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Ocular hazards of the colors used during the festival-of-colors (Holi) in India—Malachite green toxicity

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Abstract

The objective of this study was to evaluate the nature of the colors used and their toxicity to the eye upon exposure to them during celebration of Holi (our festival-of-colors). Color powders and formulations were procured at random in and around Delhi during the festival. The green/bluish-green colors reported with the higher incidence of ocular toxicity were subjected for further evaluation. Eyewash fluid collected from the patients exposed to the colors was also subjected for analysis. This study was further extended to evaluate the corneal penetration of malachite green using goat cornea in perfusion chamber. In 16/18 color samples collected, malachite green or 4-[(4-dimethylaminophenyl)-phenyl-methyl]-*N*,*N*-dimethyl-aniline was detected at different concentrations. In the eyewash fluid of four patients, HPLC estimation confirmed the presence of malachite green at concentrations of 1.3, 0.18, 3.5 and 5.4 μg in 250 ml which was responsible for its reported toxicity. The *in vitro trans*-corneal penetration studies did not show any detectable amount of malachite green in effluent fluid—*in vitro* tissue retention studies revealed that increasing the contact time increases tissue concentration. After 2 min of exposure, the tissue concentration was significantly higher. To conclude, malachite green was extensively used in our festival of Holi and has caused severe ocular irritation with epithelial defect upon exposure, though it did not penetrate through the cornea—further *in vitro* and *in vivo* studies are required on colors used in Holi.

Keywords: Malachite green; Eye toxicity; Synthetic colors; Holi festival

1. Introduction

Colors made up from natural and synthetic compounds are historically used in different parts of the world for the celebration of festivals. Documentation of relevant information is lacking regarding the type of 'attractive' colors used in the celebrations and their human toxicity upon exposure. The 'Holi' festival is celebrated mostly in the northern and eastern regions of India by throwing dry/wet colors on each other. These colors occasionally enter the eye and damage the ocular surface causing temporary visual disability, immense ocular discomfort and complications threatening vision [1,2]. Different attractive shades of colors are used in this festival and in recent days usage of natural colors (from leaf extracts) are largely emphasized by the media for alerting the public.

The usage of green color has increased with the belief that green (a 'natural' color) is safe. The objective of the present study was to evaluate the nature of colors used during the Holi festival and confirmation of the agents which are causing higher incidence of ocular toxicity. In March 2005 (Holi festival season here), a massive drive was undertaken to evaluate the type of compounds in green colors available at different regions of Delhi. The eyewash fluid of patients, who reported with eye injury to the "eye casualty" of our tertiary eye hospital at New Delhi, was collected for identification of their chemical nature.

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2. Materials and methods

2.1. Materials

Two days before the festival of Holi, a total of 82 samples were procured at random from five major markets in and around the city of Delhi. The samples were broadly classified based on the visually distinguishable colors—18 of the 82 samples having the representative green and bluish green colors, were subjected to spectral analysis using UV–vis spectrophotometer.

2.2. Methods

2.2.1. Green dye ocular toxicity

As soon as the patients reached our emergency, external eye irrigation was performed as a routine procedure using sterile saline under topical proparacaine anesthesia. The first eyewash fluid of 250 ml was collected in polyethylene tubes and preserved at −70 °C till analysis. A detailed case history was taken after extensive eyewash and the patient's eye subjected for slit-lamp biomicroscopy for epithelial defects and for foreign bodies after fluorescein staining.

2.2.2. Evaluation of colors using spectrophotometer

Accurately weighed or measured quantity of the color powders and solutions were diluted to reach the concentration of 100 μg/ml in deionized water. The solutions were subjected for vortex for 2 min and centrifugation at 7000 × *g* for 10 min at room temperature. The supernatant was further diluted and subjected to scanning (200–800 nm) using UV–vis spectrophotometer (UV-1, Thermo, USA). The peak absorbance was matched with the absorption of known dyes.

2.2.3. Chromatographic method for the estimation of malachite green from the samples

A reverse phase high performance liquid chromatography (HPLC) method was developed for the quantification as well as identification of malachite green from biological samples. For this chromatographic analysis, a quaternary gradient Surveyor HPLC (Thermo Electron Corp., USA) pump connected with a octadecylsilane C-18 column (Symmetry Shield 150 mm × 3.9 mm, 3.5 μm particle size, Waters, USA) and photodiode array detector (Finnigan Surveyor PDA, USA) were used. The mobile phase of the HPLC was optimized to elute malachite green at the retention time of 6.05 min using a gradient program (Table 1). The mobile phase consisted of HPLC grade acetonitrile (S.D. Fine-CHEM Ltd., India) and 0.02 M potassium dihydrogen phosphate, and the analyte was detected at 617 nm. The spectral match was achieved using inbuilt PDA library option using ChromQuest (Ver.4.5) software (Thermo Electron Corp., USA).

2.2.4. Analyzing eyewash fluid for the identification of malachite green

The eyewash fluids collected were subjected for solid phase extraction using C-18 cartridges (Orochem Technologies Inc., USA). The column was conditioned with 2 ml acetonitrile and 4 ml of water. Under negative pressure the eyewash fluid was passed at the speed of 1 ml/min. The trapped dye was subjected to water wash (4 ml) followed by elution with 2 ml of acetonitrile. The eluted acetonitrile was suitably diluted and subjected for identification and quantification by HPLC.

Table 1
Chromatographic conditions used for the gradient run for the separation of malachite green

Time (min)	Acetonitrile	0.02 M KH_2PO_4	Flow rate (ml/min)
0.01	20	80	1
2	40	60	1.5
5	60	40	1.5
6	50	50	1.5
8	40	60	1.5
9	20	80	1
10	20	80	1

2.2.5. Trans-corneal penetration studies of malachite green

For this experiment, simulated *in vitro* goat cornea perfusion chamber was used. Fresh goat's eyes were procured from the local slaughter house and by cutting through the limbus, the cornea was separated. Cornea was washed with saline and mounted in a temperature regulated chamber at 37 °C, malachite green standard solution (1% in saline) was instilled at the volume of 50 μl on the upper chamber, and balanced salt solution was perfused at the rate of 0.33 ml/h. The effluent fluid was collected at 15 min intervals up to 8 h. The collected effluent was subjected to HPLC estimation using the method mentioned above.

2.2.6. Tissue retention of malachite green

The goat's eye was exposed to 50 μl of 1% malachite green in the form of a single drop over the bulbar conjunctiva and the drop was allowed to spread the area for 15, 30, 60 and 120 s. For each time point four eyes were used. Brief washing was then administered with 5 ml tear buffer for 30 s to remove the free dye. Then, a circular piece (5 mm diameter) of the stained tissue was taken out using a dermal punch, weighed and homogenized with the addition of 500 μl acetonitrile. The tissue was then subjected to centrifugation at 7000 × *g* for 10 min. A 100 μl of supernatant fluid was further diluted to 1 ml with acetonitrile. A 20 μl of this final extract was injected into HPLC for the quantification of malachite green.

3. Results

3.1. Green dye ocular toxicity

Among the patients reported to the emergency unit of Dr. Rajendra Prasad Centre for Ophthalmic Sciences, All India Institute of Medical Sciences, New Delhi on 25 March 2005, 10 of them were due to Holi colors related toxicity/trauma. Among the 10 patients, 6 of them had bluish green discoloration of the conjunctiva in the upper and lower fornix. Inner surface of the lower eyelid was also highly stained with malachite green. Despite our best efforts, the eyewash fluid of only four patients could be collected out of six.

Fig. 1. Showing the malachite green stained inner surface of the lower eyelid and lower conjunctiva.

All the patients presented with severe eye irritation and pain. Most of them reported to our casualty 1–2 h after the exposure. All cases were reported with severe eye irritation starting immediately after the exposure of the malachite green (1–2 min) in the liquid form (dissolved in water). All of them were reported to wash their eye immediately after the exposure. In one case, conjunctival edema with epithelial defect was observed (Fig. 1). The eyewash did not remove the stain totally and the staining persisted up to 48 h after exposure and then resolved. The eyewash was carried out under a stream of sterile saline under topical proparacaine 0.3% anesthesia.

3.2. Chromatography method for the estimation of malachite green from the samples

The method used for the estimation of malachite showed linearity in the range of 0.015–1 μg/ml ($R^2 = 0.9998$). Solid phase extraction method used for the sample purification of malachite green showed a recovery of 95%. External calibration curve was plotted for the estimation of the unknown concentration of malachite green in the eyewash fluid as well as other samples. The representative chromatogram showing the elution of malachite green at the retention time of 6.05 min is shown in Fig. 2a. The photodiode array detector's (PDA) report of the visible spectrum of the isolated peak of malachite green at the RT of 6.05 min is shown in the Fig. 2b.

3.3. Evaluation of the green colors using UV–vis spectrophotometer and quantification using HPLC

The colored powders obtained from various places of Delhi were subjected for spectral scanning showed maximum absorbance at 617 nm for majority of the green colors. Out

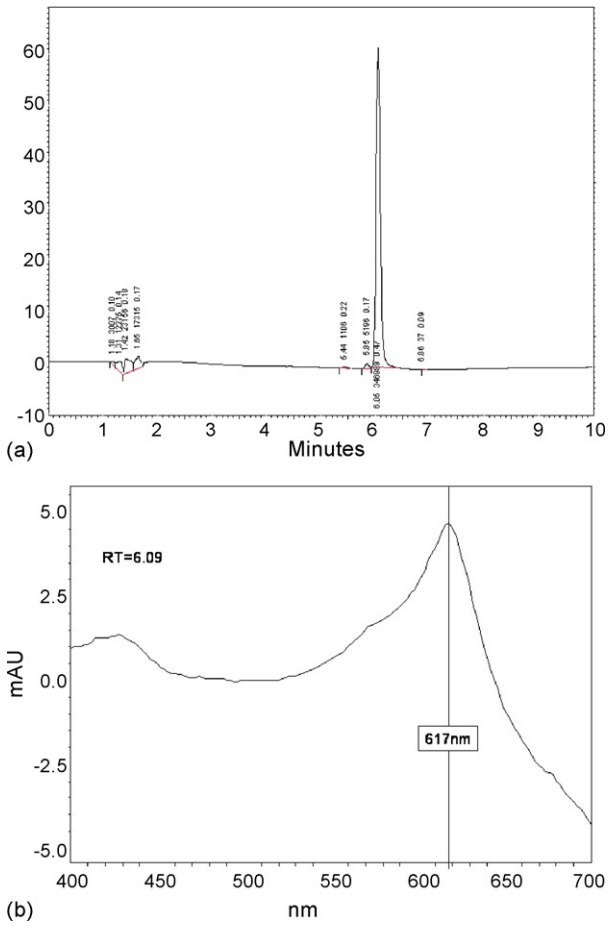

Fig. 2. (a) The representative chromatogram showing the elution of malachite green at the retention time of 6.05 min. (b) The visible spectrum recorded for the peak of malachite green at the retention time of 6.05 min in photodiode array.

of 18 samples, 16 of them found to be positive for malachite green. The reverse phase method developed for HPLC also confirmed the presence of malachite green in the color powders and formulations used in the festival. However, the amount of malachite content in the powders varied from 0.3% to 85%.

3.4. Analyzing eyewash fluid for the identification of malachite green

All four eyewash fluids showed the presence of malachite green. The amounts of malachite green found in the eyewash fluid of the four studied cases were 1.3, 0.18, 3.5 and 5.4 μg, respectively, in a volume of 250 ml of the first eyewash fluid in the hospital.

3.5. Trans-corneal penetration studies of malachite green in vitro

In the *trans*-corneal penetration study using goat cornea, at the applied concentration of malachite green (50 μl of 1% solution), no detectable concentration was observed in the effluent samples by HPLC estimation.

3.6. In vitro tissue retention of malachite green

All the samples increasingly retained malachite green beyond 1 min of exposure. Exposing the ocular tissue for 15, 30 and 60 s showed tissue levels of 4.01 ± 2.01, 4.0 ± 1.87 and 5.06 ± 0.86 μg/cm^2, respectively. However, after the exposure time of 120 s the tissue concentration of 12.6 ± 3.09 μg/cm^2 was significantly higher ($p > 0.001$) as compared to other exposure timings.

3.7. Statistical analysis

Unpaired Student's *t*-test was applied and $p < 0.05$ was considered to be statistically significant. All values are represented as mean ± S.D.

4. Discussion

Several colors are used in the festivals like Holi in India either as a dry powder or in liquid or paste form with water/oil. These are applied directly with hands or using spray guns, fluid filled balloons or splash. These colors occasionally enter the eye and damage the ocular surface causing temporary visual disability, immense ocular discomfort and complications threatening vision [1,2]. Pioneering work on Holi colors by Ghose et al. [1], was reported for their toxicity of green dye causing severe eye irritation in experimental animals. Even though, several colors are being used in this festival, so far no attempt has been made to evaluate the chemical constituent, and to document their eye toxicity profile.

Since the incidence of ocular toxicity in the patients reported to our eye emergency was higher with the green color, the chemical nature of all green colors collected from local market were subjected for analysis along with the eyewash fluid collected from the patients. From the green samples collected, 89% of them found to have malachite green at the concentration varying from 0.3% to 85%. It is a versatile triarylmethane compound having high solubility in water. It is extensively used for dyeing silk, wool, jute, leather, cotton and making microbiological media [3]. It is also reported to be a highly cytotoxic dye to mammalian cells in culture and has a potential environmental health hazard.

Clemmensen et al. [4] conducted toxicological studies on malachite green in rats, guinea pigs and rabbits after oral and topical applications. In their eye irritation studies they have administered 100 μl of (8% solution) malachite green solution in the lower conjunctival sac of the left eye of the rabbit where right eye served as a control. With the help of the ophthalmoscope, they have reported the ocular toxicity of the malachite green. Malachite green produced marked edema, substantial discharge and slight hyperemia of the conjunctiva, which disappeared after 24 h in two out of three rabbits. They have also reported that no effect on cornea or iris was observed and all rabbits were fully restituted after 8 days. In their study they have not explained the duration of exposure. However, in our patients we have recorded the duration of exposure, in most of the cases within a minute or two severe irritations prompted them to wash their eyes with water. One patient applied honey and another applied ghee (clarified butter or anhydrous butter fat) to get soothing effect. However, the patient applied ghee reported that after the application of ghee the irritation has increased. This is probably due to the hydrophobic nature of ghee, which has decreased the water solubility of malachite green thereby malachite clearance from the stained tissue is minimized.

The moderately toxic response to the acute exposure of rosaniline family of compounds is reported to be transient, usually passing within 2 days [5,6]. In our patients who suffered green toxicity, they have been asked to report to the hospital on the next day for regular follow up. In one of the patients, the eye irritation caused by the exposure of malachite solution persisted for few days (maximum 7 days). However, all patients reported a severe form of irritation on the first day even after the quick wash with water immediately after the exposure. They were all managed with topical antibiotics (as a precautionary measure to prevent infection) and artificial tears.

In our experiments the *trans*-corneal permeability of malachite green was found to be extremely low in the dose studied. Therefore, upon brief topical exposure the possibility of intraocular penetration of malachite green and its related retinal toxicity is very low. Our tissue exposure study reveals that the duration and strength of malachite green solution exposure would be having a positive correlation with its tissue levels. Our goat eye exposure experiments showed a tissue concentration of 12.6 μg in 1 cm^2 surface area after 2 min exposure of 500 μg. In most of the patients reported that the irritation started within 2 min of the exposure subsequently they have washed the excess with water. Similar, observation has also been reported by Srivastava et al. [7] that increase in toxicity of this compound with increase in exposure time, temperature and concentration. In our study, the patient's eyewash fluid concentration of malachite green varies from 0.18 to 5.4 μg in the volume of 250 ml in the four patients reported with green dye toxicity. Considering the levels found in wash fluids, the original concentration of malachite green in the eye cannot be correlated since the possibility of malachite leaching out from the adjourning area can not be ruled out while performing eyewash. Extrapolating the present finding with the green spectral match report of Ghose et al. [1], we could interpret that the green dye causing severe irritation used in Holi festival of 1994 was also malachite green.

Malachite green is extensively used as a biocide in aquaculture industry worldwide. However, it has become a highly controversial compound due to its effects on the immune system, reproductive system and its genotoxic and carcinogenic properties [8–12]. Its use has been banned in several countries and not approved by the US FDA [11]. Moreover, US FDA has nominated malachite green as a priority chemical for carcinogenicity test [13].

Dada et al. [2] also reported the chemical injury due to colors used in the festival of Holi from our hospital. They have reported that 15% of the injuries of the 40 cases were due to liquid colors. Its easy availability, attractive color, water solubility and less cost makes people misuse this color for festival celebrations. They have also suggested the toxicity of colors to be an alkali injury resulting in saponification of cell membrane. However,

the alkalinity of malachite green is unlikely be the mechanism of its ocular manifestations since its ionization constant (p*K*) is 6.9. Increasing pH decreases its ionization and at pH 10.1, it is totally unionized [14].

5. Conclusions

The green color used in the 2005 Holi festival causing severe eye irritation is found to be malachite green. The easy availability and extensive usage of malachite green in the festivals or celebrations cause severe form of ocular manifestations upon its exposure. *Trans*-corneal penetration studies revealed lack of detectable concentration of malachite green in the aqueous humor at the studied concentration showed that it is unlikely to cause intraocular toxicity. Therefore, usage of malachite green dye during the festivals should be strongly discouraged.

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