EVALUATING THE TOXICITY OF REACTIVE DYES AND DYED FABRICS WITH THE HaCaT CYTOTOXICITY TEST

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Abstract

We investigated the cytotoxicity of reactive dyes and dyed fabrics using human keratinocyte HaCaT cells in vitro. The HaCaT cells were exposed to three monochlortriazinyl dyes: yellow, red and blue with different concentrations. The HaCaT cells were also exposed to water extracts of dyed fabrics. After 72 hours exposure, the protein contents of the samples compared to the protein contents of non-exposed cells were measured. The level of protein content indicates the viability of the cells. The mean inhibitory concentration values (IC50) showed the dye concentration when the protein content of the sample was 50% of the protein content of the non-exposed cells. The mean inhibitory concentration values (IC20) when the protein content of the samples was 80% were also measured. The IC20 values show the limiting value of toxicity. The IC50 values show whether samples are clearly toxic. The IC50 value for the yellow dye was 237µg/ml and the IC20 value was 78µg/ml. The IC50 for the red dye was 155µg/ml: the red dye caused adverse effects under the lowest dye concentration (28µg/ml). The IC50 value for the blue dye was 278µg/ml and the IC20 value was 112µg/ml. Cotton fabrics dyed using these same three reactive dyes were extracted with water and the extracts were analysed using the HaCaT cell line. The viability of the cells was good, the protein content of the samples being over 80% compared to the non-exposed cells. The HaCaT cell test indicated the toxicity of pure dyes; the dyed fabrics had no adverse effect. The human keratinocyte HaCaT cells seem to be a useful tool for the study of the purity/toxicity of dyes and other substances applied to textiles.

Key words:

textile, fabric, extract, reactive dye, monochlortriazinyl, toxicity, human keratinocytes

Introduction

Information is available concerning the toxicity of textile chemicals, but there is limited data about the overall toxicity of textile chemicals and fabrics containing them. Although a chemical itself may be toxic, its presence in the finished material may not be harmful. *In vitro* tests can be useful for studying the overall toxicity of textile chemicals on their own or included in fabrics.

The manufacturing of textiles commonly utilises reactive dyes for dyeing cotton and other cellulose-based fibres. Reactive dyes have complicated chemical structures, including organic ring forms with colour-giving double bonds and form covalent bonds between reactive groups on the cellulose fibres and vinyl sulphonyl groups and chloride atoms on the dye molecules. In this study, the reactive dyes used belong to the category of monochlorotriazinyl dyes and are also called azo dyes. The dyeing process using reactive dyes is not complicated and does not cause as many environmental problems as other dyes and dyeing processes which give equivalent wet-fastness and it is because of their excellent overall properties that reactive dyes are so widely used. However, since by definition reactive dyes are chemically reactive, they may be harmful, especially when in powder form [24].

Many studies have shown that reactive dyes can cause allergic dermatoses and respiratory diseases [7,10,15,18,27]. Contact dermatitis and asthma resulting from contact with reactive dyes were also studied by Thoren *et al.* [23]. Gonzales *et al.* stated that workers in the textile industry have a two-fold

increased risk of contracting bladder cancer compared to workers in other occupations, for instance aviation, agriculture and construction [9]. The increased risk of contracting colonic and rectal cancers was also noted. However, these cancers related mostly to the synthetic fibre industry [4].

Tests for mutagenicity [6,16,21], genotoxicity [6,16], carcinogenicity [4,9] and teratogenicity [2] have been used to detect any adverse effects of textile dyes. Waste waters from the dyeing process have been tested using luminescent bacteria [25]. Kopponen *et al* have used Hepa-1 mouse hepatoma cells to detect common adverse effects of textile substances [13]. Klemola et al. have studied the toxicity of three reactive dyes and dyed fabrics using boar spermatozoa (paper submitted to AUTEX Research Journal). These studies have shown varying degrees of adverse effects. However, more information about the overall toxicity of reactive dyes and dyed fabrics is needed.

The human keratinocyte HaCaT cells have been used widely in studying, for instance, skin irritation, skin cancer, genotoxicity, mutagenicity, and cytotoxicity caused by contact with nickel and chromium [26,17,28,14]. HaCaT cells have also used in many investigations for detecting adverse effects of UV-radiation [11,19]. In addition to these studies, skin cells have been useful especially for investigating cell signalling pathways [1,22].

The aim of this study was to use HaCaT cell lines for detecting any adverse effects of three monochlorotriazinyl dyes on their own and when present in dyed cotton fabrics. These three dyes are typical components used when mixing different colours. In addition, the effectiveness of using the HaCaT cell line to give information about the toxicity of reactive dyes and fabrics dyed with them was studied.

Materials and methods

In order to study reactive dyes and fabrics dyed with them, the HaCaT cell test was modified in accordance with the hepa-1 cell test which is used to assess potential toxicity (INVITTOX protocol number 112). All dyes were dissolved in the solution of the used medium. The three reactive dyes tested with the HaCaT cells were: Drimarene blue CL-2RL, Drimarene yellow CL-2R and Drimarene red CL-5B (Clariant Ltd. Switzerland). The sample concentrations of the dyes were: 0,075%, 0,038%, 0,019%, 0,015%, 0,010%, 0,005%, 0,0025% and 0,0012% in the test wells, corresponding to 750µg/ml, 380µg/ml, 190µg/ml, 150µg/ml, 100µg/ml, 50µg/ml, 25µg/ml and 12 µg/ml respectively of pure substance. These dye concentrations were expected to cover toxic and non-toxic values.

10g sample fabrics were each washed gently without soap. The amount of dye used was 3% on 10g fabric. The dye bath contained 400 ml water with 50g Na_2SO_4/l H_2O and 20g Na_2CO_3/l H_2O. Dyeing continued for one hour at 55ºC. Na_2CO_3 was added to the dye bath ten minutes after the beginning of the dyeing process to adjust the pH. After dyeing, the fabrics were spooled in cool and warm water baths and were kept in pure boiling water for 10 minutes. Dyed cotton fabrics were extracted with sterilized water (1g/20ml H_2O). The tubes were shaken at room temperature for two hours and incubated at 37ºC for 18 hours. The samples were shaken well before centrifugation for 5 min at 4500 rpm. The fabric extracts were sterile filtered before being exposed to HaCaT cells.

Cytotoxicity test with human keratinocyte cells

HaCaT cells (obtained from the Department of Anatomy, Kuopio University) were grown as a monolayer at 37ºC in 5% CO_2 atmosphere in DMEM medium supplemented by 1% glutamine, 10% foetal calf serum and 1% penicillin/streptomycin solution. The test was carried out in 96-well plastic microplates seeded with 200µl cell suspension (5 x 10^4 cells/ml). After growing for 24 hours, the culture is usually about 60% confluent. The cells were exposed to the dye samples and the sterile filtered (0,22µm pore size) fabric extracts. Non-exposed cells with medium were used as a negative control and all results were compared to them. 2,4-dinitrophenol was used as a positive control in three concentrations: 0,5 mg/ ml DMSO was used as one control and diluted to concentrations of 0,05 mg/ml and 0,005 mg/ml medium to obtain the other two controls. After 72 hours exposure, the cells were washed twice with PBS-buffer. Before addition of sodium phosphate buffer, the viability of the cells can be observed by light microscopy in order to obtain preliminary information. Subsequently 50µl of sodium phosphate buffer (0,05mM, pH 8.0) was added to each well before freezing the plates for at least 15 min at -70ºC. After breaking the cells in the freezer, the plates were thawed for 15 min and cell viability was detected by assaying the total protein content in the cultures. 150µl of sodium

phosphate buffer was added to the wells followed by 50µl of cold fluorescamine (1.08mM in acetonitrile). The plates were allowed to stand at room temperature for 15 min before being stirred in a microtitration plate shaker for one minute. The total protein content in each well was measured by the plate- reading fluorimeter at a wavelength of 405/460nm. The protein BSA standard curves were measured in each bioassay. All processing, except protein assays, was done under sterile conditions.

The inhibitory concentration value, IC50, is the concentration (µg/ml) of the dye when the sample contains 50% of the total protein compared to the non-exposed samples. The amount of protein responds to the viability of the cells. The IC50 values for the dye samples were calculated from the curves which describe the percentages of the protein content under different dye concentrations. When the protein content of the cells was 80%, the IC20 values were measured. The IC50 and IC20 values are the concentrations (µg/ml) on the x-axis when the corresponding value on y-axis is 50% or 80% respectively. The IC20 values show the lowest toxicity and the IC50 values show the toxic values. For the fabric extracts, the results were given as percentages of protein content compared to the total protein content of non-exposed cells. The limiting value of the toxicity of the fabric extracts was set as 80% protein in the sample compared to total protein of non-exposed cells. The protein content of 80% represents the lowest concentration/ the limit value of the substance that causes adverse effects.

Results

The mean IC50 value for the yellow dye was 237µg/ml and the mean IC20 value was 78µg/ml (Figure 1).

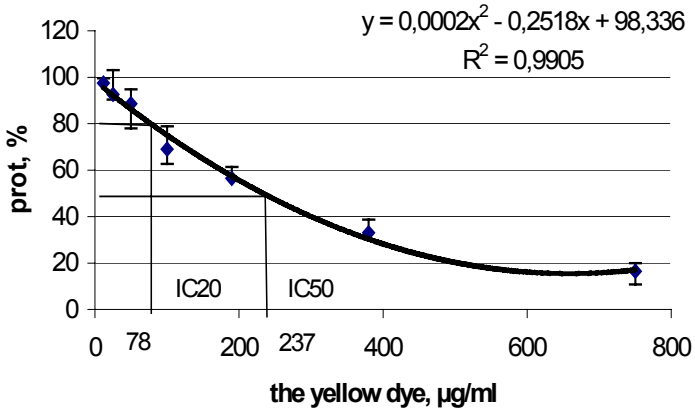

Figure 1. The IC20 and IC50 values for the yellow dye after 72 hours exposure (number of samples, n=6)

The mean IC50 value for the red dye was 155µg/ml and the mean IC20 value was 28µg/ml (Figure 2).

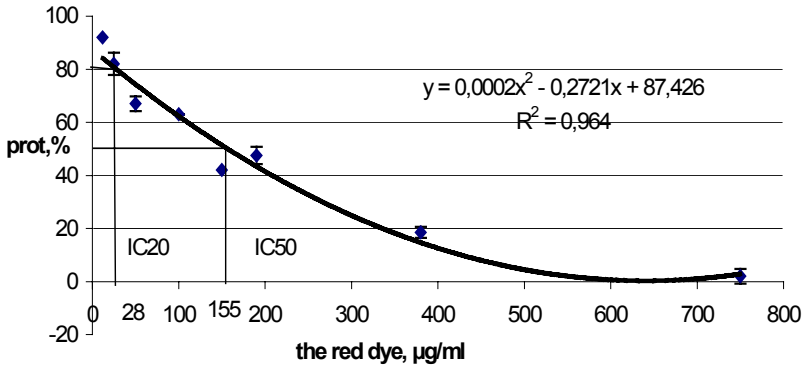

Figure 2. The IC20 and IC50 values for the red dye after 72 hours exposure (n= 5)

The mean IC50 value for the blue dye was 278µg/ml and the mean IC20 value was 112µg/ml (Figure 3).

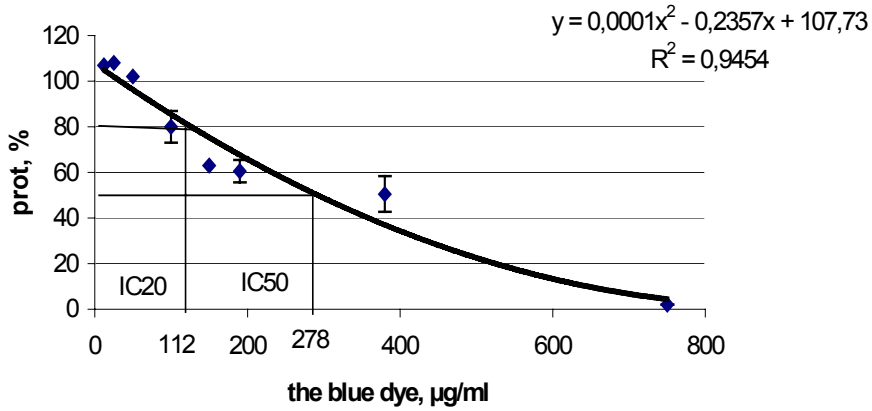

Figure 3. The IC20 and IC50 values for the blue dye after 72 hours exposure (n=4 and n=2)

The IC20 and the IC50 values were lowest for the red dye and highest for the blue dye (Figure 4).

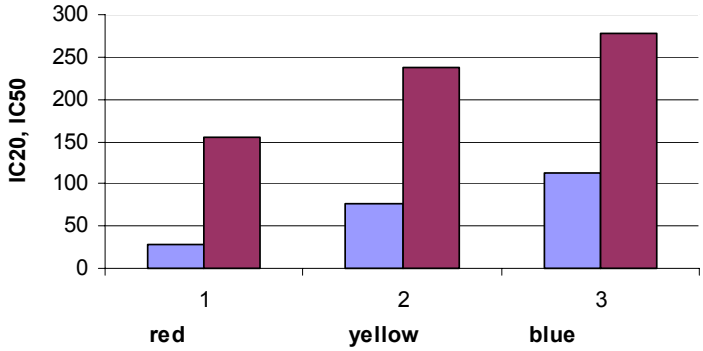

Figure 4. The IC20 and the IC50 values for three reactive dyes (red, yellow, blue)

All the fabric extracts had protein contents of more than 80% of the total protein contents of the unexposed samples, showing that none of the fabric extracts were toxic to skin cells (Figure 5).

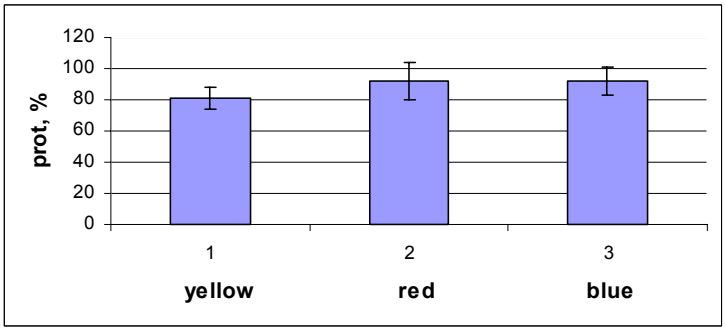

Figure 5. Values of protein content of the fabric extracts

The non-exposed cells had good viability. Using the 2,4-dinitrophenol samples as positive controls showed that, at low concentrations, the mean value of the protein content was 80% of the total protein content of the non-exposed samples. The lower control concentration of 0,05 mg/ml resulted in a 60% protein content, while the highest control concentration resulted in a 10% protein content.

For the red and blue dye samples, for all non-toxic concentrations, the coefficient of variation (C of V) values were all less than 10%. For toxic concentrations, the C of V values ranged between 7-15%. For

the yellow dye, the C of V values for the non- toxic samples were between 2-14%, and for the toxic samples were between 17-21%. For fabric extracts, the C of V values were between 9-13%.

Discussion

This study has shown that the reactive dyes were toxic in concentrations which were lower than those used in commercial dyeing processes. Reactive dyes react with cellulose under alkaline conditions [24]. The reactive dyes have studied pH values between 4,5-6,5; however, the dyes are very reactive in human keratinocyte cells. The textile dyes when used commercially contain many other chemicals in addition to the dye molecules. There are different salts, including calcium stearate, CMC and other chemicals. This means that the discussion of the results of this work relates to the toxicity of a mixture of chemicals and not just to the pure molecules: the effect of the pure dye alone cannot be evaluated and it is also not possible to know the concentration of the dye itself in the powder used as the commercial dyestuff. However, since these dyes are part of a mixture when they are used in industry, it is more useful to know the toxicity of this mixture than that of the pure dye when assessing the safety of commercial dye formulations.

The results show that the dyes are toxic, but the dyed fabrics are not. This can be explained by the fact that the pure reactive dye in powder form is very active, but after the dyeing process many of the reactive sites on the dye molecules have taken part in the formation of covalent bonds with fibre molecules [8]. These bonds are very stable and this can explain why the dyed fabric material is not toxic.

The red dye produced adverse effects under the lowest concentration of the dye: the IC20 value was 28µg/ml: the IC50 value (155µg/ml) was the lowest compared to the values for the other dyes. The blue dye and the yellow dye had IC20 values between 78-112µg/ml: IC50 values were between 237-278µg/ml, showing clear toxicity. When 80% of the cells are dead IC20 values indicate low toxicity and the results show that the red dye is very toxic even at low concentrations.

Although the dyes were toxic, extracts from the dyed fabrics were not toxic. Although the extracts contained dye (they were coloured), the results showed that they were not toxic and it can be assumed that the dye in solution had been hydrolyzed. The protein contents of all fabric samples were over 80% compared to the non-exposed cells. For some samples the C of V values showed the protein content to be under 80%. However, the mean percentage values for the protein contents of the fabric extracts showed clearly that these were non-toxic.

This study therefore supports earlier studies which have shown pure reactive dyes to have adverse effects. Allergic reactions are commonly known to cause allergic diseases for workers in industry [5,7]. Park *et al.* detected workers in the textile industry as having changes in the amounts of immunoglobulins in their blood [20]. Keneklis noticed some textile dyes are mutagenic [12]. HaCaT cells were used by Wollin *et al.* [28] to show that several azo are genotoxic. Birhanli *et al* have established that some reactive dyes are teratogenic [2].

The Chemical Safety Data Sheets [3] show the LD50 value for the yellow dye to be 5000mg/kg, higher than the 2000mg/kg values for the red and blue dyes. According to toxicity tests using activated sludge, the toxicity of the blue dye measured as EC50 (the molar concentration of an agonist which produces 50% of the maximum possible response for that agonist) was higher than 100mg/l. Using the OECD 209 method, 1984, the red and yellow dyes had IC50 values higher than 1000mg/l. Using the OECD 203 method (an acute fish toxicity test using Salmo gairneri and Oncorhynchus mykiss) the values of LC50 (the concentration of a chemical which kills 50% of a sample population) for the red and yellow dyes were found to be higher than 100mg/l.[3]. In this study, the IC50 values for all three dyes showed the limit value of toxicity to be over 100µg/ml as in the tests using activated sludge and fish. The cytotoxicity test using HaCaT cells is very sensitive and the IC20 values indicated toxicity in concentrations lower than 100mg/l.

Kopponen *et al* [13] have used hepa-1 cells to test the toxicity of textile dyes and dyed fabrics. They measured IC50 values but not IC20 values. The dyes that they used were not the same as the ones used in this study, but the results showed similar levels of dye concentration which resulted in toxicity. Klemola *et al* have used the spermatozoa motility test for studying the same textile dyes as in this work (paper submitted to AUTEX Research Journal). In this test the IC50 values were calculated after

24 and 72 hours exposure. After 24 hours exposure the spermatozoa test showed the red dye to be the most toxic: this is confirmed by this study. The IC20 values from the spermatozoa test after 24 hours exposure were higher than those from the HaCaT cell test. The spermatozoa test after 72 hours exposure had the most toxic result for the blue dye. The IC50 and the IC20 values from the HaCaT cell test showed the red dye to have the highest toxicity. Klemola *et al* (article in preparation) have also used hepa-1-mouse cells in studying these same three reactive dyes. The IC50 values from this study showed the blue dye to have the highest toxicity. The IC20 values indicated that the red dye was the most toxic. The HaCat cell line is more sensitive than the hepa-1 cell line against these dyes, showing lower concentrations for toxic values. This is because hepa-1 cells have an increased ability to metabolise foreign substances than keratinocyte cells. Although spermatozoa cells also have different metabolic abilities from keratinocytes and hepa-1-cells, the results have similarities. When using all three cell line tests together it is possible to get more precise information and the tests also support each other.

It is useful to study both the IC20 and the IC50 values when using the HaCaT cell test. The IC20 value shows the lowest toxic concentration of the sample, but the IC50 value gives extra information. The HaCaT cell test is an acute cytotoxicity test giving information after a short time of exposure. If the results show high toxicity, it is not necessary to carry out subchronic and chronic tests, which are used when the exposure time is from one month to several years.

The eco textile standard Öko-Tex-100 environmental label sets limiting values for the amounts of chemicals allowed in fabrics. The list of chemicals includes, for instance, heavy metals, pesticides and other chemicals which can remain after textile processing [29]. However, there is no biological test to assess the overall toxicity of the material. Since HaCat cells can be used for studying the overall toxicity of textile substances in addition to other cell tests and other chemical tests, they could also be used to evaluate textile substrates against the Öko-Tex-100 environmental label.

Conclusion

Human keratinocyte HaCaT cells can be used for studying the overall toxicity of textile chemicals and fabrics containing them. In addition, the HaCaT cell line could be used to provide information about the purity of different processes, as well as wastewaters and the environment which could be especially useful when developing textile products for allergic people. For instance, tests for compliance with Öko-Tex-100, for contact allergies, mutagenicity and carcinogenicity are important, but cell tests can give very useful additional information for studying the purity of textile substances.

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