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Reversible Hepatic Toxic Effect of Crocin Dyes in Rats

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Abstract—*Gardenia jasminodes* has been medically used for anti-inflammation, sedation and anti-diarrhea; The extract of this plant has been traditionally used as food colorant and referred as crocin dyes. In the present study, the possible hepatic toxicity of this dye has been evaluted on the basis of its alteration on the marker enzymes, namely, glutamic-oxaloacetic transaminase, glutamic-pyruvic transaminase, alkaline phosphatase, lactate dehydrogenase and gamma-glutamyltransferase. Crocin dyes did not affect hepatic function when they were orally administered to rats in a daily dose of 50 mg/kg for 8 days, but could induce acute hepatic discoloration. A high dose of 100 mg/kg for 2 weeeks could induce both hepatic damage and black pigmentation, but a lower dose of 10 mg/kg for 40 days did not The induced black pigmentation and the acute hepatic damage were completely reversible. In conclusion, the crocin dyes have a very low hepatic toxicity in rats, even in high experimental dosages which could hardly happen in human practice. It is therefore suggested that the crocin dyes are safe for coloring foods.

Keywords—Crocin dyes *Gardenia jasminodes*·reversible hepatic pigmentation·reversible hepatotoxicity·natural food colorant

The use of coloring agents in food processing is quite popular in all nations. It is a general tendency that the quality of a given food is generally justified by its characteristic color appearance. For years. the chemically synthetic coal tar dyes have been widely used as food colorants. These synthetic dyes are cheaper and easier to obtain. However, some of these dyes have been demonstrated to be mutagenic, teratogenic and carcinogenic in the experimental animals[1,2,3,4]. For this reason, Amaranth (FD & C red no. 2) has been officially banned. Most consumers are deeply concerned about the possible toxic effects of these synthetic dyes and they urge manufacturers to use natural food colors in their consuming food products.

Among the natural food colours, myoglobin and hemoglobin are proteinous substances and unstable; chlorophyll from vegetables are occasionally used. Flavonoids and carotenoids are two large groups of natural dyes and appear as a wide range of colors such as light yellow, yellow, orange yellow, red, pinkish red, violet and blue.

Gardenia jasminodes, a plant in the family of Rubiacease, has been widely used for anti-inflammation, sedation, decongestion and anti-diarrhea in folk medicine. The fruits of Gardenia contain natural yellow pigments called crocin dyes containing carotenoids and geniposides as major components. It seems that the crocin dyes are high economic value and used as color additives in many kinds of foods and beverages.

Since the high demand of natural food colours

in food technology, it is of necessity to evaluate the safety of crocin dyes for being used as food colorant. Hong, *et al.*[5] reported that crocin dyes could induce hepatic black pigmentation in pigs and mice whereas Miwa, *et al.*[6] and Chung, *et al.*[7]. did not make such an observation. In order to solve this discrepancy, a prolonged feeding of higher dose of crocin dyes to rats was carried out to study the hepatic toxicity. The development of hepatic pigmentation was closely observed and the serum enzymes, which are generally used in the clinical assessment of liver function, including glutamic-oxaloacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), and gamma—glutamyl transsferase (rGT) were assayed. The effect of crocin dyes on the hepatic function of rats and the relationship between the doses of dyes and the degree of black pigmentation were systematically investigated.

Materials and Methods

Extraction of Crocin Dyes

Dried fruits of *Gardenia jasminodes* were defatted with ethylether, and extracted with warm methanol in a Soxhelet apparatus. The resulting extracts were condensed under reduced pressure to dryness and gave a syrup-like residues.

Reagent Kits

GOT, GPT and rGT diagnosis kits were purchased from Boehringer Manheim GmbH Diagnostica. ALP and LDH diagnosis kits were purchased from E. Merck Co., West Germany. Coproporphyrin and bilirubin standards were purchased from Sigma Chemical Company. All the reagents used were in analytical or HPLC grade.

Effect of Crocin Dyes on the Hepatic Function of Rats

Female rate of the Sprague-Dawley strain from the Medical Animal Center of National Taiwan University were used. They were fed on Taitan Chicken Feed formulated by Taiwan Sugar Cane Co., ROC.

(a) Acute and subacute effects on hepatic functions

Eighteen rats with 240~260 g body weight were divided into three groups. The experimental rats were administered *via* gastric tube with 50 mg/kg of crocin dyes daily for 3 and 8 days. Then, they were sacrificed by decapitation, and the blood was collected for assays of GOT, GPT, ALP, rGT and LDH. The livers were excised and weighed.

(b) Chronic effect on hepatic functions

Twelve rats weighing 100~120 g were divided equally into control and experimental groups. The control animals were fed on a Taitan diet while the experimental animals were given 1% crocin dyes. Every two weeks, the rats were weighed and the growth rates were compared. Four months later, they were decapitated and the blood was collected. The enzymes and bilirubin in the serum samples were assayed and the livers were examined histologically.

Study of the Dose Response on the Hepatic Pigmentation

Eighteen rats with 150~160 g body weight were divided equally into six groups, and administered daily with crocin dyes in a dose of 0.0 (control), 0.08, 0.4, 2, 10, and 50mg/kg respectively via gastric tube. After 40 days, the livers were excised and examined for black pigmentation. The relative liver weight (liver weight/body weight) was measured and the levels of coproporphyrin, uroporphyrin and bilirubin in the urine were determined.

Study on the Reversibility of Hepatic Pigmentation

Twelve rats with 200~220 g body weight were given 100 mg/kg of crocin dyes by stomach tube. This treatment was continued for two weeks,

three rats were taken randomly to see if the livers were blackened, meanwhile the enzyme activities, bilirubin levels and the residues of the dyes were measured. The relative liver weight was also determined. When administration of crocin dyes was terminated, with 2~3 day intervals, a random group of three rats were examined as described above.

Assay of Serum Enzymes and Bilirubin

Transaminases (GOT and GPT) were determined according to the method of Reitman and Frankel[8]. For ALP assay, 0.1 ml of serum was incubated with p-nitrophenylphosphate and the p-nitrophenol thus formed was determined spectrophotometrically at 405 nm[9]. For the estimation of LDH, the fresh serum was reacted with the pyruvic acid and NADH and the rate of the reaction was measured by the method of Elliot and Wilkinson[10]. rGT was estimated according to the method of Parsijin[11]. Serum bilirubin was coupled with diazotized sulfanilic acid to form azobilirubin, the product was determined by the method of Sims and Horn[12].

Determination of Porphyrin in the Urine

The urinary porphyrins were measured by the spectrofluorometric method of Martinez and Millis [13]. Coproporphyrin was extracted from the urine at PH 4~6 into ethylacetate and back extracted into dilute HCl for measurement. Preformed uroporphyrin was determined in the aqueous layer by absorption onto alumina and then eluted off with dilute HCl. Coproporphyrin and uroporphyrin have similar fluorescent characteristics, the wavelengths of excitation and emission are 400nm and 595nm, respectively.

Determination of Residual Crocin Dyes in Rat Liver

Rats were sacrificed and a piece of liver was excised and homogenized in polytron with 15ml redistilled water. The homogenate was centrifuged at 3000 rpm for 15 minutes, and 15 ml of ice cold methanol was added to the upper layer and kept at 0°C overnight. The precipitate was discarded after centrifugation for 15 minutes. The interference of bile pigments was extracted with $BaCl_2$ and ethylacetate. The residues of crocin dyes in liver were determined spectrophotometrically at 440nm.

Results

Effect of Crocin Dyes on the Growth and Hepatic Function of Rats

The administration of 1% crocin dyes in the diet for 4 months had no significant effect on the growth of experimental animals. Oral administration of crocin dyes (50mg/kg) for 3 days had no effect on the hepatic function of rats except for the higher ALP activities as compared to normal animals (Table I). When the oral administration was continued for 8 days, serum enzyme levels were unchanged, whereas the whole liver developed black pigmentation. However, the histological sections of the livers of these animals showed no pathological alterations. Treatment of rats with 1% crocin dyes for four months in the diet resulted in slightly increased

Table I. Acute effect of crocin dyes on hepatic functions in rats

Treatment[a]	Serum GOT (mμ/ml)	Serum GPT (mμ/ml)	Serum ALP (mμ/ml)	Serum LDH (mμ/ml)	Serum rGT (mμ/ml)
Control	79.0±2.6[b]	3.9±0.6	60.0±4.6	880±80	4.8±0.4
Crocin dyes (50mg/kg)	84.0±3.5	5.2±1.8	86.0±4.7[c]	1104±118	6.8±0.9

[a] Diets and dye treatments were described in Materials and Methods.
[b] Values are presented as mean±standard error, n=6.
[c] $p<0.01$ as compared with control.

Table II. Chronic effect of crocin dyes on hepatic function in rats

Treatment[a]	Serum GOT (mμ/ml)	Serum GPT (mμ/ml)	Serum ALP (mμ/ml)	Serum rGT (mμ/ml)	Serum LDH (mμ/ml)	Bilirubin, mg/dl	
						Total	Direct
Control	79±14[b]	4.5±1.1	47.5±4.8	2.44±0.6	1288±115	0.18±0.05	0.05±0.01
Crocin dyes	92±14	6.8±1.6	78.2±7.2[c]	8.68±1.03[d]	1044±131	0.38±0.06[e]	0.16±0.06

[a] Diets and dye treatments were described in Materials and Methods.
[b] Values are presented as mean±standard error, n=6.
[c] $p<0.01$; [d] $p<0.001$; [e] $p<0.05$ as compared with control.

Table III. Dose-response of crocin dyes induced hepatic pigmentation in rats

Dose[a] (mg/kg)	Time (day)	No. of rats	Black liver	Liver weight/body weight ($\times 10^2$)
0	40	3	−	2.70±0.04[b]
0.08	40	3	−	2.95±0.11
0.4	40	3	−	2.90±0.13
2	40	3	−	2.95±0.15
10	40	3	−	3.10±0
50	3	6	−	ND
50	8	6	+	ND
50	40	3	+	3.45±0.08

[a] Diets and dye treatment were as described in Materials and Methods.
[b] Values are shown as mean±standard error. ND, not determined.

serum GOT and GPT activities, together with significantly increased ALP, rGT and total bilirubin levels (Table II).

Morphological examination showed that the whole liver was deeply blackened, and seemed to have the signs of a fatty liver. Two rats among the treated group showed white lipocolloid in their hepatic lobe. Histological sections showed a mild fatty matamorphosis with small lipid vacuoles in the cytoplasm here and there.

Dose-dependence of Hepatic Pigmentation induced by Crocin Dyes

Table III. demonstrated that continuous treatment with a daily dose of crocin dyes below 10 mg/kg for 40 days did not produc any black pigmentationg, whild a dose of 50mg/kg for 8 days induced this abnormal pigmentation. The tendency of an increasing relative liver weight in the case of a higher dose was also demonstrated, especially in a dose of 50mg/kg for 40 days. When hepatic pigmentation occurred, the amounts of bilirubin and prophyrin excreted in the urine were substantially increased in doses higher than 10mg/kg. At the same time, the urine became dark greenish when the animal ingested 50mg/kg of crocin dyes.

The reversibility of Hepatic Black Pigmentation

The treatment with a high dose of crocin dyes (100mg/kg) for 14 days produced acute hepatic damage. As shown in Table IV, all the serum enzymes assayed in the experimental animals were significantly increased as compared with the control (Table I). After day 14, the feeding of crocin dyes was stopped and the reversibility of hepatic change was investigated at different time intervals. The residues of crocin dyes in liver, the relative weight of the liver, GOT, GPT, ALP, rGT, LDH and biririubin all gradually decreased and returned to normal within

Table IV. The reversibility of the hepatic pigmentation and serum enzyme alterations

Time course[a]	Hepatic residual crocin dyes (OD/g) $\times 10^2$	Liver weight/ body weight ($\times 10^2$)	GOT (mμ/ml)	GPT (mμ/ml)	ALP (mμ/ml)	rGT (mμ/ml)	LDH (mμ/ml)	Bilirubin (mg/d*l*)
Day 14	3.9	3.7	170	118	148	130	1950	0.62
Day 16	2.9	3.1	168	100	149	98	1750	0.25
Day 19	2.2	3.0	150	99	95	88	1800	0.12
Day 21	0.6	2.5	92	98	62	86	1600	0.12

[a] Rats were fed with crocin dyes (100mg/kg diet) *ad. libitum*. The feeding was stopped on day 14 and shifted to the normal diet. The activities of serum enzymes, bilirubin, relative liver weight to body weight and hepatic residual crocin dyes were studied on day 14, 16, 19 and 21, respectively. All data are the mean values obtained from 3 experimental animals.

Table V. Effect of crocin dyes on the concentrations of urine bilirubin and porphyrins in rats

Dose of crocin dyes[a] (mg/kg)	Bilirubin[b] (mg/d*l*)	Coproporphyrin[b] (mg/24hrs)	Uroporphyrin[b] (mg/24hrs)
0	0.01±0.00	1.96±0.83	1.10±0.22
0.08	0.01±0.00	2.03±1.17	1.37±0.32
0.4	0.03±0.01	2.25±1.92	1.80±0.51
2.0	0.06±0.02	3.31±1.51	1.85±0.47
10.0	0.38±1.20	7.13±1.82	2.42±0.78
50.0	4.22±2.12	8.75±3.21	3.47±0.92

[a] Diets and dye treatments were as described in Materials and Methods.
[b] The biochemical determination were made on day 38 after crocin dyes treatment. All data were expressed as mean±standard error, n=6.

8 days (Table IV). Black pigmentation of the liver gradually returned to the normal color. These results demonstrated that high doses of crocin dyes could produce liver damage in a short time. However, the hepatic injury could recover entirely after stopping the treatment.

Discussion

Administration of 1% crocin dyes in the diet for 4 months did not affect the growth rate of rats, but could produce black pigmentation and acute liver damage as evidenced by the slight elevation of serum enzymes and the fatty metamorphosis change. Crocin dyes (50mg/kg) given continously for 8 days produced black pigmentation in rat liver, but did not induce an alteration of hepatic function. On the other hand, a high dose of crocin dyes (100mg/kg) given for 2 weeks could induce acute liver injury as manifested by the elevation in serum enzymes and black pigmentation, which was proved to be totally reversible in the present study.

A lower dose of crocin dyes (0.08-10mg/kg) for 40 days induced neither black pigmentation nor hepatomegaly (Table IV). During the period of hepatic pigmentation, the urinary porphyrin excretion increased and the urine appeared greenish black (Table V). Yellow pigmentation of rat skin was also observed, this suggested that the hepatic functional disorders caused by hepatic pigmentation may be related to the porphyria[14]. Sinec crocin dyes have a high content of carotenoids, the mechanism of liver black pigmentation may be similar to that of hypervitaminosis A[15].

Both carotenoids and crocin dyes possessed a number of hyperconjugated double bonds, which

were actively involved in the oxido-reduction of cell metabolism and possibly competed with the oxido-reduction enzymes in lipid metabolism. Therefore, the excess of crocin dyes deposited in the liver cells may retard the complete oxidation of fatty acid and induce fatty metamorphosis.

In conclusion, crocin dyes have a low toxicity to rats. Higher doses of the dyes do produce hepatic pigmentation and functional disorders. It is possible that the temporary pigment accumulation results in hepatic functional disorder, since the dose was too high and the experimental animals could not excrete it completely and then hepatic pigmentation, hepatomegaly and functional disorder followed. In lower doses of crocin dyes, rats could accomodate them and maitain a normal metabolism and hepatic pigmentation was absent even after long term feeding.

The concentrations of crocin dyes in various kinds of foods and beverages are estimated to be lower shan 1mg/kg. Therefore, the crocin dyes are generally regarded as safe for food coloring. Moreover, since the synthetic coal tar dyes have been suspected to have mutagenicities, teratogenicities and carcinogenecities, the crocin dyes should be considered as an excellent alterative source of yellow colorant in food technology.

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