EVALUATING THE TOXICITY OF REACTIVE DYES AND FABRICS WITH THE SPERMATOZOA MOTILITY INHIBITION TEST

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Abstract

In this study, the toxicity of reactive dyes and dyed fabrics was investigated using spermatozoa cells in vitro. Boar semen was exposed to different concentrations of monochlorotriazinyl dyes: yellow, red and blue. The spermatozoa cells were also exposed to extracts of dyed fabrics. After 24 and 72 hours respectively, the viability of the cells was evaluated by microscopy. The mean inhibitor concentrations IC50, showing the concentration of the dye when half of the cells are dead compared to the control sample, were calculated from the viability values. After 24 hours' exposure, the IC50 value calculated for the yellow dye was 135µg/ml, and after 72 hours 60µg/ml. The IC50 value for the red dye was 124µg/ml after 24 hours, and 46µg/ml after 72 hours. The IC50 value for the blue dye after 24 hours was 127µg/ml. After 72 hours, the blue dye caused high toxicity: more than half the cells were dead. Cotton fabrics dyed using these three reactive dyestuffs were extracted by water and analysed by the spermatozoa motility inhibition test. The viability of the cells when exposed to fabric extracts was good. However, after 72 hours' exposure, the standard deviation and coefficient of variation values for cell viability of fabric extracts were large. The spermatozoa inhibition test indicated the toxicity of pure dyes, the dyed fabrics having no adverse effects. The spermatozoa test seems to be useful when screening different substances and when used in addition to other tests. The spermatozoa motility inhibition test can be used for textile material studies.

Key words:

textile, fabric, extract, reactive dye, monochlortriazinyl, toxicity, spermatozoa

Introduction

Textile manufacture utilises a wide range of chemicals which can be harmful to the environment, to people working in textile processing and to consumers. There is information about the adverse effects on textile workers and the effects of pure chemicals, but there is limited information about the overall toxicity of dyed and finished materials [3]. Although a chemical itself may be toxic, its presence in the finished material may have no adverse effect. To study the overall toxicity of textile chemicals and fabrics, *in vitro* tests can be used. Spermatozoa cells have previously been used for studying the purity of indoor building materials exposed to moisture damage [1]. Our laboratory also uses these cells for studying the harmful effects on fibre-based food related materials. The *in vitro* spermatozoa motility inhibition test is practical and cheap in laboratory work, and it has a good response to acute toxicity.

Docker et al. [5] showed that 15% of workers handling reactive dyes had work-related respiratory and nasal symptoms. Nilsson et al. [17] noticed that there were symptoms of asthma, rhinitis and dermatitis in workers exposed to reactive dyes. Dyes containing anthraquinone or azo structures are known to cause allergic contact dermatitis [7, 23]. However, it is noted that dermatological problems associated with dyes in textiles occur relatively rarely. The cause of skin reactions is difficult to trace

because the dye usually acts as a delayed sensitiser, and as such does not cause an immediate response [11, 12].

Reactive dyes are commonly used on cotton as they have good wet-fastness, which depends on converting soluble substances into relatively insoluble compounds in the fibre [21]. Reactive dyes have complicated chemical structures, including organic ring forms with colour-giving double bonds. Typical of reactive dyes is the formation of a stable covalent bond between the hydroxyl groups of the cellulose fibres and the reactive groups of the dye [9]. In this study, the reactive dyes used belong to the category of monochlorotriazinyl dyes, and are also called azo dyes. The dye molecule reacts with vinyl sulphonyl groups and chloride atoms of the dye, forming a bond with cellulose [21].

Because reactive dyes are chemically very reactive, they are commonly harmful, especially in powder form. After reaction, the dye is stable [21]. To detect any adverse effects of textile dyes, tests for mutagenicity [16, 19], genotoxicity [6, 19], carcinogenicity [4, 10] and teratogenicity [2] have been conducted. Kopponen et al. [14] have used Hepa-1 mouse hepatoma cells to detect the common adverse effects of textile substances. However, more information is needed about the overall toxicity of reactive dyes and dyed fabrics.
Different textile dyes have been evaluated, for instance, using the frog embryo teratogenesis assay-Xenopus (FETAX), [2]. Mutagenicity tests with human keratinocytes have also been used in studying the toxicity of reactive dyes [24]. Waste waters from the dyeing process have been tested using luminescent bacteria [22]. The Ames test is also used in studying waste waters in industrial textile areas. All these studies have shown varying degrees of adverse effects of dyes [16].

The Chemical Safety Data Sheets 2001/58/EY show the results of several tests made on dyes [3], including irritation tests for rats, rabbits and guinea pigs. In addition, environmental studies have been made measuring DOC- and COD values and toxicity for fish, microbes and water fleas. In the area of textiles, all tests after OECD are made only for chemicals used for textiles. There are no tests for textile fabrics and fibres based on OECD standards. However, information about the purity of dyed and finished materials is especially important, for instance for allergic consumers.

The Öko-Tex-100 textile standard assesses whether textile products with an eco-label contain harmful amounts of certain compounds, for instance heavy metals [26], and many analyses have been carried out on eco-labelled fabrics. However, this standard does not require any common biological tests to detect the adverse effects of the material.

Andersson [1] has used spermatozoa cells in developing a bioassay for toxicity. She noted that the spermatozoa *in vitro* test was useful when detecting hazardous substances in indoor building materials exposed to moisture damage and containing complex microbial communities of bacteria and fungi. Spermatozoa have simple metabolisms compared to somatic cells. They are completely dependent on their surrounding environment for nutrients and removing toxic end products due to low concentrations of detoxifying enzymes in the cytosol. Many physiological processes in spermatozoa are controlled by membrane potentials and ion fluxes [15]. The motility of semen can be measured, including hyperactivation and inhibition. Inhibition of motility is a consequence of membrane depolarisation [8]. Hyperactivation of motility in connection with capacitation is associated with membrane hyperpolarisation [25].

The aim of this study was to use boar semen for detecting the adverse effects of three reactive dyes which represent the typical components used when mixing different basic colours. Additionally, cotton fabrics dyed using these reactive dyes were analysed. The usefulness of the spermatozoa motility inhibition test to give information about the toxicity of reactive dyes and dyed fabrics was studied.

Materials and methods

The spermatozoa test was modified for dye and textile samples based on the test by Andersson [1]. Boar semen for testing was obtained from the Pieksämäki Insemination Centre , Finland. Three reactive dyes were tested: Drimarene blue CL-2RL, Drimarene yellow CL-2R and Drimarene red CL-5B (Clariant Ltd. Switzerland). All the dyes were dissolved in distilled, sterilised water. The concentrations used were 0.05%, 0.1%, 0.2%, 0.3%, 0.5%, 0.75% and 1.0% respectively, corresponding to the dye concentrations expected to cover toxic and non-toxic values.

Valinomycin dissolved in DMSO was used as the positive control in several concentrations: 2ng, 4ng, 8ng and 16ng/2 ml of boar semen. Plain semen and water were used as negative controls to show the level of normal values. DMSO used as solvent with valinomycin was also tested. Fabric samples were prepared using commercial plain-weave bleached cotton fabrics typically used for sheets. 10g sample fabrics were each washed gently without soap. The amount of dye used was 3% of 10g fabric. The dye bath had 400ml water, 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 dyeing 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 kept in pure boiling water for 10 minutes. The dyed cotton fabrics were extracted with sterilised 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 semen, using 2ml of semen with 40µl of the dye samples or fabric extracts. The sample concentrations of 1%, 0.75%, 0.5%, 0.3%, 0.2%, 0.1% and 0.05% in the spermatozoa test tubes corresponded to 196µg/ml, 147µg/ml, 9 µg/ml, 59µg/ml, 3 µg/ml, 20µg/ml and 1µg/ml respectively of pure substance. All samples were compared to the control sample of plain semen. Exposure continued for 24 and 72 hours at room temperature. The tubes were inverted once a day. Before analysis, the tubes were gently mixed manually. All processing was made under sterile conditions.

After 24 and 72 hours' exposure, the sperm motility was measured and compared to that of the sperm in the plain semen controls. Water and DMSO were also detected. After gently mixing the tubes, 200µl samples were taken to small plastic tubes for incubation at 37ºC for five minutes before microscopic analysis. The temperature of the pre-warmed objective glasses used in the microscopic analyses was also 37ºC. The samples were gently mixed, and the amount of living cells was qualitatively observed by light microscopy (100x to 400x magnification), and the results were stated as the viability capacity percentages of living cells. The optimal value of the plain control sample was set at 50% because semen always contains dead and damaged cells. In the evaluation, the following observations of activity were made for cells: speed of movement, ability to move forward, damage that can be detected by a microscope, rotation and vibration, dead cells. The toxicity limit is considered to be 25% of unexposed cells: levels of 25% and below represent toxicity. The limiting value of 25% therefore represents the IC50 (inhibitory concentration) value when 50% of the original living cells are dead at the end of the exposure time.

The results were evaluated according to the following categories: 50%: cells move forward, have strong vibration and high activity; 40 - 45%: cells move forward, have strong vibration but less activity than controls; 30-35%: some vibration left, many dead cells, most of the cells rotating, only some moving forward very slowly; 20-25%: most of the cells dead, some rotating; 10-15%: only some cells moving slowly, most of the cells dead; 5%: isolated cells vibrate slowly. The results were recorded within these 5% boundaries. CV values were calculated for all results to evaluate their reliability.

Results

The inhibitory concentration value (IC50) is the concentration (µg/ml) of the dye when 50% of the cells in the test are dead. The IC50 values for the dye samples were calculated from the curves which describe the viability percentages of the cells under different dye concentrations. The IC50 value is the concentration (µg / ml) on the x-axis when the corresponding value on the y-axis is 25%. The results from testing different dye and fabric extract samples are shown in Figures 1-4. The plain semen showed a viability of 50% through the whole exposure time (72 h), and all the samples were compared to it.

The IC50 values for the yellow dye were 135µg/ml after 24 hours' exposure and 60µg/ml after 72 hours (Figures 1A and B)
The IC50 values for the red dye were 124µg/ml after 24 hours' exposure and 46µg/ml after 72 hours (Figures 2A and B).
The IC50 values for the blue dye were 127µg/ml after 24 hours' exposure. After 72 hours' exposure, the blue dye was toxic in all concentrations, and it was not possible to calculate IC50. More than half of the cells were dead; for instance, when the concentration of the dye was 20µg/ml, only 21% of the cells were still alive (Figures 3A and B).
The fabric extracts showed that over half of the cells were still living after 24 hours' exposure. After 72 hours' exposure, the mean values of viability did not show any toxicity either (Figures 4A and B).

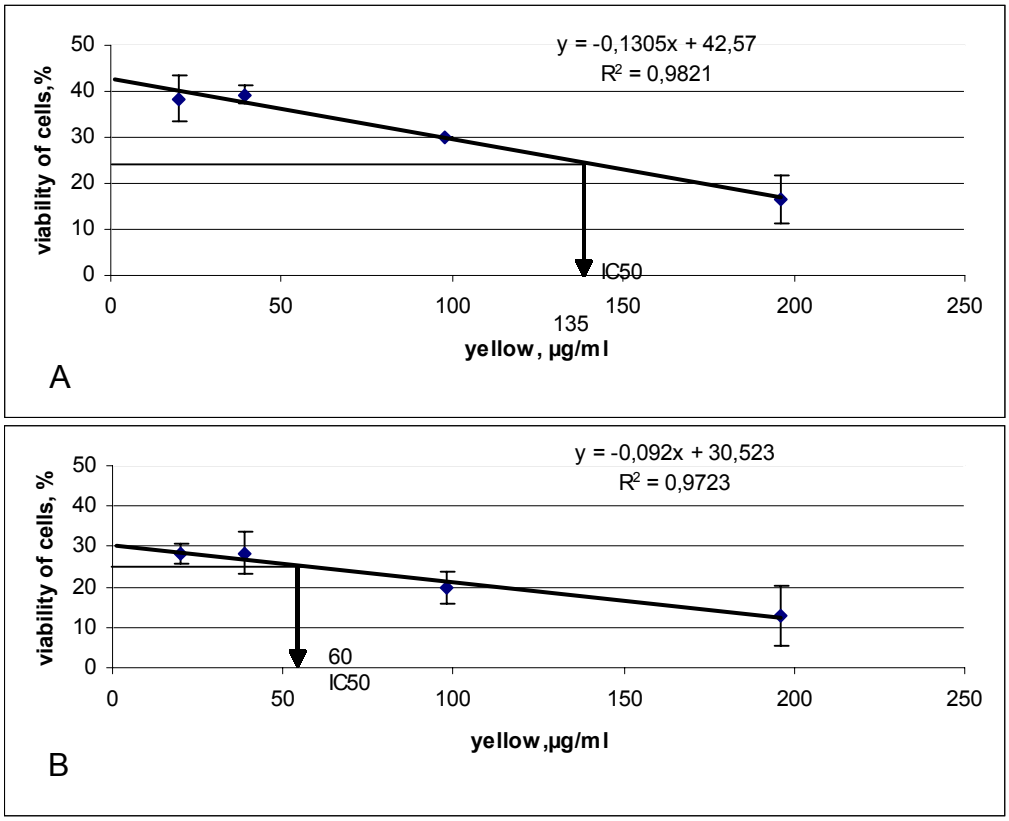

Figure 1. The IC50 value after 24 (A) and 72 (B) hours' exposure represents the value when 50% of cells are dead (number of samples, n= 4 to 9)

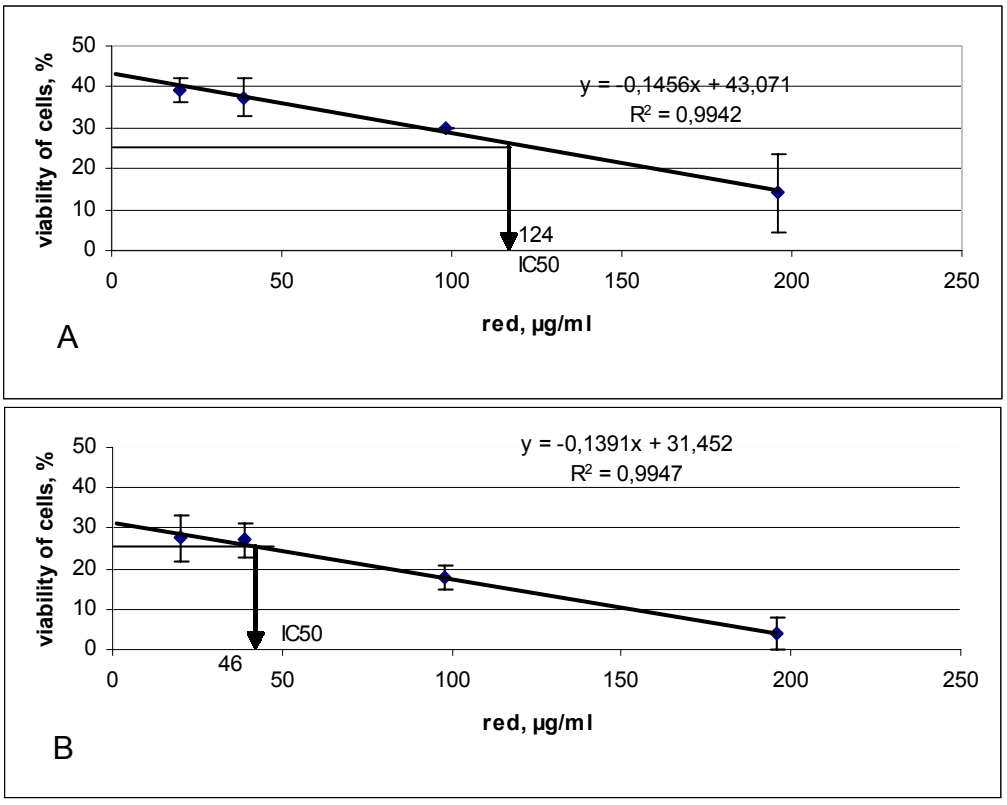

Figure 2. The IC50 value of the red dye after 24 (A) and 72 (B) hours' exposure. (n = 4-9 after 24 hours, n = 3 to 9 after 72 hours)

Valinomycin was used as the positive control. The results showed that, in low concentrations of valinomycin, cells had good viability, and in the highest concentrations all cells died. The volume of 40µg DMSO and water had no effects on the semen.

It is significant to note that after 24 hours' exposure, some of the cells were completely broken; however, some of the cells had good viability. Many cells were totally broken after 72 hours, although living cells were also found.

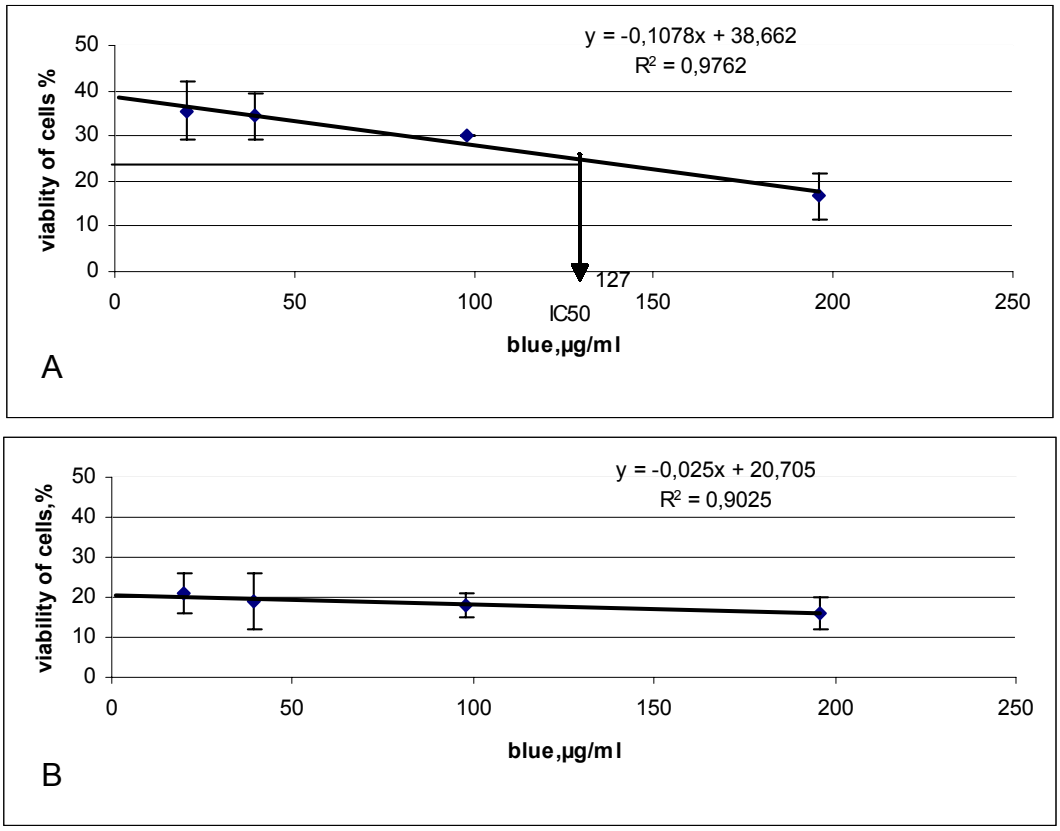

Figure 3. The IC50 value of the blue dye after 24 (A) and 72 (B) hours' exposure. All samples had mean values of viability lower than 25%. (n = 4 to 10 after 24 hours, n = 3 to 9 after 72 hours)

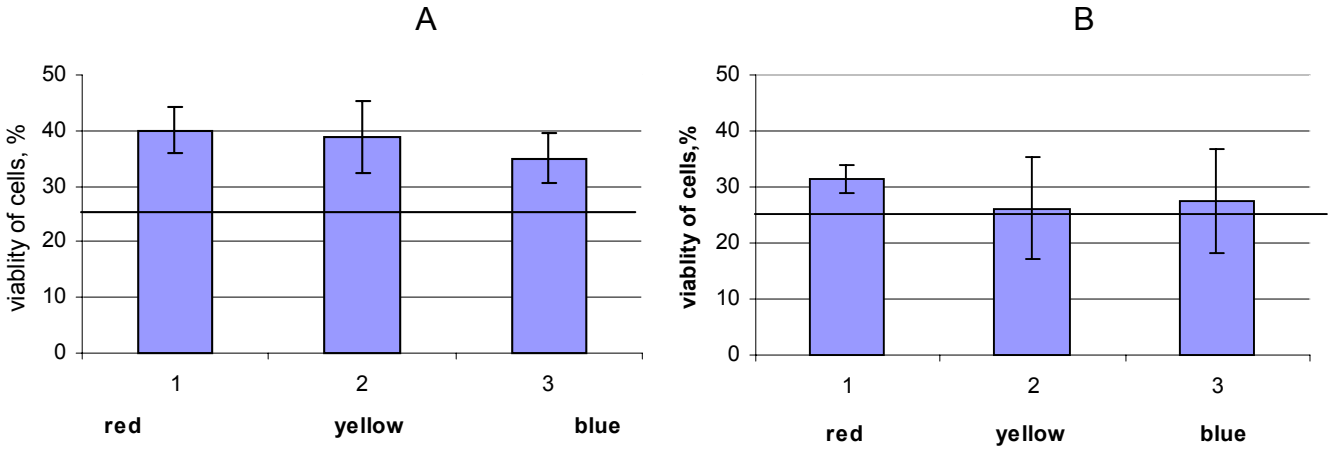

Figure 4. Viability (%) of spermatozoa cells exposed to fabric extracts for 24 (A) and 72(B) hours (number of samples n = 4 (red fabric), n = 8 (yellow fabric), n = 6 (blue fabric)

After 24 hours' exposure for the non-toxic concentrations, the C of V values ranged from 0 to 18%. Toxic results after 24 hours' testing had C of V values ranging between 31 to 69%. After 72 hours' exposure, the results had C of V values between 9 to 37% for the non-toxic results; the toxic results had C of V values of 0 TO 58%. For fabric extracts, C of V values after 24 hours were between 10 and 16% and after 72 hours, between 8 and 35%.

Table 1. Response of reactive dyes in the boar spermatozoa motility inhibition test; + positive response = toxic to cells; - negative response = not toxic to cells

		YELLOW		RED		BLUE	
	concentr.						
dye	µg/ml	24h	72h	24h	72h	24h	72h
	1	-	-	-	-	-	+
	20	-	-	-	-	-	+
	39	-	-	-	-	-	+
	59	-	-	-	+	-	+
	98	-	+	-	+	-	+
	147	+	+	+	+	+	+
	196	+	+	+	+	+	+
valino-	2 ng	-	-				
mycin	4 ng	-	+				
	8 ng	+	+				
	16 ng	+	+				
DMSO		-	-				
unexposed cells		-	-				

Discussion

The results clearly show the difference in toxicity between 24 and 72 hours' exposure. After 24 hours' exposure, the yellow dye was less toxic than the red and blue dyes. However, the differences between toxicity values were low; for instance, the yellow and red dyes showed a difference of only 11µg/ml. After 72 hours' exposure the blue dye indicated a toxic result, and it was not possible to measure the IC50 value. In all blue samples more than half of the cells were dead. The toxicity of the blue dye may partly be dependent on the copper-complex structure of the dye molecule [3]. The yellow dye showed the least toxicity, but the IC50 value was only 60µg/ml after 72 hours' exposure. There were many broken cells in all dye samples, although some of the cells were still alive.

Reactive dyes react with cellulose under alkali conditions [21]. Pure dyestuffs have pH values between 4.5 and 6.5. However, the dyes are very reactive in semen. In this study, reactive dyes are toxic in low concentrations, lower than those used in commercial dyeing processes.

This study showed that the fabric extract samples were not toxic. Although colour in the extracts showed that some dye was present in them, they were non-toxic. It can be assumed that the extra dye in the solution was hydrolysed. After 24 hours' exposure, the results showed that the fabrics were not toxic. After 72 hours' exposure there were variations in the results, but the mean values showed non-toxicity.

The textile dyes do not contain only dye molecules, but they also include compounds for forming granulates, chemicals for storage and so on. In addition to dyes there are other chemicals that can have toxic effects. This means that the discussion of the results of this work relates to the toxicity of a mixture of chemicals, and not to the pure dye molecules: from this work alone, the effect of pure dye cannot be evaluated. It is also not possible to know the exact concentration of the dye itself in the powder form of the commercial dyestuff. In powder form, reactive dyes are in a very active form, and it is understood that they have toxicity in high concentrations. After the dyeing process, the dye molecule has a permanent covalent bond between the fibre and the dye molecule [21]. The bond is very stable, which may explain why the dyed fabric material is not toxic. In this study, the results showed that pure dyes may be toxic, but dyed fabrics are not.

The toxicity of pure reactive dye has been noted in many earlier studies, and the spermatozoa test supports these. Keneklis [13] assumed that since the properties that enable the dyes to react with textile fibres also enable them to bind to body protein, the health hazard resulting from exposure to such substances is significant. Many studies have also statistically proved that reactive dyes have effects which lead to increasing IgG and IgE blood values in workers who have contact with them [18, 20]. It is clear that it is the reactivity of these dyes which causes health hazards, but the dye no longer has a toxic effect after reacting with the fibre.

The three dyes, according to Chemical Safety Data Sheets, have LC_{50} values greater than 100 mg/l based on the OECD 203 method (an acute fish toxicity test using *salmo gairdneri* and *oncorhynchus mykiss*) [3]. In this study, the results after 24 hours' exposure show similar levels. The results of the spermatozoa test after 72 hours' exposure show toxicity under lower concentrations than the results based on the OECD 203 method. Kopponen et al [14] used hepa-1 cells in studying the toxicity of reactive dyes. The IC50 values found here were also at the same level as in this study, but it is not possible to compare results exactly because Kopponen et al. did not use exactly the same reactive dyes as in this study. However, the IC50 values from previous studies show some similarities, and support the possibility that this test can be used for studying the toxicity of textile dyes and dyed fabrics. One alternative in the future is to use this spermatozoa test instead of animal tests, which could be significant when studying the toxicity of materials.

Using a boar spermatozoon as a test cell can lead to variations in the results if the quality of the cells differs. However, the results can always be compared to a control sample. During this study, all the control samples showed good results. The sample volumes and evaluation method used were the same as our laboratory now uses for other fibre-based materials. The results of this study may later be compared to other studies.

The spermatozoa test is not very difficult to perform, although the exposure time is three days. The test is qualitative, and variation may occur between persons due to the subjective nature of the evaluation being used. In this study, several researchers studied the same samples; however, the difference between their results was only 5% when the method was evaluated for use.

The spermatozoa motility inhibition test is a biological test, and the results are dependent on actions of membrane potentials and ion fluxes [15]. The test does not give any information about the chemistry of cell metabolism, nor does it show any information about reaction mechanisms. The test is very sensitive; for instance, the temperature must be exact and the process must be carried out under sterile conditions. However, the spermatozoon test is useful since it is sensitive enough to provide first-hand information about the purity/toxicity of materials.

The cells are easy to handle and they are quite cheap. The laboratory conditions for the process can also be easily arranged at relatively low cost. Although the test does not establish the reasons for toxicity, the use of semen can provide extra information when used together with other tests. Andersson [1] has used the spermatozoa motility inhibition test for detecting toxins, and this study shows the test to be useful in studying the toxicity of reactive dyes and fabrics. 24 hours' exposure is sufficient to indicate whether a material is toxic, and this can be confirmed by the longer exposure period of 72 hours.

The spermatozoa test gives high standard deviation and coefficient of variation values, especially for toxic samples. The results are read in bands of 5%, and this also has an effect on the variation of the results. However, despite theses variations, the test can be used reliably for screening samples and for use with other tests (Table 1).

Conclusion

The results suggest that in addition to assessing the toxicities of textile dyes and fabrics, it is important to determine the overall toxicities of various chemicals. The spermatozoa toxicity test is an interesting addition to other tests used to screen textile samples and chemicals for use in manufacture. This bioassay may provide useful information for the development of less harmful textile processes and products, although it cannot replace the final tests for allergy, mutagenicity, carcinogenicity and

subacute toxicity. The results clearly showed that the reactive dyes in this study were toxic, but dyed fabrics had no adverse effects on sperm cells.

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