Vol. 12/1 (2012) 12-19

JOURNAL OF NATURAL REMEDIES

Crocetin ameliorates TNBS inducd chronic colitis in rats by inhibiting expression of Cyclooxygenase-2

Hamid Ali Kazi, Zhi yu Qian. Lei song, Ruhui yang.

1. Department. of pharmacology, China pharmaceutical university, Nanjing, P.R.China.

2. Department. of pharmacology, China pharmaceutical university, Nanjing, .P.R.China.

3. Professor zhi yu qian, NO: 24 Tong Jia Xiang. Dept: of pharmacology, China pharmaceutical university, Nanjing, China.

Abstract

The aim of this study was to explore the possible mechanisms of crocetin affecting rat colitis induced by 2, 4, 6-trinitrobenzene sulfonic acid (TNBS). Rats were treated with crocetin. 5-aminosaliylic acid was used as a positive control. Change of body weight was evaluated . Leukocyte infiltration was detected by myeloperoxidase activity assay. The expression of cyclooxygenase-2 was detected by RT-PCR and Western blot. Inflammatory cytokines were determined by RT-PCR. Local concentration of prostaglandin E_2 in colon mucosa was determined by ELISA. Crocetin decreased the macroscopic scores and myeloperoxidase activity. Crocetin also reduced the expression of COX-2 and inflammation cytokines. In addition, treatment with crocetin increased the PGE_2 level. Moreover, the effects of crocetin were almost comparable with that of positive control drug. Crocetin has therapeutic effects on TNBS-induced colitis; the mechanisms seem to be related to COX-2 inhibition and PGE_2 improvement.

Keywords: Crocetin, TNBS, Colitis, COX-2, PGE_2, 5-ASA.

1. Introduction

Ulcerative colitis is characterized by chronic recurrent ulceration of the colon. Frontline drugs that are currently used for the treatment of ulcerative colitis include derivatives of 5-aminosalicylic acid, glucocorticoid and immunosuppressives. Crocetin, a kind of carotenoid is a pigment found in the dried stigmas of saffron (*Crocus sativus* L.) and the fruits of Gardenia jasminoides Ellis. The yellow color of crocetin is used in many foods as a natural colorant. Saffron and the fruits of Gardenia jasminoides Ellis have been used as traditional herbal medicine and crocetin is one of the major active ingredients of these herbal medicines. Since ancient times saffron is known as the Royal Spice and even nowadays it's the most expensive spice in the world. In animal studies, it has been shown to have multiple pharmacological actions, including the inhibition of tumor formation [1]. antihyperlipidemia [2].

* Corresponding author
Email: 1523967778@qq.com

antiatherosclerosis [3]. protection against hepatic damage [4]. has excellent antioxidant effect [5].

Mechanisms by which crocetin exerts its pharmacological effects are thought to involve down-regulating the expression of intracellular adhesion molecule-1 [6]. interference with the activity of transcription factors such as Nuclear factor kappa B [7]. and inhibition of mRNA Expression for Tumor Necrosis Factor-α, Interleukin-1β, and Inducible Nitric Oxide Synthase [8].

2. Methods and Materials

2.1 Plant material and preparation of crocetin

Crocetin (Fig.1) is a major ingredient originally found in the dried stigma of *Crocus sativus* L (Saffron). To avoid the high costs of saffron, the fruit of Gardenia jasminoides Ellis (Rubiaceae) has been used as a substitute resource of the active compound for more than fifteen years in our laboratory. The fruit of Gardenia jasminoides Ellis was used to extract the crocetin (98%, HPLC) by the method of Liang [9].

2.2 Experimental animals

Albino Sprague-Dawley rats weighing 200-220 g were obtained from Animal Laboratory Center Nanjing, China and kept at constant temperature (23 ± 2°C) and humidity (50%-70%) in a 12 h light and dark cycle. The rats were allowed to adapt to laboratory environment for one week before experiment with free access to standard rodent chow and tap water.

2.3 Induction of colitis

Colitis was induced by TNBS using the modified method described by Morris [10]. In short, rats were fasted one day before induction of colitis. Each rat was anesthetized with ether, and TNBS (Sigma, St. Louis, MO) 25 mg in1 ml of 30 % ethanol was then instilled via a rubber catheter inserted 6 cm into the colon *via* the anus. The rubber catheter was modified with numerous holes in the last 3 cm of its length. The instillation procedure required only a few seconds and the rats were maintained in a vertical position for 2 min to prevent solution leakage. Control rats received 30% ethanol of the same volume using the same technique. All the rats were checked daily for behavior and body mass. The experiments were approved by the Institution's Animal Care and Use Committee.

2.4 Treatment protocol

Crocetin (> 98%, HPLC) was from our lab. The rats were randomly divided into four groups. Control group (n = 15, receiving ethanol only and no treatment), TNBS group (n = 15, receiving TNBS and no treatment), Crocetin +TNBS group (n = 15,receiving 50 mg/kg/day ig). positive control group 5-Aminosalicylic acid+TNBS group (n=15, 5-ASA 100 mg/kg/day, ig); On day 1, the rats were fasted, colitis was induced and treatment with crocetin and 5-aminosalicylic acid was begun on day 2 till the end of experiment. Control group received same volume of 30 % ethanol rectally while crocetin and 5-Aminosalicylic acid were dissolved in 30% ethanol everyday before administration until the end of the experiment for two weeks. Later the rats were sacrificed.

2.5 Assessment of severity of colitis

After rapid removal of the colon, specimens were flushed with ice cold PBS, cut open and photographed. The photographs of the colonic specimens were then scored by a blinded observer unaware of the treatment. Scores were assessed by using the following damage scoring system [10]. 0: no damage; 1: localized hyperemia without ulcers; 2: linear ulcers with no significant inflammation; 3: linear ulcer with inflammation at one site; 4: two or more sites of ulceration and/or inflammation; 5: two or more major sites of inflammation and ulceration

or one major site of inflammation and ulceration extending more than 1 cm along the colon.

2.6 Assessment of leukocyte infiltration

Myeloperoxidase activity was assessed as a marker of neutrophil infiltration. Rat colon samples were snap frozen in liquid nitrogen and stored at -80°C before myeloperoxidase activity assessment. The tissue was thawed, weighed and homogenized in PBS, the homogenate was centrifuged and the pellet was again homogenized in PBS containing 5 g/L hexadecyl-trimethylammonium bromide (HETAB) and 10 mmol/L EDTA. This homogenate was subjected to three cycles of freezing/thawing and brief sonication. A sample of homogenates was added to reaction volume (containing 80 mmol/L PBS, pH 5.4, 5 g/L HETAB and 1.6 mmol/L 3, 3', 5, 5'-tetramethylbenzidine). The mixture was incubated at 37°C for 5 min and the reaction was started by the addition of H_2O_2 The complete reaction mixture was incubated for exactly 3 min at 37°C and terminated by the sequential addition of catalase and sodium acetate. The changes in absorbance at 655 nm were measured with a spectrophotometer.

2.7 Semi-quantitative RT-PCR

For RT-PCR, rat colon samples were snap-frozen in liquid nitrogen and stored at-80°C before ribonucleic acid (RNA) preparation. Total RNA was isolated using the TRIzol method (Life Technologies, Canada). Concentration of the RNA was detected from A260, and the integrity of the RNA was verified by electrophoresis on formaldehyde gels. Total RNA was reverse-transcribed into complementary deoxyribonucleic acids (cDNAs) using a first strand cDNA synthesis kit (Fermentas, Life Sciences). The resultant cDNAs were subjected to PCR for measurement of messenger RNAs (mRNAs). The PCR products were subjected to agarose gel electrophoresis and the abundance of each mRNA was normalized to that of GAPDH. The sequences of all primers used inthis project are as following:

GAPDH: sense: 5'ATGGGTGTGAACCACGAGAAA-3',

anti-sense: 5'GGATACATTGGGGGTAGGAA-3'(330 bp);

CYCLOOXYGENASE-2: sense: 5'-TACAAGCAGTGGCAAAGGC-3'

anti-sense: 5'-CAGTATTGAGGAGAACAGATGGG-3'(304 bp);

TNF-α sense: 5'-TACTGAACTTCGGGGTGATTGGTCC-3',

anti-sense: 5'-CAGCCTTGTCCCTTGAAGAGAACC-3'(295 bp);

IFN-γ sense: 5'AGCCTAGAAAGTCTGAAGAAC-3',

anti-sense:5'ACCGACTCCTTTTCCGCTTCCT-3'(387 bp)

iNOS: sense:5'-TGAAGCACATGCAGAAATGAGTACCG-3',

anti-sense: 5'-CCGTCAGAGGTAACTGTTTACACG-3' (464 bp)

2.8 Western blot analysis

Western blot analysis was performed by standard methods. Briefly, frozen tissue samples were homogenized in Tris- HCl buffer containing a cocktail of protease inhibitors and insoluble materials removedby centrifugation at 4°C. The solubilized lysates were resolved by sodium

dodecyl sulfate (SDS)-PAGE electrophoresis under reducing conditions at a concentration of 50 µg protein of each sample per lane. Nitrocellulose membranes were incubated overnight with rabbit anti-serum directed against COX-2 (Santa Cruz Biotechnology). Immunodetection with secondary peroxidase-conjugated antibody and chemiluminescence was performed according to the manufacturer's protocol (Santa Cruz Biotechnology). Density of the products was quantified.

2.9 Enzyme-linked immunosorbent analysis (ELISA)

Colonic mucosal samples kept at -80°C were weighed and homogenized on ice in 1 mL Tris-HCl buffer containing 5.6 mmol/L indomethacin (pH 7.4), the homogenate was vortexed thoroughly for at least 2 min. After centrifugation, the concentration of PGE_2 in supernatants was immediately determined with competitive ELISA kits (R&D), according to the manufacturer's instructions. Prostaglandin E_2 levels were normalized to microgram of protein. The detection limit of PGE_2 was 39 ng/L.

2.10 Statistical analysis

Data were expressed as mean ± S.E.M The statistical significance was evaluated by one-way analysis of variance (ANOVA) followed by Dunnett's test. $P < 0.05$ was considered statistically significant.

3. Results

3.1 Effect of crocetin on Body mass

Administration of TNBS dramatically decreased body mass of all groups.(data not shown) Most of them decreased more than 25 g after 3 day. Control group showed transient and slight loss of body weight (less than 10 gm and recovered quickly. From day 3 it began to show significant difference from TNBS group ($P < 0.05$). At the end of the experiment, except for TNBS group, body weight of the other groups reached the initial level. Slight difference was observed among control, crocetin and positive control group at the end of the experiment. Six rats from TNBS group, while three rats from each crocetin and positive control (5-aminosalicylic acid) groups died during the course of experiment.

3.2 Effect on Macroscopic scores

TNBS induced colitis was characterized by thick and stiff colonic wall due to edema, or/ and fibriform-proliferation. Ulcers were scattered along the colon and linked with each other, bleeding or redness was observed in whole or partial colon. Necrosis of epithelium, distortion of crypts, damage to glands and infiltration of inflammatory cells were observed. The macroscopic score in crocetin group was significantly lower than those in TNBS group ($P < 0.01$) Treatment with crocetin and 5-Aminosalicylic acid decreased edema. Ulcers in crocetin treated group were much smaller and superficial, most of the ulcers were healed and granulation or fibroplasias could be seen.(Table 1)

3.3 Leukocyte involvements

Myeloperoxidase activity was significantly ($P < 0.01$) increased in TNBS-treated animals compared with control group (102 020±17 16nkat/g and 250 382±42 34 nkat/g tissue protein, respectively (Table 2), which was consistent with the macroscopic index. Treatment with crocetin (50 mg/kg/day) significantly reduced the degree of polymorphonuclear and neutrophil infiltration in rats with TNBS-induced colitis.

3.4 COX-2 and PGE2 expression

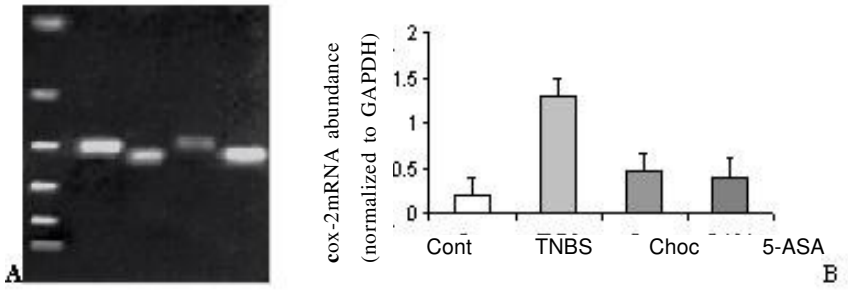
Weak expression of COX-2 mRNA could be detected in normal colon (Fig. 2)After colitis was induced by TNBS, its expression increased

more than 10-fold.Treatment with crocetin decreased its expression by 70%, but was still higher than normal ($P < 0.05$). Western blot analysis showed the similar changes of protein in colon mucosa (Fig. 2). After colitis was induced, Prostaglandin E_2 decreased and its expression was increased after crocetin treatment.

3.5 Expression of inflammatory mediator genes

Abundance of Interferon-γ, Tumor necrosis factor-α and iNOS mRNA in TNBS group was significantly elevated compared to control group.5-ASA and crocetin treatment suppressed the increased mRNA efficiently, but its level was still higher than normal (Table 2).

Fig. 1. Crocetin



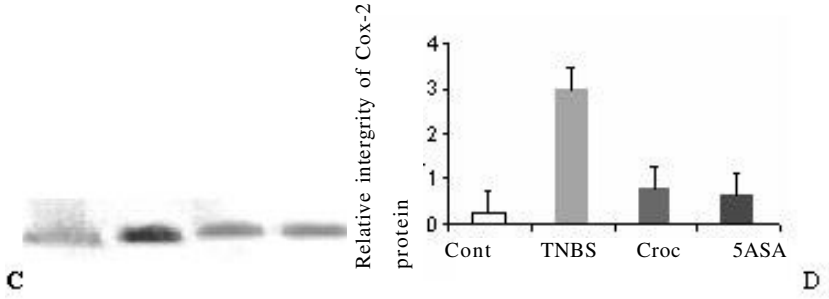


Fig. 2. COX-2 expression in rats with TNBS -induced colitis. **A**: COX-2 mRNA; **B**: COX-2 products; **C** and **D**: COX-2 protein (*M,*73 000) (mean ± S.E.M, Dunnett's *t* test).$P < 0.01$ *vs* control; $P < 0.01$ *vs* TNBS

Table 1:

Effect of Crocetin treatment on colonic mucosa following administration of TNBS

	Colonic Erosion Scale					
	0	1	2	3	4	n
Control group	8	7	0	0	0	15
TNBS group	0	4	3	5	3	15
Crocetin group	5	7	1	1	1	15
5-ASA group	6	8	1	0	0	15

Macroscopic scores graded from 0 to 4 as described by Morris *et al* (1989)
The scores for crocetin group were significantly lower than that of TNBS group rats ($P<0.01$).

Table 2:

	Tissue PGE2 (ng/g)	protein MPO (knat/g)	TNF-α	mRNA IFN-γ	iNOS
Control	177.3±28	102 020±17 16	0.0895±0.009	0.0384±0.014	0.9304±0.1179
TNBS	79.5±12	250 382±42 34	0.9861±0.043	1.0212±0.058	7.5690±0.9350
Crocetin	191.2±16	122 691±22 17	0.2967±0.026	0.0565±0.016	2.8638±0.2347
5-ASA	193.5±19	120.211±27.25	0.2119±0.035	0.0443±0.011	2.1012±0.2115

Concentration of PGE_2, activity of MPO in colon tissue and mRNA in inflammatory response (mean ± S.E.M).

4. Discussion

Treatment with crocetin reduced the severity and extension of damage induced by TNBS, decreased the extent of colitis and increased the survival rate and the incidence of adhesions. The rats treated with crocetin had improved histological image and less neutrophil infiltration in colon mucosa. The expression of COX -2 increased in colitis, suggesting that over-expression of cyclooxygenase-2 may result in neoplasia [11]. Some reports indicate that selective COX-2 inhibitors exacerbate colitis [12]. but others indicate that they have therapeutic effects on colitis [13]. In our study, the expression of COX-2 increased in colon following the induction of colitis, suggesting that high expression of COX-2 in the colon is associated with high myeloperoxidase activity and cytokine production. Crocetin decreased the level of COX-2, indicating that decreasing the expression of COX-2 may benefit in colitis.

Prostaglandin E_2, an inflammatory mediator, participates in epithelium repairing and exerts anti inflammation effects including suppression of neutrophil function or prevention of mast cell degranulation. Recently, prostaglandin E_2 has been implicated in the inhibition of the productionof Interferon-γ, Interleukin-2 and Interleukin-12 [14]. Level of prostaglandin E_2 can manifest the exacerbation or amelioration of ulcerative colitis [15]. Treatment with crocetin increased the level of Prostaglandin E_2 though its limiting enzyme COX-2 decreased, indicating that crocetin can change the expression spectrum of prostaglandins in colitis. TNBS-induced chronic intestinal inflammation mimics human ulcerative colitis, showing high interferon-γ, tumor necrosis factor-α and iNOS, but low levels of Interleukin-4 and Interleukin-5 [16]. Treatment with crocetin decreased Interferon-γ, Tumor necrosis factor-α and

iNOS , which is consistent with our previous evaluation of effects of crocetin on murine acute colitis, in which 50 mg/kg/day dose proved to be the most effective among 25,50 and 100 mg/kg/day doses of crocetin,7 leading us to adopt only 50 mg/kg/day dose in this study of rat model of chronic colitis. Since prostaglandin E_2 is capable of inhibiting Th1 cytokines, the decreased Interferon-γ, tumor necrosis factor-α and iNOS in this study may be due to prostaglandin E_2. Patients of ulcerative colitis are at risk of developing dysplasia and neoplasia. So besides reducing chronic colitis, crocetin is also a chemopreventive drug hence reduces cancer cell proliferation and tumor progression by a known mechanism [17]. in various studies.

The free radical induction theory of colitis proposes that 5-aminosalicylic acid is serving not just as an anti-inflammatory, but also as a free radical trap, destroying the hydroxyl and other radicals that may damage colonic epithelial barrier [18]. The pharmacokinetic and toxicological studies are evident that crocetin is safe even after chronic use and it has no toxicological effects on vital organs in both animal and human studies [19-20].

The paradigm of single target therapy is fast changing, and drugs that target multiple pathways are becoming common. It is now becoming increasingly apparent that the underlying molecular bases for some human diseases are far more complex and frequently involve immune, genetic as well as environmental factors [21-22]. With the completion of the human genome project and with our advancing knowledge about the function of individual genes and the different signaling pathways they regulate, it has become imperative to adopt a multi-target-based drug development paradigm for the treatment of complex human diseases [23].

5. Conclusion

crocetin attenuates ulcerative colitis in rats by decreasing the expression of COX-2 and increasing prostaglandin E_2. It seems crocetin can down regulate the expression of inflammatory genes in colitis induced by TNBS by multiple systemic pathways.

References

1.Wang, C.J., Cheng, T.C., Liu, J.Y., Chou, F.P., Kuo, M.L., Lin, J.K.Molecular Carcinogenesis (1996) 17, 235-240. PMID: 8989917.

2. Lee, I.A., Lee, J.H., Baek, N.I., Kim, D.H. *Biological and Pharmaceutical Bulletin* (2005) 28, 2106-2110

3. Zheng, S., Qian, Z.-Y., Tang, F. Liang,sheng. .*Biochemical Pharmacolog* (2005). 70, 1192-1199. PMID: 16154540

4. Wang, C.J., Shiow, S.J., Lin, J.K. Carcinogenesis (1991) 12, 459-462. PMID: 1672627.

5. Kanakis, C.D., Tarantilis, P.A., Tajimir-Riahi, H.A., Polissiou, M.G. *J. Agric. Food Chem.* (2007)55, 970–977.

6. Xiang M, Qian ZY, Zhou CH, Liu J, Li WN. *Journal of Ethnopharmacology* (2006) 107 25–31. PMID: 16529888

7. Hamid A, kazi, Zhi yu qian , *The Saudi journal of gastroenterology* (2009) (15)3; 181-7, PubMed ID: DOI: 10.4103/1319-3767.54750

8. Ya ng R, Tan X, Thomas AM, Shen J, Qureshi N, Morrison DC, *et al . Journal of Parenteral and Enteral Nutrition*, (2006) Vol. 30, No. 4, 297-301.PMID: 16804126

9. Liang Xi, Zhiyu Qian, Xiangchun Shen, Na Wen, Yabing Zhang**.** *Planta Med* (2005)71: 917-922

10. Morris GP, Beck PL, Herridge MS, Depew WT, Szewczuk MR, Wallace JL. Gastroenterology; (1989) 96:795-803. PMID: 2914642

11. Brown JR, DuBois RN. *J Clin Oncol* (2005)23: 2840-2855

12. Radaelli F, Feltri M, Meucci G, Spinzi G, Terruzzi V, Minoli G. Dig Liver Dis; (2005) 37: 372-376. PMID: 15843089.

13. Martin AR, Villegas I, Alarcon de la Lastra C. Inflamm Res; (2005) 54: 145-151 PMID:15883736.

14. Harris SG, Padilla J, Koumas L, Ray D, Phipps RP, Trends Immunol; (2002) 23:144-150

15. Tanaka K,I, Shintaro S, Tomoaki I, Yuichi T, Yasuhiro A, Tohru M. *European Journal of Pharmacology* (2009) 603, 120–132. PMID: 19101538.

16. Egi H, Hayamizu K, Yoshimitsu M, Shimamoto F, Oishi K, Ohmori I, *et al* . Cytokine (2003) 23: 23-30 PMID: 12901851.

17. Animesh D, Smita M, Gopal D, Kakali D, Snigdha B, Peter, V, V, *et al*. *Mol Cancer Ther* (2009.)February 8:315-323.

18. Pravda J *World J. Gastroenterol.* (2005)11 (16): 2371–84. PMID 15832404

19. Asai, A., Nakano, T., Takahashi, M., Nagao, A. *J. Agric. Food Chem.* (2005) 53, 7302–7306.

20. Umigai, N.Murakamia, K..Ulit M.V b, Antoniob L.S., M.Shirotori, H,Morikawa *et al.phytomedicine*

21. Loktionov, A., *J. Nutr. Biochem*., (2003) 14, 426 –451.

22. Reich, D. E., Lander, E. S., *Trends Genet*., (2001) 17, 502 –510.

23. Keith, C. T., Borisy, A. A., Stockwell, B. R. . *Nat. Rev. Drug Discov*., (2005) 4, 71–78. PMID: 15688074