability of the 5 Joule/cm^2 of UVA irradiated cells was 95.7% ±2.36 compared with non-irradiated cells (0 Joule/cm^2) and > 80% viability of the cells meets the quality criteria of OECD Test Guideline No. 432, (Figure 1).[13]In order to check the accuracy of the 3T3 NRU phototoxicity test under our laboratory conditions, an experiment was performed with chlorpromazine, which was selected as a positive control, in line with OECD guidelines. According to the OECD Guideline 432, chlorpromazine should have a PIF value greater than 14.4, and the MPE value should be in the range of 0.33-0.63. The IC50 values for *+UV* and *–UV* should be 0.1-2.0 µg/mL and 7.0-90.0 µg/mL, respectively.[13] The results of the 3T3 NRU phototoxicity test with positive control showed that the PIF value for chlorpromazine is 27.7 ±4.2, the MPE value is 0.50 ±0.11, the IC50 values are 1.7 ±0.28 µg/mL at (*+UV*) and 47 ±6.9 µg/mL at (*–UV*). These values were found to comply with the OECD Guideline 432 limits.

Tattoo inks phototoxicity evaluation with 3T3 NRU phototoxicity test is shown in Table 1. Based on our results, the red, black and blue colors of three different commercially available tattoo inks did not exhibit phototoxic potential with the 3T3 NRU phototoxicity test. The IC50 values could not be determined *+UV* and *–UV* conditions since tattoo inks did not show cytotoxic activity on BALB/c 3T3 cells. Consequently, PIF values could not be calculated, and MPE values were < 0.1, which predicts the absence of phototoxic effect, (Table 1).

DISCUSSION

While tattooing has been practiced throughout the world for many centuries, the popularity of tattooing has increased significantly in recent years due to decorative reasons. Inflammatory, infectious and neoplastic complications may be seen after tattooing and a part of the beforementioned complications is related to allergy and hypersensitivity to tattoo inks.[14] In addition, sensitivity to the sun around the tattooed area is common and 20% of individuals suffer from tattoo-associated complications.[6] Despite the increasing number of tattooed individuals, there are not a sufficient number of toxicological and pharmacokinetic evaluations of the intradermal use of inks and colorants used for tattooing.[3]

Tattoo inks mainly contain pigments, dyes, water, solvent additives such as glycerin, alcohol, and ethylene glycol, preservatives, stabilizers, and pH regulators.[15] The coloring agents that are used in the inks can vary depending on the brand and the color.[2] The tattoo ink manufacturers are not obliged to disclose the chemical composition and exact ingredients in their inks which cause potential uncertainty in the evaluation of the toxic effects of the pigments, solvents, and binders that are used in the tattoo inks.[1]

The black tattoo inks mainly contain carbon. Besides, black tattoo inks may contain mutagenic and carcinogenic compounds such as carbon black, polyaromatic hydrocarbons (PAHs), and phenols. PAHs under UV irradiation can generate singlet oxygen which can result in tattoo-associated complications.[15] The blue inks may contain elements such as cobalt, aluminum, copper, and it is reported that cobalt-containing ink compositions cause more skin reactions and irritations than copper-containing ink compositions. Red tattoo inks may contain azo pigments and elements such as cadmium and iron for coloring.[16] Tattoo-related skin allergies are especially observed when red tattoo ink is used. Red tattoo-related skin allergies are supposed to be related to azo compounds.[17] The azo compounds that are used as pigments, under high energetic radiation and heat can cause the production of the aromatic amines and aromatic amines considered allergic sensitizers.[15] Also, often tattoo ink contains azo dyes with unknown compositions.[11,18] The possible local metabolism of these unknown azo dyes into porphyrins may cause photo-sensitizing effects on the skin.[19]

Phototoxicity describes the tissue reactions caused by light and it is the toxic response of the skin that develops due to the exposure to light of a substance that is applied to the organism systemically or subcutaneously.[13] The phototoxic reactions are characterized by skin irritation or exaggerated sunburn-like symptoms such as erythema, tenderness, pruritus and edema in patients.[20]

The NRU test is based on the retention of the NRU dye in the viable cell's lysosomes, and the amount of the dye in the lysosomes is proportional to the cell viability.[21] However the alterations in the lysosomal membranes may affect the NRU test results and when the NRU test is used to evaluate the toxicity of substances that specifically target lysosomes, results may indicate artificially high cytotoxicity. This can be considered as the main limitation of the NRU test for cytotoxicity studies.[22,23] The 3T3 NRU phototoxicity test is a validated and regulatory accepted test for the prediction of phototoxicity.[13] Therefore, the aim of the study was to evaluate the phototoxic potential of the black, blue and red colors of the most widely used three different commercially available tattoo ink brands by *in vitro* NRU phototoxicity test according to OECD 432 guideline. It has been reported that red, blue, and black tattoos cause more sun-related complaints than other colors.[24] A clear relationship between having tattoos and skin cancer development has not been established today. There are case reports of the development of cancer types such as melanoma, basal cell carcinomas, squamous cell carcinomas, and keratoacanthomas in persons with tattoos. However, these cancer developments are most likely not only a result of having a tattoo but multifactorial.[25,26]

Our results showed that the black, blue and red colors of three different commercially available brands were found to be not phototoxic by *in vitro* NRU phototoxicity test while the chlorpromazine (positive control) result was within the range recommended by OECD 432 guideline.

A recent study was conducted to evaluate the phototoxic effects of different tattoo pigments. In this study, cadmium sulfide, carbazole, cadmium selenide, mercury (II) sulfide, chromium oxide, and cobalt aluminate were examined by using the 3T3 NRU phototoxicity test and 3D human reconstructed skin model. The results of this study showed that only carbazole and cadmium sulfide exhibited phototoxicity potential with the 3T3 NRU phototoxicity test, and this result could only be confirmed for carbazole with the 3D human skin model.[27] Similar to

our study, the findings of this study do not address the phototoxicity potential of red (cadmium selenide and mercury (II) sulfide) and blue (cobalt aluminate) pigments. Regensburger et al.[28] studied the 20 well-known polycyclic aromatic hydrocarbons (PAHs) content of 19 commercially available black tattoo inks. Many of the PAHs have been shown to be carcinogenic and have mutagenic activity. Furthermore, under UV irradiation they can generate singlet oxygen which can contribute the tattoo associated complications.[18,29,30] In the study, they evaluated the phototoxicity of PAH extracts from black tattoo inks through mitochondrial activity in human keratinocytes and found that some of the extracts caused singlet oxygen generation with UVA irradiation which might be indicative of phototoxic reactions.[28]

Another study is conducted to make *in vitro* and *in vivo* toxicological evaluations of the blue, green, red and black tattoo inks. According to the study, the red and green tattoo inks showed higher *in vitro* and *in vivo* toxicity due to containing azo compounds while black tattoo ink was found to be the safest.[31] Wamer and Jun-Jie[32] evaluated cytotoxic and photocytotoxic activity of eighteen TiO_2 containing permanent makeup inks in human dermal fibroblasts as inhibition of colony formation. They did not determine cytotoxicity among inks but did observe eight inks were phototoxic under UVA irradiation. It is reported that the higher the PIF values obtained from the 3T3 NRU phototoxicity test results in the higher the probability of finding phototoxic *in vivo*. Thus, it is suggested that guideline thresholds should be reconsidered for the better translation of the results to *in vivo*. Additionally, the lack of a barrier system is a known limitation of the 3T3 NRU phototoxicity test for testing topical agents.[33]In conclusion, our study results do not indicate the phototoxic potential of examined black, blue, and red colors of three different commercially available brands. However, the 3T3 NRU phototoxicity test results should be verified by other phototoxicity test methods in order to evaluate the phototoxicity of tattoo inks correctly. Also, it should be considered that the data obtained as a result of the phototoxicity test do not provide information about other toxicological properties of tattoo inks. However, evidence from some studies suggests that tattoo ink compositions are variable, and some of these ink compositions can cause cytotoxic and phototoxic reactions. Thus, it is crucial to have better regulations on tattoo ink compositions to minimize their risk of complications.

Conflict of Interest

The authors have no conflict of interest to declare.

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Tables and Figures:

Table 1: Phototoxic evaluation of the red, black and blue colors of three different commercially available tattoo inks with Phototox Version 2.0 software (ZEBET Germany).

	IC50				
Substance	***-UV***	***+UV***	**PIF ±SD**	**MPE ±SD**	**Evaluation**
Clorpromazine (positive control)	47 ±6.9 µg/mL	1.7 ±0.28 µg/mL	27.7 ±4.2	0.50 ±0.11	phototoxic
Eternal - Black	–	–	1	-0.361±0.07	non-phototoxic
Eternal - Red	–	–	1	-0.351±0.02	non-phototoxic
Eternal - Blue	–	–	1	-0.229±0.01	non-phototoxic
Intenze - Black	–	–	1	-0.559±0.02	non-phototoxic
Intenze - Red	–	–	1	-0.159±0.01	non-phototoxic
Intenze - Blue	–	–	1	-0.126±0.01	non-phototoxic
Tang Dragon - Black	–	–	1	-1.330±0.01	non-phototoxic
Tang Dragon - Red	–	–	1	-1.155±0.04	non-phototoxic
Tang Dragon - Blue	–	–	1	-0.535±0.03	non-phototoxic

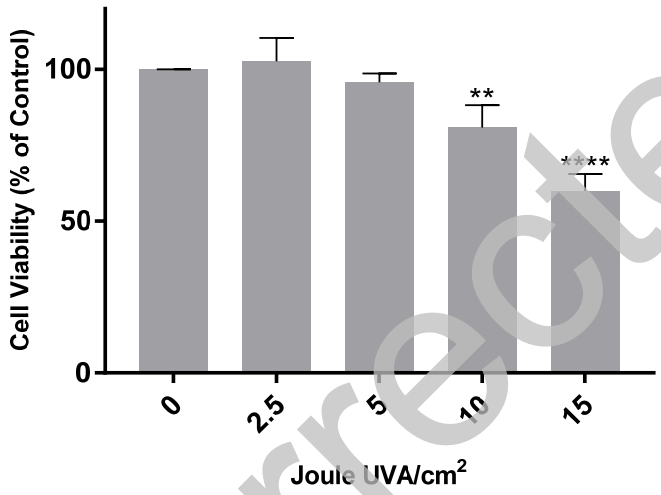

Figure 1: Photosensitivity of the BALB/c 3T3 fibroblast cells. ***p*<0,01, *****p*<000.1