Effects of Crocetin on protecting DNA Against Oxidative Damages Induced by Free Radical Generation Systems

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ABSTRACT **AIM** To research the protecting effects of crocetin on DNA damages induced by OH· from free radical generation systems containing Vc or H_2O_2. **METHODS** The protecting effects of crocetin on DNA was detected by the methods of UV-spectrometer, TBA reaction and agarose gel electrophoresis, induced by Cu^{2+} and H_2O_2 or Vc and Fe^{2+} injuring DNA base or DNA desoxyribose. **RESULTS** DNA was damaged by OH· . The absorbance of DNA base at the wavelength of 260 nm decreases. Trace metal ion such as Cu^{2+} or Fe^{2+} could accelerate the reactions. Crocetin can prevent DNA damages induced by these factors. **CONCLUSION** Crocetin has certainly antioxidant effects.

KEY WORDS Crocetin; OH· ; Vitamin C; H_2O_2; DNA base; Damage; Protection; Free radical generation system

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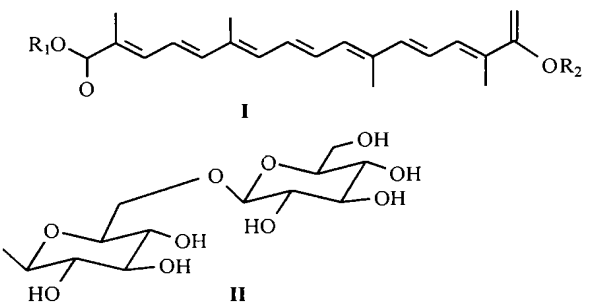
Crocus sativus L, a noble Chinese herbal medicine, has strong physiological activities[1] with stigma as a major pharmaceutical part. As major active ingredients in *Crocus sativus* L, crocetin and crocin have multi-unsaturated conjugate olefine acid structure, belonging to carotenoid species[2] (see Fig 1). It is confirmed that crocin cannot be absorbed well after ig in rat, rabbit, dog and human. However crocetin can be absorbed well. crocetin can reduce the number of cancer cells of many kinds and delay the morbidity[3]. Researches on oncology show that crocetin has antineoplastic activity. It can inhibit the cutaneum carcinoma of gymno-rat induced by 9, 10-dimethyl-1, 2-benzanthracene and outstandingly inhibit the cutaneum carcinoma induced by TPA[4]. It is proved by pharmacology experiments that Crocetin can obviously decrease the side effects of cisplatin and cyclphsophamicle (chemotherapeutical drugs), when used together with cisplatin or cyclophosphamicle[5]. Moreover, crocetin can eliminate oxygen free radical as well[6] and has a remarkable protection against the liver injury by aflatoxin B_1 and other species of acute toxicants[7]. Our experiment shows that crocetin has good curative effects on anti- myocardial ischemia. However, it is rarely domestically or abroad reported whether it protects DNA in vitro most. Hydroxy radical (OH·) is the activest and the most toxic free radical of oxygen free radicals. Thus, we study the protection of crocetin on DNA against OH· induced by two different free radical generation systems(FRGS).



R_1= R_2= H, ⊨ α-crocetin R_1= R_2= II ⊨ crocin

Fig 1. The structure of crocetin and crocin

1 Materials

2-Thiobarbituric acid, 1, 1, 3, 3-tetraethoxypropan, Agarose, Calf Thymus DNA are purchased from Sigma, Vitamin C, DMSO, Mannitol, KI are AR grades. Plasmid PUC_{19} is extracted in our laboratory. And crocetin is a red

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crystal (purity > 80%, HPLC) made in our lab, dissolved in 0.1% DMSO.

2 Methods and Results

2.1 *Effects of crocetin on the DNA chain broken by two FRGS*[8]

2.1.1 *Preparation of Plasmid PUC*19[9]

A ring of mono-colony of *E. coli* is inoculated in LB culture medium (200 ml), and cultured for one night. Bacteria liquid (10 ml per tube) is centrifuged at the speed of 4000 r/min for 10 min and the upper liquid is removed. After dispersion and deposition with solution I (50 mmol/L Glucose, 25 mmol/L Tris-HCl, 10 mmol/L EDTA pH 8.0), add 400 μl of solution II (0.2 mol/L NaOH, 1% SDS), and mix well. Then it is put into ice-bath for 3 min. Add 300 μl of solution III (3 mol/L potassium acetate, 5 mol/L acetate) and mix well, then put into in ice-bath for 3 min again. Centrifuge at the speed of 12000 r/min for 5 min. Take out the upper liquid, add twice volume of the liquid of ethanol and deposit it for 2 min. Centrifuge it at the speed of 12000 r/min for 5 min, and remove the upper liquid. Dry the deposit, and add 70 μl of TE solution (10 mmol/L Tris-HCl, 1 mmol/L EDTA pH 8.0) to the limit. At last, all the extracted plasmids are combined with the final volume to be 840 μl.

2.1.2 *The system of Cu^{2+} and H_2O_2*

In the solution of Tris-HCl (20 mmol/L, pH 8.0), H_2O_2 is 0.03%, the concentration of Cu^{2+} is 200 μmol/L, the concentrations of crocetin are 20, 10, 5 μmol/L and the concentration of KI is 110 mmol/L. Dilute the extracted plasmid PUC19 by loading buffer (0.25% Bromophenol blue, 40% Saccharose), transfer 5 μl of the dilution, and keep it at 37℃ for 30 min. Electrophoresis voltage is 100 V with 1.2% agarose gel. And Electrophoresis buffer is 0.04 mol/L Tris-Acetate, 0.001 mol/L EDTA. After electro-phoresis, agarose gel is put into 0.5 μg/ml ethidium bromide dyeing solution for 30 min at the room temperature. Then agarose gel is taken out and washed with distilled water. The washing time is 10 min. Take photos and record under UV light (see Figure 2).

Fig 2. Effects of crocetin on DNA chain broken by the system of Cu^{2+} and H_2O_2

2.1.3 *The system of Vc and Fe^{2+}*

In the solution of Tris-HCl (20 mmol/L, pH 8.0, the concentration of Vc is 1.25 mmol/L, the concentration of Fe^{2+} is 200 μmol/L, that of KI is 110 mmol/L, and that of the crocetin is 20, 10, 5 μmol/L. Dilute the extracted plasmid PUC19 by one time, transfer 5 μl of the dilution, and keep it at 37℃ for 30 min. The conditions of electrophoresis and Ethidium Bromide dyeing are as identical as above. Take photos and record under UV light (see Figure 3).

Fig 3. Effects of crocetin on DNA chain broken by the system of Fe^{2+} and Vc

It is showed in the two figures that double chains broke in the model group, while the crocetin solution (20 μmol/L) had a remarkable protective activity: in the systems of H_2O_2 & Cu^{2+}, only one chain broke, and in the systems of Vc& Fe^{2+}, some parts of double chains did not break at all. In both free radical generation systems, the crocetin solutions (10, 5 μmol/L) have certain protective activities.

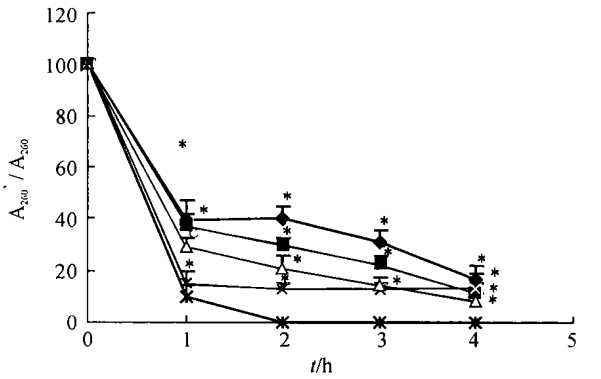
2.2 *Effects of crocetin on DNA base injuries induced by two FRGS*

2.2.1 *The systems of Cu^{2+} and H_2O_2*

In the buffer solution of PBS, the final concentration of thymus DNA from calf is 60 mg/L with 0.3% H_2O_2, 25 μmol/L Cu^{2+} and 0.5 mol/L mannitol. The final concentrations of the Crocetin solutions are separately 20, 10, 5 μmol/L, respectively. Keep the solutions at 37℃, sampling it at certain time each and determine the absorbances at the wavelength of 260 nm (see Fig 4). Statistical analysis of data was carried out by using Students' *t* test.

2.2.2 *The system of Vc and Fe^{2+}*

In the buffer solution of PBS, the concentration of thymus DNA from calf is 60 mg/L, that of Vc is 0.05 mmol/L, that of Fe^{2+} is 5 μmol/L, that of DMSO is 1.7 mol/L, and the concentrations of the crocetin solutions are 20, 10, 5 μmol/L respectively. Keep the solutions at 37℃, sampling it at certain time each and determine the absorbances at the wavelength as 260 nm (see Tab 1). Statistical analysis of data was carried out by using Students' *t* test.



—◆— 20μmol/L,—■— 10μmol/L,—▲— 5μmol/L,
—×— Man,—*— Model

Fig 4. Effects of crocetin on DNA base damage induced by Cu^{2+} and H_2O_2

* $P < 0.01$ *vs* Model (n= 6, A_{260}: 0 h; A_{260}': T h

Tab 1. Effects of crocetin on DNA base injury induced by Fe^{2+} and Vc (A_{260}'/A_{260}, n= 6)

Time (min)	Crocetin (20μmol/L)	Crocetin (10μmol/L)	Crocetin (5μmol/L)	DMSO (1.7 mol/L)	Model
0	100.00± 5.36	100.00± 5.36	100.00± 5.36	100.00± 5.36	100.00± 5.36
15	46.43± 1.52*	45.19± 0.80*	45.17± 1.08*	43.94± 0.72*	39.66± 0.96
30	47.17± 2.23*	45.23± 1.71*	42.98± 1.04*	43.07± 0.43*	38.14± 1.99
45	45.83± 1.29*	43.57± 1.14*	43.07± 1.13*	41.82± 1.07*	38.14± 1.04
75	44.04± 0.47*	42.84± 1.86*	41.87± 0.73*	42.01± 0.74*	37.22± 0.79
135	46.48± 0.07*	43.94± 0.42*	43.71± 1.28*	42.47± 0.90*	37.31± 1.54

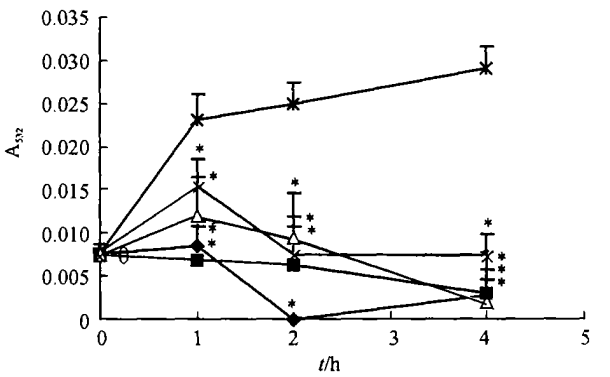
* $P < 0.01$ *vs* model, A_{260}= 0 h, A_{260}'= T h

It is shown in two figures that under the action of OH· produced by the two free radical systems, the DNA absorbance of model group at the wavelength of 260 nm decreases with the extending of time, especially in the period of 0~ 1 h, explaining that the conjugate structure of base is destructed. The 20, 10, and 5μmol/L of crocetin could obviously inhibit the fall of the DNA absorbance at 260 nm, which proved that crocetin protected the conjugate structure of DNA base.

2.3 *Effects of Crocetin on DNA desoxyribose injuries induced by two FRGS*

2.3.1 *The system of Cu^{2+} and H_2O_2*

In the buffer solution of PBS, the concentration of thymus DNA from calf is 240 mg/L, 0.6 percent of it is H_2O_2, the concentration of Cu^{2+} is 50μmol/L, that of mannitol is 0.5 mol/L, and the concentrations of crocetin solutions separately are 20, 10, 5 μmol/L. Keep the solutions at 37℃, sample it at certain time each and determine MDA by the method of Thiobarbital acid[5] (TBA) (see Fig 5). Statistical analysis of data was carried out by using Students' *t* test.



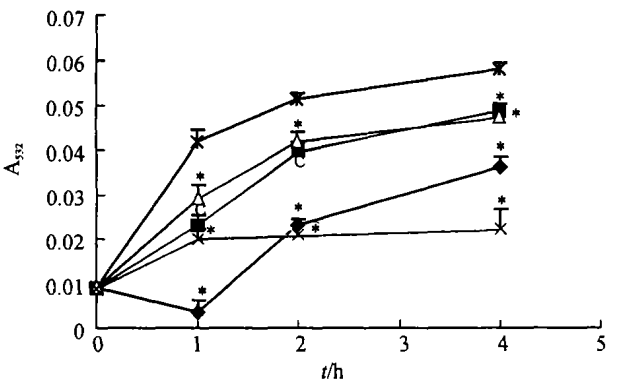
—◆— 20μmol/L,—■— 10μmol/L,—▲— 5μmol/L,
—×— Man,—*— Model

Fig 5. Effects of crocetin on DNA desoxyribose damage induced by Cu^{2+} and H_2O_2

* $P < 0.01$ *vs* Model ($n = 6$)

2.3.2 *The system of Vc and* Fe^{2+}

In the buffer solution of PBS, the concentration of thymus DNA from calf is 240 mg/L, Vc is 0.05 mmol/L, Fe^{2+} is 5μmol/L, DMSO is 1.7 mol/L, and the concentrations of Crocetin solutions are 20, 10, and 5μmol/L respectively. Keep the solutions at 37 ℃, sample it at certain time each and determine MDA by the method of Thiobarbital acid (TBA) (see Fig 6). Statistical analysis of data was carried out by using Students' *t* test.



—◆— 20μmol/L,—■— 10μmol/L,—▲— 5μmol/L,
—×— Man,—*— Model

Fig 6. Effects of crocetin on DNA desoxyribose damage induced by Fe^{2+} and Vc

* $P < 0.01$ *vs* Model ($n = 6$)

By means of TBA reaction, it is determined the relative content of malonaldehyde analogues produced by oxygen free radical attacking to desoxyribose of DNA. Absorbtion of model group at 532 nm increased rapidly, proving that the desoxyribose has been oxidized, with the amount of malonaldehyde analogues as oxidation product increased sharply, while, the 20, 10 and 5μmol/L of crocetin solution groups could obviously reduce the value of A_{532nm}, that is, crocetin could obviously inhibit the desoxyribose oxidation induced by OH·.

3 Discussion

It is proved that the process of carcinogenesis is a complex and multi-stages process. Chromosome transgenation is resulted from DNA injury and cancer gene expression started by free radical reactions, thus make body suffer from cancers[10].

Fenton reaction produced OH·

Ascorbic acid + $2H^+$ + $2O_2^-$· ⟶ H_2O_2 + Dehydroascorbic acid

Fe^{2+} + H_2O_2 ⟶ Fe^{3+} + OH· + OH^-

It could be seen from the above reaction equation, a small amount of ascorbic acid may clean superoxide anion free radical ($2O_2^-$·) and possess protected property. But with the amount of ascorbic acid increased, the system produced mmore H_2O_2 and react with Fe^{2+} to generate hydroxide radical (OH·). The characteristic of this free radical is unstable, short half-life period, and high injury activity. The injury on DNA by OH· can be included in following three major aspects:

1. OH· attacks PUC19, break double chains or break single chain.

2. OH· attacks DNA base, destruct its conjugate structure and decrease the absorbance of DNA base at 260 nm.

3. OH· attacks desoxyribose of DNA, generate ribose radical by dehydroreacting, open deoxyribose ring and increase the absorbtion of malonaldehyde anlogues at 532 nm.

Crocetin is a kind of carotenoid, which can inhibit the injury of biology molecule induced by free radicals[11]. Researchs *in vitro* prove that crocetin can significantly eliminate OH°, whose effects are as good as mannitol, a specific scavenger. Crocetin plays a significant inhibition role in the double chains breakage of DNA destruction of base conjugation structure and the breakage and oxidation of saccharide ring from desoxyribose caused by free radical generation systems. DNA protectioni and strong eliminate OH° function of crocetin may play an important role in the occurrence and development of tumor.

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西红花酸对自由基发生系统诱导 DNA损伤的保护作用

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摘 要 目的 研究西红花酸对不同自由基发生系统诱导的 DNA损伤的保护作用。方法 通过葡聚糖凝胶、TBA反应、紫外碱基吸收来检测西红花酸对 DNA的保护作用。结果 DNA被 OH° 进攻损伤后,碱基在 260 nm处紫外吸收下降, DNA脱氧核糖氧化破坏,其氧化产物与 TBA反应在 532 nm处出现特征吸收, DNA的链结构破坏,痕量的金属离子可以加速 DNA的损伤。西红花酸可以显著抑制 DNA的上述损伤。结论 西红花酸具有很强的抗自由基作用,对 DNA具有保护作用。

关键词 西红花酸; OH°; Vc; 过氧化氢; DNA碱基;损伤;保护;自由基发生系统