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Crocetin improves endothelium-dependent relaxation of thoracic aorta in hypercholesterolemic rabbit by increasing eNOS activity

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ABSTRACT

Our previous studies have proven that crocetin (CCT), extracted from *Gardenia jasminoides* Ellis, possesses the anti-atherosclerotic effect. Because endothelial dysfunction strongly contributes to the initiation and progression of atherosclerosis, the present study aims to investigate whether CCT is capable of improving this dysfunction and to explore the possible mechanisms. Endothelial dysfunction was induced by in vivo feeding high cholesterol diet (HCD) to rabbit and by in vitro treating bovine aortic endothelial cells (BAECs) with oxidized LDL (oxLDL). Endothelium-dependent relaxation (EDR) evoked by acetylcholine (Ach) and endothelium-independent relaxation (RIDR) mediated by sodium nitroprusside (SNP) of thoracic aorta isolated from rabbit were measured. The results indicated that the EDR in HCD alone treated rabbits was seriously impaired and the maximal relaxation induced by Ach ($10^{-5.5}$ M) was only 54% that in control rabbit fed with regular diet. Oral complementation with CCT (15, 30 mg/kg) dose-dependently improved this impairment and restored the maximal relaxation to 68% and 80% that in control group, respectively. However, the EIDR maintained comparable in all groups. Complementation with CCT (15, 30 mg/kg) simultaneously increased serum level of nitric oxide (NO), upregulated vessel activity and mRNA expression of endothelial NO synthase (eNOS) as well as vessel cyclic GMP (cGMP) content compared with those in rabbit treated with HCD alone. Inducible NOS (iNOS) activity remained unchangeable in all groups. In BAECs, oxLDL treatment decreased NO production, downregulated both activity and mRNA expression of eNOS. While those decrease or downregulation were inhibited by co-treatment with CCT (0.1, 1, 10 μM) in a dose-dependent manner. These findings suggested that CCT significantly restored the EDR of thoracic aorta in hypercholesterolemic rabbit, which might be explained by its action to increase the vessel eNOS activity, leading to elevation of NO production.

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1. Introduction

The vascular endothelium plays an important role in the control of blood flow and thus tissue oxygenation by means of the release of nitric oxide (NO) [1]. Consequently, an impairment of this endothelial function appears to play a key role in several cardiovascular diseases, particularly in atherosclerosis (AS) [2,3]. The impairment already occurs at an early state of the disease [4], preceding macroscopically visible lesions

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which are characteristic of advanced states [5]. Unlike the vascular lesions, defects of endothelial function can be detected not only in conduit arteries but also in resistance vessel [6]. Earlier studies [7,8] confirmed that while receptor-mediated endothelium-dependent relaxation (EDR) to acetylcholine (Ach) as well as receptor-independent responses to calcium ionophore A23187 was lost, endothelium-independent agents such as the NOs donor sodium nitroprusside (SNP) elicited normal vasodilation in thoracic aorta of hypercholesterolemic rabbit. These observations implied the site of inhibition of EDR to be distal to receptor-mediated events and proximal to the activation of the vascular smooth muscle, suggesting that impaired enzymatic synthesis of NOs could be one of the mechanisms causing endothelial dysfunction. NO is produced by an enzyme known as NO synthase (NOS) which converts L-arginine to L-citrulline and NO [9,10]. There are two isoforms of NOS in endothelial cells, endothelial NOS (eNOS) and inducible NOS (iNOS). NO originated from eNOS is thought to play a pivotal role in maintaining the vasorelaxation. Ooboshi et al. [11] reported that overexpression of eNOS gene in AS animals improved the EDR by increasing NO production. Otherwise, chronical administration of NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) to rabbits resulted in reduced NO production and completely abolished the EDR evoked by Ach [12]. Therefore, alleviation of endothelial dysfunction by increasing NO production would be beneficial to prevention and treatment of AS. It is likely that oxidized low density lipoproteins (oxLDL) which accumulate in the arterial wall in hypercholesterolemia subjects play a causal role in the reduction of NO production through downregulation of eNOS mRNA expression [13,14], suggesting a possible mechanism by which oxLDL contributes to impairment of EDR and consequently to AS plaque formation.

Crocetin (CCT), a carotenoid extracted from *Gardenia jasminoides* Ellis, is widely used for the prevention and treatment of cardiovascular diseases, largely depending on its anti-oxidation effect [15]. We have previously reported that CCT exerted the anti-AS action on animal models of both rat and rabbit [16,17]. The mechanism exploration showed that the inhibition of oxLDL production and consequently the alleviation of injury to vascular cells caused by oxLDL might be partly responsible for the anti-AS action of CCT [18]. Because endothelial dysfunction strongly contributes to the initiation and progression of atherosclerosis, the present study aims to investigate whether CCT is capable of improving this dysfunction of thoracic aorta isolated from hypercholesterolemic rabbit and to explore the possible mechanisms.

2. Materials and methods

2.1. Drugs and chemicals

Crocetin (CCT) was extracted from *G. jasminoides* Ellis in our laboratory and its purity is over 98% (assayed by high performance liquid chromatography, HPLC) (chemical structure shown as Fig. 1). Phenylephrine (PE), acetylcholine hydrochloride (Ach), sodium nitroprusside (SNP), indomethacin were from Sigma (USA). DMEM culture medium was from Gibico Company. Newly born bovine serum (NBS) was provided by Shanghai Weike Biochemical Reagent Co. Ltd. (China). Low density lipoprotein (LDL) was purchased from Nanjing Military Hospital (China). Kits for determinations of nitric oxide (NO), endothelial NO synthase (eNOS), inducible NOS (iNOS) and protein were provided by Nanjing Jiancheng Bioengineering Company (China). Kit for cyclic guanine monophosphate (cGMP) was the product of TCM University (China). Tripul was the product of Roche Company. dNTP, Moloney murine leukemia virustranscriptase (MMLV), Taq DNA polymerase, RNAsin, oligo(dT)15 primer and oligonucleotides for eNOS (both rabbit and bovine) and GAPDH were from Sangong Biotechnology (Shanghai, China). The following primer pairs were used—rabbit eNOS: sense, 5′-GCT GCG CCA GGC TCT CAC CTT C-3′; antisense, 5′-GGC TGC AGC CCT TTG CTC TCA A-3′; BAECs eNOS: sense, 5′-GAG CCA CAG AGC AGA CGG AG-3′; antisense, 5′-CAC TCT CTC GGA GGT GGA TG-3′; GAPDH: sense, 5′-ATC ACC ATC TTC CAG GAG CG-3′; antisense, 5′-CCT GCT TCA CCA CCT TCT TG-3′. Other chemicals used were analytical grade.

2.2. Animal preparations

Male New Zealand rabbits weighing 2.0–2.5 kg were provided by Animal Experimental Center of China, Pharmaceutical University (China) and all experimental procedures were performed in accordance with the Guidelines of Animal Experiments from the Committee of Medical Ethics, National Health Department of China (1998). The rabbits were randomly divided into four groups of eight animals each: control group, HCD group, HCD + CCT (30) group and HCD + CCT (15) group. Except for the rabbits in control group fed with regular diet, those in other groups were fed with high cholesterol diet (HCD) containing regular diet (94.8%), lard (4%) and cholesterol (1.2%) for 8 weeks. In the CCT groups, the diet was prepared daily by mixing CCT (30 and 15 mg/kg body weight), respectively, into 20 g HCD and by checking that rabbits completely consumed their food. All animals received 120 g of food daily. All animals were anesthetized with sodium pentobarbital (30 mg/kg i.v.) and sacrificed by exsanguinations at the end of experiment.

2.3. Arterial rings preparation and protocol

At the end of the experiment, rabbits were anaesthetized with sodium pentobarbital and sacrificed by exsanguinations from abdominal aorta. The thoracic aorta isolated from rabbits was placed in ice cold Krebs solution (mM) (NaCl 118.3; KCl 4.7; $CaCl_2$ 2.5; $MgSO_4$ 1.2; KH_2PO4 1.2; glucose 11.1 and $NaHCO_3$ 24.9, pH 7.4), cleaned from connective tissues and cut into transverse rings of 3 mm long. Special care was taken to avoid damage to the endothelium. Each ring was then suspended vertically in the organ chamber (volume 20 ml) between two

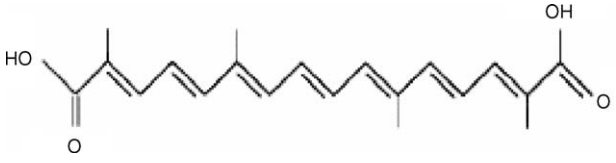

Fig. 1 – Chemical structure of CCT.

stainless steel hooks in Krebs solution maintained at 37 °C and aerated with 95% oxygen and 5% carbon dioxide. One of the hooks was fixed to a stand while the other was attached to an isometric force transducer (Hugo Sachs, Germany) and tension was recorded on a recorder (Linearcorder WR 3320, Japan). Rings were initially stretched until resting tension reached 2 g and then allowed to equilibrate for 45 min. During this period, resting tension was continuously monitored and, if needed, readjusted to 2 g by further stretching. To prevent the synthesis of vascular prostaglandins, all experiments were done in the presence of 10 μM indomethacin.

The rings from each group were preconstricted with phenylephrine (1×10^{-6} M) and then exposed to relaxant effects evoked by increasingly cumulative concentrations of either Ach (1×10^{-8} to 1×10^{-5} M) or SNP (1×10^{-8} to 1×10^{-5} M) when the constrictive plateau was reached.

2.4. Serum NO content and NOS activities in abdominal aorta

The blood was collected at weeks 0, 2, 4, 6, 8, respectively, from marginal ear vein and from which the serum was separated by centrifugation at 3000 rpm. Serum NO content was determined according to the Griess method which measures nitrite concentration after conversion of nitrate to nitrite with nitrate reductase. Nitrite concentrations were determined at an optical density of 554 nm by comparison to standard solutions of sodium nitrite. After the rabbit was killed, abdominal aorta was sectioned and homogenized in phosphate buffer solution (PBS) solution with final concentration 10% (g/v) and the homogenization was centrifuged at 3000 rpm for 10 min. The supernatant was used for determinations of total NOS (tNOS) and inducible NOS (iNOS) activities and protein content using kits following the instructions of manufactory. Endothelial NOS (eNOS) activity was the difference between tNOS and iNOS. In this method, NOS catalyzes L-arginine and O_2 to produce NO which reacts with a substance, forming a compound with blue color determined at 530 nm. The fact that eNOS is calcium-dependent while iNOS is not can distinguish the eNOS from iNOS by adding specific inhibitor of eNOS (calcium ion chelator) in the case of determination of iNOS. All NOS activity was expressed in U/mg protein.

2.5. cGMP content in abdominal aorta

The supernatant of abdominal aorta homogenate obtained in Section 2.4 was used for determinations of both cGMP and protein content by radioimmunological assay following the instruction of manufactory and the cGMP content was expressed in pmol/mg protein.

2.6. Preparation of oxLDL

Whole blood was obtained by venipuncture from healthy volunteers after 12 h of fasting and processed for LDL separation within 1 day by sequential flotation in NaBr solution containing 1 mg/ml EDTA. Cu^{2+}-modified LDL (1.0 mg protein/ml) was prepared by exposure of LDL to 5 mM $CuSO_4$ for 18 h at 37 °C. The extent of LDL oxidation was determined by thiobarbituric acid-reactive substances (TBARS) and the TBARS content of oxLDL used in our experiment was 19.2 nmol/100 mg protein versus 0.56 nmol/100 mg protein in the native-LDL preparation. Protein was measured by the Comas protein assay reagent.

2.7. Culture of bovine aortic endothelial cells (BAECs) and protocols

Bovine thoracic aorta was filled with 0.25% trypsin in D-hanks solution. After about 10-min incubation at 37 °C, dissociated bovine aortic endothelial cells (BAECs) were collected, washed by centrifugation at 1000 × *g* for 10 min, and re-suspended in DMEM containing 20% NBS. Cells were seeded in flasks and monitored until 75% of the cell clumps adhered (24 h). Nonadhering cells were poured off, and the adhering cells were incubated in DMEM with 20% NBS at 37 °C under an atmosphere of 5% CO_2 and 95% air. The cells used in this study had undergone 2–3 passages. Confluent BAECs were digested using 0.25% trypsin and seeded into 24- or 6-well plate with cell number of 5×10^5 or 5×10^6/well, respectively. Cells were divided into five groups: control group, oxLDL group, oxLDL + CCT (0.1, 1, 10 μM) groups. After pretreatment of the cells in oxLDL + CCT (0.1, 1, 10 μM) groups with CCT (0.1, 1, 10 μM) for 12 h followed by replacing culture medium with fresh one, the cells in oxLDL and oxLDL + CCT (0.1, 1, 10 μM) groups were incubated with oxLDL (final concentration 50 μg/ml) for 24 h. Culture medium and cells treated were used for the following experiments.

2.8. NO content in culture medium and NOS activities of BAECs

After the BAECs in 24-well plate were treated as described in Section 2.7, NO content in culture medium and the activities of tNOS and iNOS in BAECs lysed using 1% triton solution were determined using kits as described in Section 2.4. eNOS activity was the difference between tNOS and iNOS activity and expressed as U/mg cell protein.

2.9. eNOS mRNA expressions of rabbit thoracic aorta and BAECs

Total mRNA from rabbit thoracic aorta or BAECs in 6-well plate were isolated by using Tripul isolation reagent and used for RT-PCR assay. Briefly, after total mRNA was quantified by use of an ultraviolet (UV) spectrophotometer (DU-640, Beckman, USA), reverse transcription to cDNA was accomplished by priming 2 μg of total RNA samples with MMLV and oligo(dT) 15 primer. The products were then used for the following PCR amplification: the PCR reaction mixture was in a 25-μl volume containing 2.5 mM dNTP 1 μl, 10-PCR buffer (20 mM $MgCl_2$, 500 mM KCl, 1.5 M Tris–HCl, pH 8.7), 2.5 μl cDNA, 200 nM of the appropriate rabbit or bovine eNOS-paired primers and 1.25 U of Taq DNA polymerase. Amplification was performed in a Perkin-Elmer Gene Amp PCR System 9600 and consisted of 30 cycles of denaturation at 95 °C, 30 s, annealing at 53 °C, 30 s (rabbit eNOS and GAPDH) or at 62 °C, 30 s (BAECs eNOS and GAPDH) and extension at 72 °C, 30 s (terminal extension at 72 °C, 7 min) after an initial denaturation step at 95 °C, 10 min. The PCR-amplified products were electrophoresed on a 1.5%

agarose gel and visualized with ethidium bromide staining. eNOS mRNA band was normalized with band of relative internal reference GAPDH mRNA. Relative intensity of band was analyzed by Bio-Rad software (Bio-Rad Company) and expressed as the ratio to GAPDH mRNA band.

2.10. Statistical analysis

Relaxations (%) were expressed as the percentage of the contraction induced by 1×10^{-6} M of phenylephrine. All results were expressed as mean ± S.D. The results were analyzed with Student's *t*-test for paired observations. $P < 0.05$ was considered to be a significant probability.

3. Results

3.1. Endothelium-dependent relaxation (EDR) of thoracic aorta evoked by Ach

Ach (10^{-8} to $10^{-5.5}$ M) caused EDR of thoracic aorta in a concentration–response manner in all the groups studied (Fig. 2). However, EDR in HCD group was seriously impaired compared with that in control group at the corresponding point ($P < 0.05$ or <0.01). The maximal relaxation induced by Ach ($10^{-5.5}$ M) in HCD group was only 54% that in control group. Complementation of CCT (15, 30 mg/kg) significantly improved this impairment and restored the maximal relaxation to 68% and 80% that in control group, respectively ($P < 0.05$ or <0.01), suggesting that CCT improved the dysfunction of EDR impaired by HCD. No significant difference at the point of Ach ($10^{-5.5}$ M) was found between control group and HCD + CCT (30) group ($P > 0.05$).

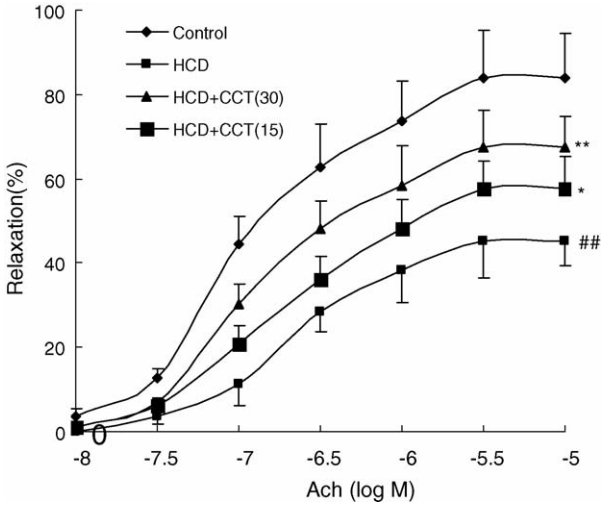

Fig. 2 – Endothelium-dependent relaxations (EDR) induced by Ach of isolated aortic rings ex vivo from rabbits fed regular diet (control) or a diet enriched with 1.2% cholesterol (HCD) or a HCD + CCT (15, 30 mg/kg) for 8 weeks. EDR was measured as described in Section 2. Value represents mean ± S.D. of eight rings per group. $^{\#\#}P < 0.01$ vs. control group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. HCD group.

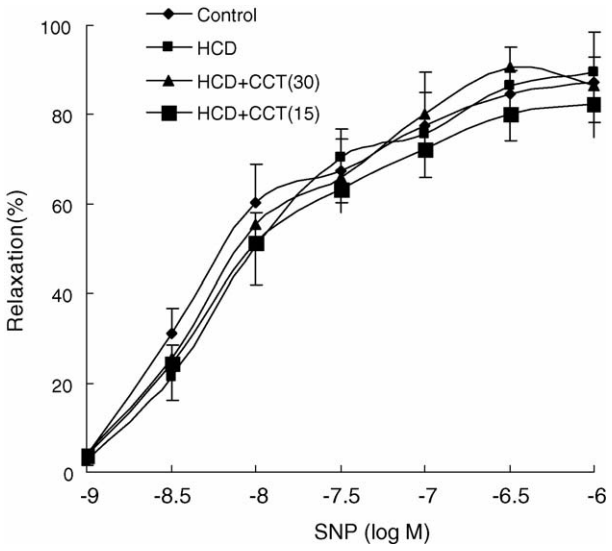

Fig. 3 – Endothelium-independent relaxations (EIDR) induced by SNP of isolated aortic rings ex vivo from rabbits fed regular diet (control) or a diet enriched with 1.2% cholesterol (HCD) or a HCD + CCT (15, 30 mg/kg) for 8 weeks. EIDR was measured as described in Section 2. Value represents mean ± S.D. of eight rings per group.

3.2. Endothelium-independent relaxation (EIDR) of thoracic aorta evoked by SNP

SNP (10^{-9} to $10^{-6.5}$ M) induced EIDR of thoracic aorta in a concentration–response manner in all the groups studied and no significant differences were found among the groups ($P > 0.05$), suggesting that EIDR maintained normal in hypercholesterolemia rabbits and that CCT had no influence on EIDR (Fig. 3).

3.3. Serum NO content

Eight weeks' feeding of HCD alone to rabbit caused a gradual reduction in serum NO content by 10%, 25%, 39% and 53% at weeks 2, 4, 6, 8, respectively, compared with that in control group. While those reductions were significantly inhibited by complementation of CCT ($P < 0.05$ or <0.01), with inhibited percentages of 9%, 14%, 32% and 36% for CCT (15 mg/kg) and 13%, 24%, 47% and 72% for CCT (30 mg/kg), respectively, compared with HCD at the corresponding week (Fig. 4).

3.4. cGMP content in abdominal aorta

Eight weeks' feeding of HCD alone to rabbit was shown to reduce cGMP content in abdominal aorta by 56% compared with that in control group ($P < 0.01$). Co-feeding of CCT (15, 30 mg/kg) significantly increased the cGMP content by 40% and 66% compared with HCD, respectively ($P < 0.05$ or <0.01) (Fig. 5). No significant difference in vessel cGMP level was found between control group and HCD + CCT (30) group ($P > 0.05$).

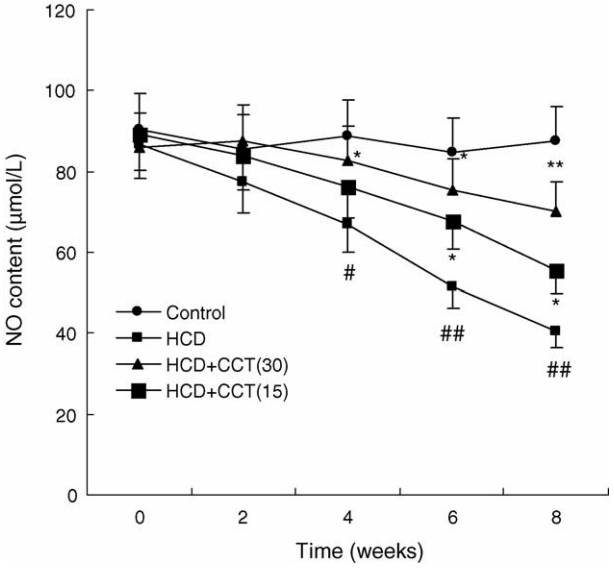

Fig. 4 – Serum NO content of rabbits fed regular diet (control) or a diet enriched with 1.2% cholesterol (HCD) or a HCD + CCT (15, 30 mg/kg) at weeks 0, 2, 4, 6, 8. NO was measured as described in Section 2. Value represents mean ± S.D. of eight samples per group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. control group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. HCD group.

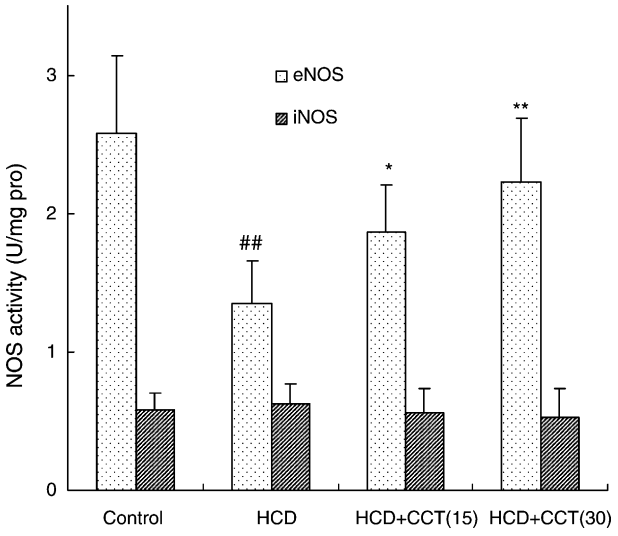

Fig. 6 – Activities of eNOS and iNOS in abdominal aorta of rabbits fed regular diet (control) or a diet enriched with 1.2% cholesterol (HCD) or a HCD + CCT (15, 30 mg/kg) for 8 weeks. NOS activities were measured as described in Section 2. Value represents mean ± S.D. of eight samples per group. $^{\#\#}P < 0.01$ vs. control group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. HCD group.

3.5. *NOS activity in abdominal aorta*

Exposure of rabbits to HCD for 8 weeks was shown to lower eNOS activity of abdominal aorta to nearly half of that in control group ($P < 0.01$). Complementation of CCT (15, 30 mg/kg) significantly increased eNOS activity by 38% and 65% compared with HCD, respectively ($P < 0.05$ or <0.01). Both HCD and CCT had no effect on iNOS activity, suggesting that changes of serum NO content might be attributed to changes of eNOS activity rather than iNOS activity (Fig. 6). No significant difference in eNOS activity was found between control group and HCD + CCT (30) group ($P > 0.05$).

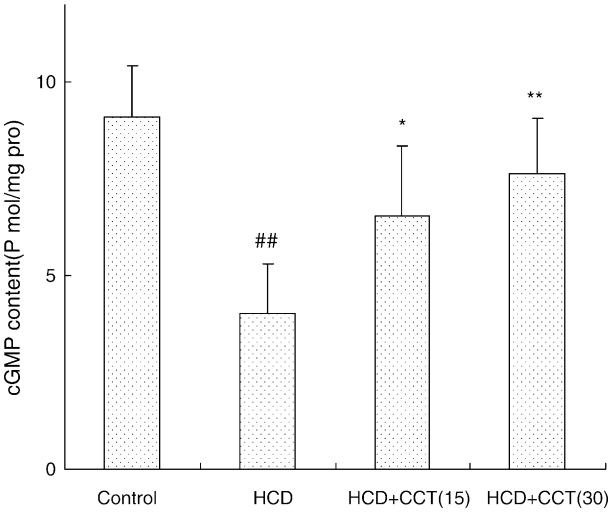

Fig. 5 – Vessel cGMP content of rabbits fed regular diet (control) or a diet enriched with 1.2% cholesterol (HCD) or a HCD + CCT (15, 30 mg/kg) for 8 weeks. cGMP was measured as described in Section 2. Value represents mean ± S.D. of eight samples per group. $^{\#\#}P < 0.01$ vs. control group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. HCD group.

3.6. *eNOS mRNA expression of thoracic aorta*

HCD feeding to rabbit was found to downregulate the eNOS mRNA expression in thoracic aorta by 43% with respect to control group ($P < 0.01$). Co-feeding of CCT (15, 30 mg/kg) significantly increased the eNOS mRNA expression by 30% and 53% compared with HCD alone, respectively ($P < 0.05$ or <0.01) (Fig. 7). No significant difference in eNOS mRNA expression was found between control group and HCD + CCT (30) group ($P > 0.05$).

3.7. *NO content in culture medium and NOS activities in BAECs*

Incubation of BAECs with 50 μg/ml oxLDL was found to reduce both NO content in culture medium and the eNOS activity in BAECs by 42% and 45% compared with the control, respectively ($P < 0.01$). Those reductions were remarkably inhibited by pretreatment with CCT (1, 10 μM) ($P < 0.05$ or <0.01). Both oxLDL and CCT had no effect on iNOS activity. (Fig. 8).

3.8. *eNOS mRNA expression of BAECs*

Incubation of BAECs with 50 μg/ml oxLDL was shown to downregulate the mRNA expression of eNOS in BACEs by 39% compared with control ($P < 0.01$). This downregulation was

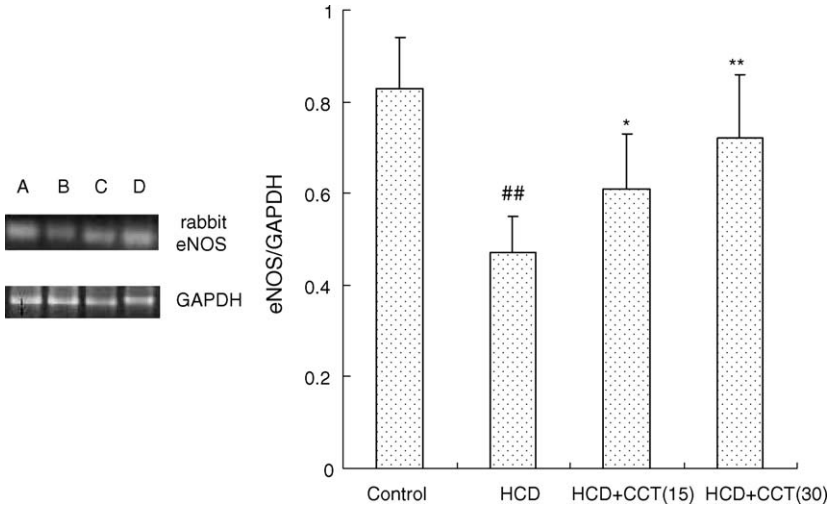

Fig. 7 – eNOS mRNA expression in thoracic aorta of rabbits fed regular diet (control) or a diet enriched with 1.2% cholesterol (HCD) or a HCD + CCT (15, 30 mg/kg) for 8 weeks. eNOS mRNA expression was measured as described in Section 2. Representative electrophoresis photo of eNOS and GAPDH mRNA expression (left). (A) control; (B) HCD; (C) HCD + CCT (15); (D) HCD + CCT (30). The analyzed mRNA expression of eNOS and GAPDH by image analyzer (right). Value represents mean ± S.D. of eight samples per group. $^{\#\#}P < 0.01$ vs. control group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. HCD group.

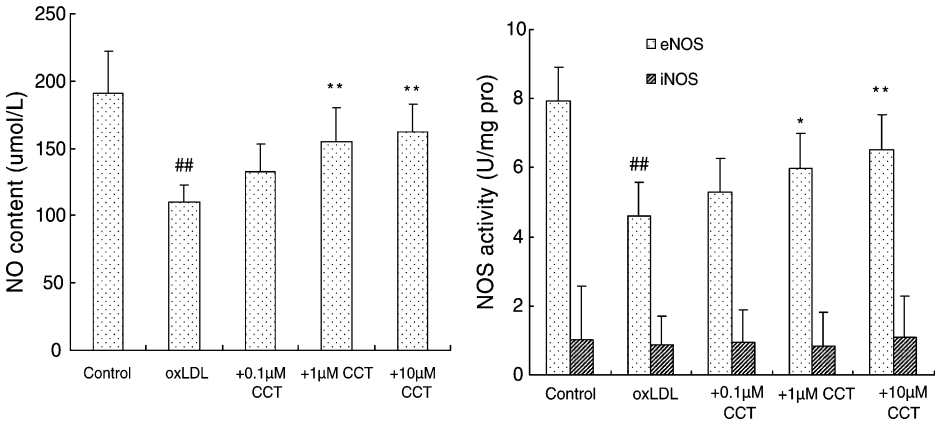

Fig. 8 – NO content in culture medium (left) and NOS activities in BAECs (right). NO and NOS were measured as described in Section 2. Value represents mean ± S.D. of eight samples per group. $^{\#\#}P < 0.01$ vs. control group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. oxLDL group.

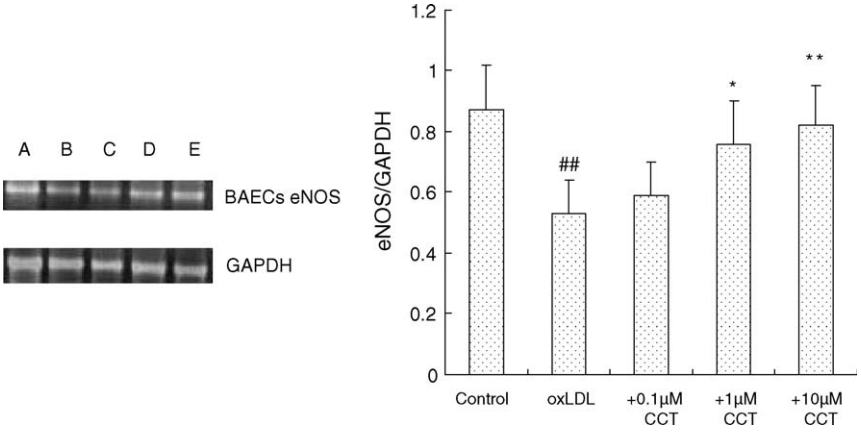

Fig. 9 – eNOS mRNA expression in BAECs was measured as described in Section 2. Representative electrophoresis photo of eNOS and GAPDH mRNA expression (left). (A) control; (B) oxLDL; (C) oxLDL + CCT (0.1 μM); (D) oxLDL + CCT (1 μM); (E) oxLDL + CCT(10 μM). The analyzed mRNA expression of eNOS and GAPDH by image analyzer (right). Value represents mean ± S.D. of six samples per group. $^{\#\#}P < 0.01$ vs. control group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. oxLDL group.

inhibited by pretreatment with CCT (0.1, 1, 10 μM) by 11%, 43%, and 55%, respectively (Fig. 9). No significant difference in eNOS mRNA expression was found between control group and oxLDL + CCT (10 μM) group ($P > 0.05$).

4. Discussion and conclusion

The major findings of this study were that dietary supplementation of high cholesterol diet (HCD) fed rabbits with crocetin (CCT) restored the systemic NO production and improved endothelium-dependent relaxation (EDR) in response to acetylcholine (Ach) of aortic rings ex vivo.

It is well established that eNOS–NO–cGMP pathway gets involved in the Ach induced EDR. The biding of Ach to the outer surface of an endothelial cell signals a rise in cytosolic Ca^{2+} concentration that activates eNOS. The NO formed in the endothelial cell catalyzed by eNOS diffuses across the plasma membrane and into the adjacent smooth muscle cells, where it bines and stimulates guanylyl cyclase, the enzyme that synthesizes cGMP, which is an important second messenger similar in structure to cAMP. Cyclic GMP leads to a decrease in cytosolic Ca^{2+} concentration, which causes relaxation of the muscle cell and dilation of the blood vessel.

It is well known that vascular rings of atherosclerotic animals show an endothelium injury, which is the first step in atherosclerotic plaque development [19]. This injury leads, among other things, to an alteration in the normal functionality of endothelium with a decrease in EDR induced by agonists, such as Ach [8]. The present study showed that the EDR of thoracic aorta isolated from hypercholesterolemic rabbit in response to Ach was significantly impaired and the maximal relaxation induced by Ach ($10^{-5.5}$ M) was only 54% that in control rabbit fed with regular diet. The result is well in agreement with the reported studies [20]. Co-feeding of CCT (15, 30 mg/kg) dose-dependently improved this impairment and restored the maximal relaxation to 68% and 80% that in the control, respectively. Taken together with our previous study [17] that CCT attenuated the atherosclerotic pathologies of rabbit, the present study suggested that improvement of EDR might be involved in the anti-atherosclerotic effect of CCT.

However, the present study and others showed that the endothelium-independent relaxation (EIDR) in response to sodium nitroprusside (SNP), which spontaneously releases NO, was not affected by the atherogenic diet [4,21]. Those implied that, on one hand, the reduced responsiveness of the atherosclerotic arteries to Ach was not a result of an inability of the vessels to relax; on the other hand, the impairment of EDR was due, at least in part, to reduced production of NO by endothelial cells. This was confirmed by the present study demonstrating that impaired EDR was accompanied by decreased NO production, consequently leading to reduced vessel cGMP production. Supplementation with CCT improved the EDR without affecting EIDR, suggesting that the action of CCT on vasorelaxation might be attributed to the vessel endothelia instead of direct effect on vessel smooth muscle. Furthermore, the beneficial effect of CCT on the EDR might be mediated by an enhanced formation of NO observed in the present study, leading to increased cGMP production.

NO is produced by an enzyme known as NOS which converts L-arginine to L-citrulline and NO [9,10]. There are two isoforms of NOS in endothelial cells which are eNOS and iNOS. eNOS originated NO is thought to play a pivotal role in maintaining the vasorelaxtion. Ooboshi et al. [11] reported that overexpression of eNOS gene in atherosclerotic animals improved the EDR by increasing the NO production. Otherwise, simultaneous administration of HCD with L-NAME, an eNOS inhibitor, to rabbits resulted in a further reduction of NO production and completely abolished the EDR by Ach [12]. oxLDL, the oxidized form of LDL which is the main carriers of cholesterol, is presumed to play a pivotal role in initiation and progression of AS. In deed, Zheng et al. [18] in our group reported that atherosclerotic areas in AS rabbit model positively correlated with plasma level of oxLDL and the susceptibility of LDL to in vitro oxidation. In addition, Simon et al. [22] reported that oxLDL not LDL significantly impaired the EDR of pig right coronary artery ex vivo, suggesting a potential role of oxLDL in impairment of the EDR seen in hypercholesterolemia and atherosclerosis. This is further supported by in vitro study that oxLDL downregulates the eNOS expression in human coronary artery endothelial cells [23]. In order to explore the underlying mechanism by which the NO production was increased by CCT, activities of eNOS and iNOS in both hypercholesterolemic rabbit vessels and oxLDL treated BAECs were measured. The results showed that CCT increased the activities of eNOS rather than iNOS in both in vivo and in vitro studies, suggesting that the increase of NO production was exclusively attributed to the increased eNOS activity. Furthermore, the facts that CCT upregulated the eNOS mRNA expressions in both in vivo and in vitro studies were in accordable with its action on eNOS activity and NO production. Taken together with our previous reports and others, inhibition of oxLDL production and consequently the alleviation of injury to endothelial cells caused by oxLDL might explain the upregulation of eNOS in both activity and mRNA levels by CCT. The exact mechanisms need further elucidated.

Conclusively, the present study demonstrated that CCT could restore EDR of thoracic aorta isolated from hypercholesterolemic rabbit, which might be partly explained by increased vessel activity of eNOS, leading to increasing of NO production. Functionally, this might be one of the mechanisms by which CCT ameliorate atherosclerosis. These observations should offer further insight into the anti-atherosclerotic mechanisms of anti-oxidants and provide a potential target for anti-oxidants in the prevention and treatment of atherosclerosis.

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REFERENCES

[1] Moncada S, Palmer RM, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev 1991;43:109–42.

[2] Jimenez A, Arriero MM, Lopez-Blaya A, Gonzalez-Fernandez F, Garcia R, Fortes J, et al. Regulation of endothelial nitric oxide synthase expression in the vascular wall and in mononuclear cells from hypercholesterolemic rabbits. Circulation 2001;104:1822–30.
[3] Runnie I, Salleh MN, Mohamed S, Head RJ, Abeywardena MY. Vasorelaxation induced by common edible tropical plant extracts in isolated rat aorta and mesenteric vascular bed. J Ethnopharmacol 2004;92:311–6.
[4] Verburen TJ, Jordens FH, Zonnekeyn LL, VanHove CE, Coene MC, Herman AG. Effect of hypercholesterolemia on vascular reactivity in the rabbit. Circ Res 1986;58: 552–64.
[5] Zeiher AM, Drexler H, Wollschlager H, Just H. Endothelial dysfunction of the coronary microvasculature is associated with coronary blood flow regulation in patients with early atherosclerosis. Circulation 1991;84:1984–92.
[6] Zeiher AM, Drexler H, Saurbier B, Just H. Endothelium-mediated coronary blood flow modulation in humans. Effects of age, atherosclerosis, hypercholesterolemia, and hypertension. J Clin Invest 1993;92:652–62.
[7] Keaney Jr JF, Xu A, Cunningham D, Jackson T, Frei B, Vita JA. Dietary probucol preserves endothelial function in cholesterol-fed rabbits by limiting vascular oxidative stress and superoxide generation. J Clin Invest 1995;95:2520–9.
[8] Kang SY, Kim SH, Schini VB, Kim ND. Dietary ginsenosides improve endothelium-dependent relaxation in the thoracic aorta of hypercholesterolemic rabbit. Gen Pharmacol 1995;26:483–7.
[9] Moncada S, Higgs A. The L-arginine-nitric oxide pathway. N Engl J Med 1993;329:2002–12.
[10] Sessa WC. The nitric oxide synthase family of proteins. J Vasc Res 1994;31:131–43.
[11] Ooboshi H, Toyoda K, Faraci FM, Lang MG, Heistad DD. Improvement of relaxation in an atherosclerotic artery by gene transfer of endothelial nitric oxide synthase. Arterioscler Thromb Vasc Biol 1998;18:1752–8.
[12] Cayatte AJ, Palacino JJ, Horten K, Cohen RA. Chronic inhibition of nitric oxide production accelerates neointimal formation and impairs endothelial function in hypercholesterolemic rabbits. Arterioscler Thromb 1994;14:753–9.
[13] Liao JK, Shin WS, Lee WY, Clark SL. Oxidized low-density lipoprotein decreases the expression of endothelial nitric oxide synthase. J Biol Chem 1995;270:319–24.
[14] Cominacini L, Pasini AF, Garbin U, Davoli A, Tosetti ML, Campagnola M, et al. Oxidized low density lipoprotein (ox-LDL) binding to ox-LDL receptor-1 in endothelial cells induces the activation of NF-kappaB through an increased production of intracellular reactive oxygen species. J Biol Chem 2000;275:12633–8.
[15] Tseng TH, Chu CY, Huang JM, Shiow SJ, Wang CJ. Crocetin protects against oxidative damage in rat primary hepatocytes. Cancer Lett 1995;97:61–7.
[16] Deng YX, Qian ZY, Tang FT. Effects of crocetin on experimental atherosclerosis in rats. Chin Tradition Herbal Drugs 2004;35:777–81.
[17] Zheng S, Qian Z, Tang F, Sheng L. Suppression of vascular cell adhesion molecule-1 expression by crocetin contributes to attenuation of atherosclerosis in hypercholesterolemic rabbits. Biochem Pharmacol 2005;70:1192–9.
[18] Zheng S, Qian Z, Sheng L, Wen N. Crocetin attenuates atherosclerosis in hyperlipidemic rabbits through inhibition of LDL oxidation. J Cardiovasc Pharmacol 2006;47:70–6.
[19] Hattori Y, Kawasaki H, Abe K, Kanno M. Superoxide dismutase recovers altered endothelium-dependent relaxation in diabetic rat aorta. Am J Physiol 1991;261:H1086–94.
[20] Guerra Jr R, Brotherton AF, Goodwin PJ, Clark CR, Armstrong ML, Harrison DG. Mechanisms of abnormal endothelium-dependent vascular relaxation in atherosclerosis: implications for altered autocrine and paracrine functions of EDRF. Blood Vessel 1989;26:300–14.
[21] Osborne JA, Siegman MJ, Sedar AW, Mooers SU, Lefer AM. Lack of endothelium-dependent relaxation in coronary resistance arteries of cholesterol-fed rabbits. Am J Physiol 1989;256:C591–7.
[22] Simon BC, Cunningham LD, Cohen RA. Oxidized low density lipoproteins cause contraction and inhibit endothelium-dependent relaxation in the pig coronary artery. J Clin Invest 1990;86:75–9.
[23] Mehta JL, Li DY, Chen HJ, Joseph J, Romeo F. Inhibition of LOX-1 by statins may relate to upregulation of eNOS. Biochem Biophys Res Commun 2001;289:857–61.