

This article was downloaded by:

On: 22 January 2011

Access details: *Access Details: Free Access*

Publisher *Taylor & Francis*

Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK



## Journal of Asian Natural Products Research

Publication details, including instructions for authors and subscription information:

<http://www.informaworld.com/smpp/title~content=t713454007>

### Crocetin attenuates norepinephrine-induced cytotoxicity in primary cultured rat cardiac myocytes by antioxidant *in vitro*

Xiang-Chun Shen<sup>ab</sup>, Zhi-Yu Qian<sup>c</sup>, Ya-Juan Wang<sup>c</sup>, Jin-Ao Duan<sup>b</sup>

<sup>a</sup> Research Division of Basic and Clinical Pharmacology, Guiyang Medical College, Guiyang, China <sup>b</sup>

Jiangsu Key Laboratory for TCM Formulae Research, Nanjing University of Chinese Medicine,

Nanjing, China <sup>c</sup> Department of Pharmacology, China Pharmaceutical University, Nanjing, China

**To cite this Article** Shen, Xiang-Chun , Qian, Zhi-Yu , Wang, Ya-Juan and Duan, Jin-Ao(2009) 'Crocetin attenuates norepinephrine-induced cytotoxicity in primary cultured rat cardiac myocytes by antioxidant *in vitro*', Journal of Asian Natural Products Research, 11: 5, 417 – 425

**To link to this Article: DOI:** 10.1080/10286020902858838

**URL:** <http://dx.doi.org/10.1080/10286020902858838>

PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: <http://www.informaworld.com/terms-and-conditions-of-access.pdf>

This article may be used for research, teaching and private study purposes. Any substantial or systematic reproduction, re-distribution, re-selling, loan or sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.

## Crocetin attenuates norepinephrine-induced cytotoxicity in primary cultured rat cardiac myocytes by antioxidant *in vitro*

Xiang-Chun Shen<sup>ab\*</sup>, Zhi-Yu Qian<sup>c</sup>, Ya-Juan Wang<sup>c</sup> and Jin-Ao Duan<sup>b</sup>

<sup>a</sup>Research Division of Basic and Clinical Pharmacology, Guiyang Medical College, Guiyang 550004, China; <sup>b</sup>Jiangsu Key Laboratory for TCM Formulae Research, Nanjing University of Chinese Medicine, Nanjing 210046, China; <sup>c</sup>Department of Pharmacology, China Pharmaceutical University, Nanjing 210009, China

(Received 27 November 2008; final version received 2 March 2009)

This study aims to investigate the protective role of crocetin, a natural antioxidant, against cytotoxicity produced by exposure to norepinephrine (NE) in primary cultured rat cardiac myocytes. Reactive oxygen species (ROS) and Ca<sup>2+</sup> in cells were evaluated by fluorescence microplate reader using 6-carboxy-2',7'-dichlorofluorescein and fluoro-3-acetoxymethyl ester, respectively. Lipid peroxidation was quantified using thiobarbituric acid-reactive substances. The activities of superoxide dismutase (SOD) and contents of glutathione (GSH) were detected by xanthine/xanthine oxidase-mediated ferricytochrome *c* reduction assay, and recycling effect of glutathione disulfide with GSH reductase and NADPH, respectively. The apoptotic cells were assayed by fluorescein diacetate (FDA)–ethidium bromide (EB) two-staining method. Intracellular accumulation of ROS, Ca<sup>2+</sup>, and products of lipid peroxidation resulting from NE were significantly reduced by crocetin. Preincubation of primary cultured rat cardiac myocytes with crocetin remarkably prevented the decrease in SOD activity and quantities of GSH induced by NE. The percentage of NE-induced apoptosis in the cells was decreased by FDA–EB two-staining assay after pretreated with crocetin. The results showed that crocetin may ameliorate NE-induced injury in cardiac myocytes by enhanced SOD activity and increased quantities of GSH, decreased lipid peroxidation and Ca<sup>2+</sup> in cells, and apoptosis death ratio that may represent the cellular mechanisms for its cardioprotective role.

**Keywords:** crocetin; norepinephrine; cardiac myocytes; intracellular Ca<sup>2+</sup>; apoptosis; oxidative stress

### 1. Introduction

It is well known that the sympathetic nervous system plays an integral role in the rapid regulation of myocardial function. In addition, catecholamines released by the sympathetic nervous system can exert important tonic effects on the biology of cardiac myocytes that comprise the major portion of the myocardium by mass [1]. Recently, many reports have found out that

the subhypertensive doses of norepinephrine (NE) can not only reduce the cardiac noradrenergic-nerve terminal profiles, but also produce cardiomyocyte apoptosis in animals [2]. NE probably exerts these effects via increasing oxidative stress and free radical scavengers may be protective against NE-induced injury [3].

Saffron, the dried stigmas of *Crocus sativus* L., is an extremely expensive spice. It is an important flavoring and coloring

\*Corresponding author. Email: shenxiangchun@126.com

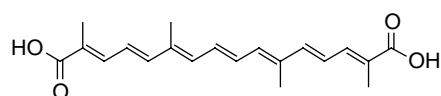


Figure 1. Chemical structure of crocetin.

food agent and is usually applied to treat various diseases in traditional and modern medicine in different areas of the world, especial in China [4]. Saffron has been successfully cultivated in some places of China, and was used as antianginal traditional Chinese medicine for a long time. *Trans*-crocetin (crocetin), a dicarboxylic 20-carbon carotenoid derivative (Figure 1), is a primarily bioactivity ingredient in the extracts from *C. sativus* L. (saffron) [5,6]. In our previous research, crocetin has been characterized as an antioxidant, and proved to protect against myocardium injury by different methods [7,8]. We also found that it significantly ameliorated and/or reversed the cardiac hypertrophy induced by constriction of the abdominal aorta and NE in rats [9,10]. In the present study, we investigated the protective effects of crocetin on primary cardiac myocytes that were exposed to 1.0  $\mu$ M NE. The production of lipid peroxide and accumulation of reactive oxygen species (ROS), contents of glutathione (GSH), activities of superoxide

dismutase (SOD), accumulation of  $\text{Ca}^{2+}$ , and apoptotic cells were evaluated.

## 2. Results and discussion

### 2.1 Results

#### 2.1.1 Crocetin reduced lipid peroxidation

The levels of lipid peroxidation were detected by the contents of malonaldehyde (MDA) in the cells. The results presented in Table 1 are consistent with the notion that cells that have been exposed to NE have an increased levels of MDA ( $2.25 \pm 0.25$  in 1.0  $\mu$ M NE vs.  $1.73 \pm 0.19$  in control,  $p < 0.01$ ), which indicated the NE-induced oxidative stress. Preincubation with crocetin prevented the production of lipid peroxidation ( $1.80 \pm 0.16$ ,  $1.91 \pm 0.28$ , respectively, in the presence of 0.1, 1.0  $\mu$ M crocetin vs.  $2.25 \pm 0.25$  in 1.0  $\mu$ M NE;  $p < 0.01$ ,  $p < 0.05$ ).

To determine whether crocetin could prevent NE-induced ROS generation and oxidative stress as resulting, the levels of ROS production in the cells were determined using the fluorescence probe 2',7'-dichlorofluorescein (DCF). Cultures exposed to NE displayed increased intensity of DCF-labeled cells when compared with untreated control cultures. Cultures pretreated with 0.1 and 1.0  $\mu$ M crocetin

Table 1. Effect of crocetin on NE-induced intracellular accumulation of ROS and MDA in primary cultured rat cardiac myocytes.

Drugs ( $\mu$ M)	MDA (nmol/mg prot.)	Increase fluorescence (%)
Control	$1.73 \pm 0.19$	$9.13 \pm 2.60$
NE 1.0	$2.25 \pm 0.25^a$	$60.73 \pm 10.52^a$
Puerarin 1 mg/ml + NE 1.0	$1.81 \pm 0.28^b$	$32.16 \pm 8.87^c$
Crocetin 1.0 + NE 1.0	$1.80 \pm 0.16^c$	$25.43 \pm 6.52^c$
Crocetin 0.1 + NE 1.0	$1.91 \pm 0.28^b$	$35.24 \pm 13.28^b$
Crocetin 0.01 + NE 1.0	$1.97 \pm 0.40$	$40.26 \pm 14.15$

The cells were plated and grown for 36–48 h in cultured medium with 0.5% fetal bovine serum (FBS), then were switched to fresh medium in the presence of 0.01, 0.1, and 1.0  $\mu$ M crocetin or 1 mg/ml puerarin. After 0.5-h incubation, 1.0  $\mu$ M NE was added and the cells were incubated for an additional 24 h for MDA and intracellular ROS levels assay. The values shown are the mean  $\pm$  SD of three experiments (five to six cultures per experiments).

<sup>a</sup>  $p < 0.01$  vs. control.

<sup>b</sup>  $p < 0.05$  vs. NE-alone cells.

<sup>c</sup>  $p < 0.01$  vs. NE-alone cells.

were significantly reduced intensity of DCF-labeled cells when compared with only NE-treated cultures.

### 2.1.2 Free radical scavengers

The activities of SOD and content of GSH were evaluated to ascertain whether crocetin protected against the antioxidant enzymes and increased the content of the antioxidative compound in NE-induced cell injury. As shown in Table 2, SOD activity and GSH levels were significantly decreased in cells exposed to NE, and crocetin protected on the free radical scavengers in the cells exposed to NE.

### 2.1.3 Crocetin inhibited NE-induced $[Ca^{2+}]_i$ increase in cardiac myocytes

In Krebs's solution containing calcium, 1.0  $\mu$ M NE exposure induced an increase in the fluorescence from  $331.83 \pm 49.90$  in controls to  $416.28 \pm 41.79$  in NE-exposed cells. When the cells were pretreated with 1.0  $\mu$ M crocetin, the fluorescence was reduced from  $416.28 \pm 41.79$  to  $345.14 \pm 49.46$ , while the other two dose crocetin showed a trend of decrease in the values of fluorescence, but there was no significant difference compared with NE-exposed cells. In calcium-free Krebs's solution, 1.0  $\mu$ M NE could also induce the increase in fluorescence ( $238.50 \pm 41.10$

vs.  $303.57 \pm 54.98$ ,  $p < 0.05$ ), and crocetin at 0.01, 0.1, and 1.0  $\mu$ M did not remarkably decrease fluorescence ( $p > 0.05$ ; Figure 2). The results indicated that NE-induced increase in intracellular  $Ca^{2+}$  was not dependent on extracellular  $Ca^{2+}$ ; however, crocetin mainly prevented the cytosolic released  $Ca^{2+}$  related to extracellular  $Ca^{2+}$ .

### 2.1.4 Crocetin protected primary cultured rat cardiac myocytes against NE-induced apoptosis

The percentage of apoptotic cells was measured by fluorescein diacetate (FDA) and ethidium bromide (EB) two-staining assay (Figure 3). Our results showed that treatment of cells with 1.0  $\mu$ M NE for 24 h significantly induced the orange fluorescence cells, indicating apoptotic cells, whereas crocetin significantly attenuated NE-induced apoptotic cell (Figure 4).

## 2.2 Discussion

In Asia, particularly in China, saffrons have been extensively used for treatment of cardiovascular diseases such as angina pectoris and congestive heart failure for quite a long time in clinic. Studies on the effectiveness of these products have been undertaken, but only limited scientific evidence has been reported for their traditional therapeutic use and mechanisms.

Table 2. Effect of crocetin on NE-induced SOD activity and GSH contents in primary culture cardiac myocytes.

Drugs ( $\mu$ M)	SOD (IU/mg prot.)	GSH ( $\mu$ g/mg prot.)
Control	$92.91 \pm 4.62$	$174.42 \pm 8.04$
NE 1.0	$82.28 \pm 12.59^a$	$150.29 \pm 11.57^a$
Puerarin 1 mg/ml + NE 1.0	$95.78 \pm 5.97^b$	$178.58 \pm 14.91^b$
Crocetin 1.0 + NE 1.0	$96.83 \pm 8.60^b$	$175.42 \pm 10.97^b$
Crocetin 0.1 + NE 1.0	$90.92 \pm 8.09$	$151.74 \pm 15.84$
Crocetin 0.01 + NE 1.0	$91.82 \pm 8.75$	$149.90 \pm 20.38$

The SOD activity and GSH contents were determined in Materials and methods section. Briefly, SOD activity was measured by xanthine/xanthine oxidase-mediated ferricytochrome *c* reduction assay and the contents of GSH were determined by recycling glutathione disulfide (GSSG) with GSH reductase and NADPH. The values shown are the mean  $\pm$  SD of three experiments (five to six cultures per experiments).

<sup>a</sup> $p < 0.05$  vs. control.

<sup>b</sup> $p < 0.05$  vs. NE-alone cells.

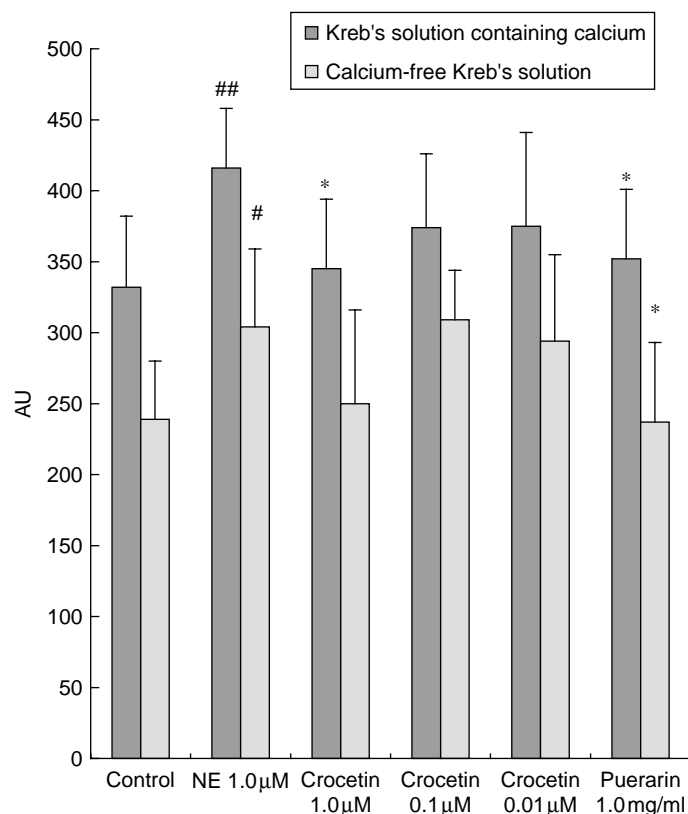


Figure 2. Effect of crocetin against cytosolic  $[Ca^{2+}]_i$  increased by  $1.0 \mu M$  NE in primary culture cardiac myocytes. The cells were treated as per the legend of Table 1.  $[Ca^{2+}]_i$  was measured by Fluo/3AM. The fluorescence was measured at emission wavelength of 535 nm and excitation wavelength of 435 nm on a 1420 Victor<sup>2</sup> V. The values shown are the mean  $\pm$  SD of three experiments (five to six cultures per experiments). <sup>#</sup> $p < 0.05$ , <sup>##</sup> $p < 0.01$  vs. control, and <sup>\*</sup> $p < 0.05$  vs. NE-alone cells.

Therefore, the present study was performed in an attempt to clarify pharmacological activity of crocetin isolated from *C. sativus* L. As well known, pharmacological effects of crocetin to the cardiovascular systems have been highlighted the most attention, but the cardiac myocytes protective activity and elucidation of the mechanism(s) are of the interest in this paper.

Recently, some reports have shown that an important mechanism of NE-induced toxicity of cardiac myocytes is involved in oxidative stress [11,12]. Accumulating evidence suggests that oxidative stress plays an important role in mediating pathologic responses in cardiac myocytes and various cardiovascular

system diseases [13,14]. It has been known for some time that excessive generation of ROS is associated with cell injury in a variety of pathological conditions. Many cellular compounds are easily oxidized, and lipid peroxides have been used as a marker for oxidative stress. GSH is the first line of defense against ROS, a decrease in GSH is known to be deleterious for the cells [15,16]. SOD is an important free radical scavenger, especially in protected mitochondrial function, and catalyzes the dismutation of the superoxide anion that was mainly produced by mitochondria. Our results indicated that the contents of MDA (the production of lipid peroxide) were significantly increased in the cells

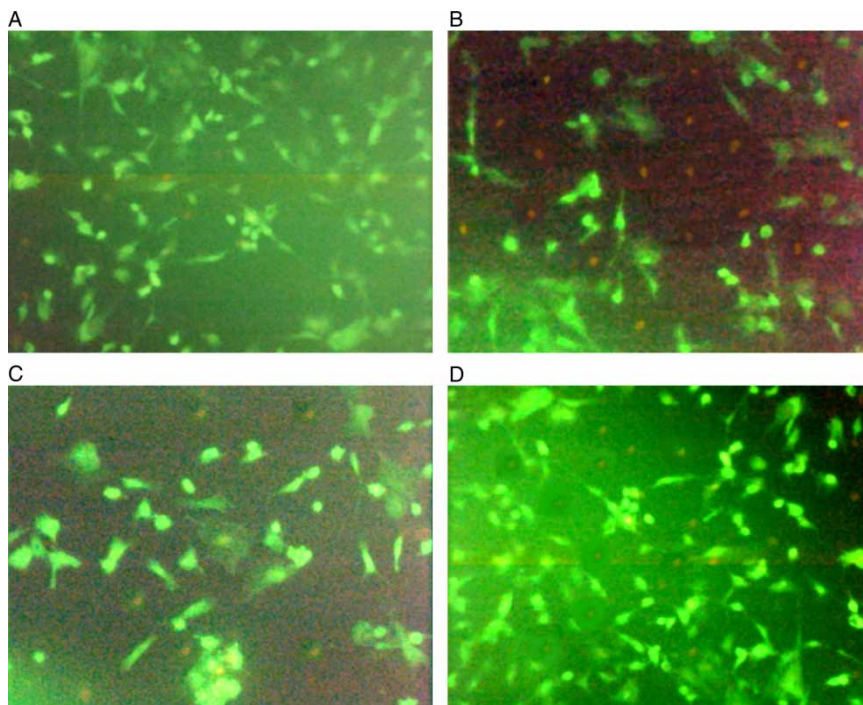


Figure 3. [Colour online] Protected effect of crocetin against NE-induced apoptosis in primary cardiac myocytes as determined by FDA–EB two-staining assay. (A) Control; (B) primary cardiac myocytes exposed to 1.0  $\mu\text{M}$  NE; (C) primary cardiac myocytes pretreated with 1 mg/ml puerarin for 30 min followed by exposure to 1.0  $\mu\text{M}$  NE for additional 24 h; and (D) primary cardiac myocytes pretreated with 1.0  $\mu\text{M}$  crocetin for 30 min followed by exposure to 1.0  $\mu\text{M}$  NE for additional 24 h. The orange fluorescence cells were apoptotic cells and the green fluorescence cells were living cells.

exposed to NE for 24 h, and preincubation with crocetin prevented the oxidative stress and GSH exhaustion, and elevated SOD activity.

In addition to the increase in the MDA contents, NE exposure caused an elevation of intracellular  $\text{Ca}^{2+}$  levels. The occurrence of large increases in intracellular  $\text{Ca}^{2+}$  represents detrimental insults from oxidative stress imposed by NE in the cells. Sustained elevated  $\text{Ca}^{2+}$  level in cells may impair mitochondrial function, and activate phospholipase, protease, and endonucleases, leading to irreversible membrane, organelles, and chromatin damage, and eventually to cell death. Therefore,  $\text{Ca}^{2+}$  plays an important role in the development of NE injury. Pretreatment with crocetin blocked NE-induced  $\text{Ca}^{2+}$  influx. Crocetin may

have (a) prevented membrane injury and (b) directly acted on the  $\text{Ca}^{2+}$  channel to prevent its opening. The precise mechanism remains to be investigated.

Evidences have indicated that the apoptotic mechanism is involved in various heart disorders. The finding of increased myocyte apoptosis in myocardium from patients with heart failure was particularly provocative, since it is known that sympathetic nervous system activity is increased in such patients and the use of  $\beta$ -adrenergic receptor antagonists has had a dramatic effect on both the clinical outcomes of patients with heart failure and the progression of the underlying myocardial failure [17]. Our results indicated that apoptotic cells increased when the cells were exposed to 1.0  $\mu\text{M}$  NE, and preincubation with crocetin significantly decreased

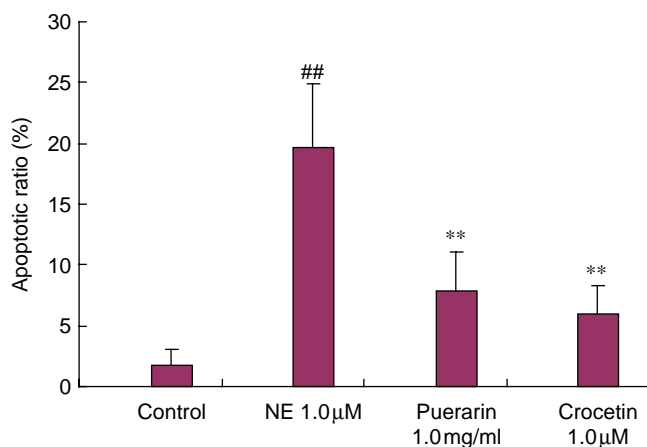


Figure 4. Protected effect of crocetin against NE-induced apoptotic ratio in primary cardiac myocytes. The cells were treated as per the legend of Table 1. At least 300 cells were counted for each determination. The apoptotic ratio was expressed as the percentage (%) of the total cells (orange fluorescence cells + green fluorescence cells), according to the equation % apoptotic ratio = (orange fluorescence cells/total cells)  $\times$  100. The values shown are the mean  $\pm$  SD of three experiments (three to four cultures per experiments). ## $p$  < 0.01 vs. control, \*\* $p$  < 0.01 vs. NE-alone cells.

the apoptotic ratio. From these results of our research, crocetin may decrease the apoptotic ratio in NE-induced primary cultured rat cardiac myocytes injury by antioxidant.

In summary, the NE-caused apoptotic cell death in primary cultured rat cardiac myocytes may depend on oxidative stress. Crocetin protected against primary cultured rat cardiac myocytes from NE-induced injury by interrupting the cell death cascade at three distinct steps: (1) blocking ROS production, (2) elevating SOD activity and GSH contents, and (3) inhibiting  $\text{Ca}^{2+}$  influx. The elucidation of characterization of injury caused by NE and the modulation by crocetin may provide additional insights into the molecular basis of chemoprotective effects of this antioxidant and will also aid in the development of potential drugs or regimens to control and prevent NE-induced cardiovascular disorders.

### 3. Materials and methods

#### 3.1 Materials

Crocetin (molecular formula:  $\text{C}_{20}\text{H}_{34}\text{O}_4$ ), which was purified in the lab of Prof. Zhi-Yu

Qian, was confirmed by HPLC, UV, NMR, and MS analyses. Its purity, determined by HPLC method, was >98.0%. Thiobarbituric acid-reactive substances (TBARS) were purchased from Fluka (Buchs, Switzerland) and Dulbecco's modified Eagle's medium (DMEM) was the product of Gibco RBL (Grand Island, NY, USA). EB was purchased from Amresco (Solon, OH, USA). Tetraethoxypropane, FDA, bromodeoxyuridine (BrdU), 6-carboxy-2',7'-dichlorofluorescein diacetate (DCFH-DA), and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Fluo/3AM was purchased from Calbiochem Novabiochem (La Jolla, CA, USA). All other chemicals were of analytical purity grade.

#### 3.2 Methods

##### 3.2.1 Neonatal rat cardiac myocytes culture

Primary cultures of neonatal rat cardiac myocytes were prepared as previously described with some modifications [18]. Briefly, 10–15 1-day-old Sprague–Dawley pups were decapitated under aseptic

conditions. The hearts were removed and rinsed thrice with cold  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -free phosphate-buffered saline (PBS). The atria and aorta were discarded, and the ventricles were dissected, minced into 1–3-mm<sup>3</sup> fragments and subjected to trypsin digestion. After enzymatic dissociation, the cells were preplated for 1.5–2 h to selectively enrich for cardiac myocytes. The resultant suspension of cardiac myocytes were plated onto gelatin-coated 24- or 96-well plates at a density of  $1 \times 10^6/\text{ml}$ , cultured in high-glucose DMEM supplemented with 10% heat-inactivated FBS, 2 g/l BSA, 15 mM HEPES (pH 7.4), 100 IU/ml penicillin, and 100  $\mu\text{g}/\text{ml}$  streptomycin and were incubated at 37°C in a humidified atmosphere containing 5%  $\text{CO}_2$ –95%  $\text{O}_2$  room air. Then, Brdu (100  $\mu\text{M}$ ) was added during the first 48 h to inhibit proliferation of non-myocytes. The myocytes were then incubated in DMEM containing 0.5% FBS without Brdu, and all experiments were done 36–48 h after this incubation. A certain concentration of crocetin (in the results, the concentration is final in medium) was added in the medium for cell preincubation. Crocetin was freshly prepared as solution in dimethylsulfoxide (DMSO) and diluted with PBS before the experiment. Control cultures were performed in the presence of DMSO under the same culture conditions. The crocetin was preincubated for 30 min before NE stimulation. Cardiac myocytes were stimulated for 24 h. Assays for lactate dehydrogenase, SOD, lipid peroxide, GSH, cellular morphology of apoptotic cells, and apoptotic ratio were calculated by taken into account of cell number.

### 3.2.2 Measurement of activities of SOD

Activities of SOD were measured by xanthine/xanthine oxidase-mediated ferricytochrome *c* reduction assay as described by Yang *et al.* [19]. One unit of SOD activity was defined as the amount that reduced the absorbance at 550 nm by 50%. The medium was discarded after

cells were stimulated by 1.0  $\mu\text{M}$  NE. Cells were washed with ice-cold PBS and lysed in 50 mM  $\text{KH}_2\text{PO}_4$ – $\text{Na}_2\text{HPO}_4/0.1$  mM EDTA buffer (pH 7.8) using a Pasteur pipette. The supernatant was collected after centrifugation and assayed for SOD activity. SOD activity was calculated using a concurrently run standard curve and expressed per milligram of protein. Protein concentration in cell homogenates was measured using the Coomassie blue method, and BSA was used as a reference standard [20].

### 3.2.3 Determination of the contents of GSH in cells

For determination of the contents of GSH in cells, cells were washed with ice-cold PBS and lysed using ice-cold 0.1% Triton X-100 in 100 mM sodium phosphate buffer (pH 7.4). The proteins were subsequently precipitated with 5% perchloric acid and pelleted by centrifugation at 2600g for 15 min. The supernatants were neutralized with 0.3 M KOH in 20 mM HEPES and used for GSH estimation. Total GSH concentration was chemically determined by recycling GSSG with GSH reductase and NADPH [20]. GSH content was calculated from a standard curve.

### 3.2.4 TBARS assay

After treatment with crocetin, the medium was replaced with PBS and cells were lysed in PBS using a Pasteur pipette and freeze thawing. The suspension was transferred to a 10-ml test tube and then centrifuged (1000g for 30 min). The pellets were resuspended in 5 ml of PBS containing 0.002% butylated hydroxytoluene and recentrifuged at 1000g for 3 min. The pellet was used to quantify lipid peroxides using the TBARS assay [21]. The concentrations of TBARS were calculated using tetraethoxypropane as a reference standard.



### 3.2.5 Detection of intracellular ROS

DCF was used as a measure probe of the oxidative stress induced by NE [22]. DCFH-DA was prepared in ethanol and diluted into culture medium to a final concentration of 100  $\mu\text{M}$ . After the treatment with crocetin, cells plated in 96-well plates were incubated with 100  $\mu\text{M}$  DCFH-DA in the loading medium in 5%  $\text{CO}_2/95\%$  air at 37°C for 30 min to establish a stable intracellular level of the probe. And then, DCFH-DA was removed and the cells were washed once with DMEM. The fluorescence from each well was captured, digitized, and stored on a computer using 1420 Victor<sup>2</sup> V (Perkin Elmer Life Science, Waltham, MA, USA). The excitation filter was set at 485 nm and the emission filter was set at 530 nm. The percentage increase in fluorescence per well was calculated by the formula  $[(F_{t30} - F_{t0})/F_{t0} \times 100]$ , where  $F_{t0}$  is the fluorescence at time 0 and  $F_{t30}$  is the fluorescence at time 30 min.

### 3.2.6 Measurement of intracellular $\text{Ca}^{2+}$ ( $[\text{Ca}^{2+}]_i$ )

$[\text{Ca}^{2+}]_i$  was monitored using the fluorescent  $\text{Ca}^{2+}$ -sensitive dye, fluoro-3-acetoxymethyl ester (Fluo/3AM) [23]. The medium was discarded, and then cells were gently rinsed thrice with D-Hanks' solution. The confluent monolayer of cardiac myocytes in the 96-well plates was preloaded with Fluo/3AM (5  $\mu\text{M}$ ) in Krebs' solution containing calcium or calcium-free Krebs' solution with 1% BSA, respectively, for 30 min at room temperature in the dark, and then 30 min at 37°C in a humidified incubator. The fluorescence was measured at emission wavelength of 535 nm and excitation wavelengths of 435 nm on a 1420 Victor<sup>2</sup> V.

### 3.2.7 FDA and EB two-staining detection of apoptotic cell

After the cell was exposed to NE 24 h, the medium was replaced with PBS and

washed thrice. Cell apoptosis was assayed by EB and FDA uptake after incubation with final concentrations of 10  $\mu\text{g}/\text{ml}$  EB and 5  $\mu\text{g}/\text{ml}$  FDA for 5 min. The images were obtained with fluorescence inverted microscope (Leica-DMIRB, Wetzlar, Germany), and at least 300 cells were counted for determination. EB and FDA were simultaneously excited at 480 nm and a 530 nm long-pass filter was used [24,25]. The green fluorescence was detected when FDA was uptaken by viable cells and orange fluorescence of EB was uptaken only by apoptotic cells. The apoptotic ratio was expressed as the percentage (%) of the total cells (orange fluorescence cells + green fluorescence cells), according to the equation % apoptotic ratio = (orange fluorescence cells/total cells)  $\times$  100.

### 3.2.8 Statistical analysis

Results are presented as mean  $\pm$  SD. The statistical significance of differences among experimental groups was evaluated for statistical significance with one-way analysis of ANOVA followed by Duncan's multiple range.

### Acknowledgements

This research was supported by a grant from National Natural Science Foundation of China (No. 30701024), High-ranking Intellectual Special Assistant Foundation of Guizhou Province (No. TZJF-2006-13), Science and Technology Foundation of Guizhou Province (No. 2007-2133), Provincial Key Technologies R&D Program of Guizhou (No. 2007-1035), and Jiangsu Postdoctoral Science Foundation (No. 0801036B). The authors are grateful to Prof. Wen-Yong Huang for valuable comments on the manuscript.

### References

- [1] Y. Xiang and B.K. Kobilka, *Science* **300**, 1530 (2003).
- [2] F. Qin, J. Shite, and C.S. Liang, *Am. J. Physiol. Heart Circ. Physiol.* **285**, H822 (2003).

- [3] Y.C. Fu, C.S. Chi, S.C. Yin, B. Hwang, Y.T. Chiu, and S.L. Hsu, *Cardiovasc. Res.* **62**, 558 (2004).
- [4] N. Li and Y.W. Kwan, *J. Chromatogr. A* **849**, 349 (1999).
- [5] G.L. Xu, Z.N. Gong, W.P. Yu, L. Gao, S.Y. He, and Z.Y. Qian, *Basic Clin. Pharmacol. Toxicol.* **100**, 31 (2007).
- [6] W.Y. Gao and D.Y. Zhu, *Chin. Tradit. Herb. Drugs* **30**, 389 (1999).
- [7] T.Z. Liu, Y. Lu, C.Y. Bao, and Z.Y. Qian, *J. China Pharm. Univ.* **33**, 505 (2002).
- [8] W.P. Yu, Z.Y. Qian, G.L. Xu, and C.X. Shen, *Chin. Tradit. Herb. Drugs* **35**, 68 (2004).
- [9] X.C. Shen and Z.Y. Qian, *Acta Pharm. Sin.* **39**, 172 (2004).
- [10] X.C. Shen and Z.Y. Qian, *Pharmazie* **61**, 348 (2006).
- [11] J.K. Amin, L. Xiao, D.R. Pimental, P.K. Pagano, K. Singh, D.B. Sawyer, and W. Colucci, *J. Mol. Cell. Cardiol.* **33**, 131 (2001).
- [12] G.M. Kuster, D.R. Pimentel, T. Adachi, Y. Ido, D.A. Brenner, R.A. Cohen, R. Liao, D.A. Siwik, and W.S. Colucci, *Circulation* **111**, 1192 (2005).
- [13] F. Qin, N.K. Rounds, W. Mao, K. Kawai, and C.S. Liang, *Cardiovasc. Res.* **51**, 736 (2001).
- [14] M.K. Gupta, T.V. Neelakantan, M. Sanghamitra, R.K. Tyaqi, A. Dinda, S. Maulik, C.K. Mukhopadhyay, and S.K. Goswami, *Antioxid. Redox. Signal.* **8**, 1081 (2006).
- [15] M. Neri, D. Cerretani, A.I. Fiaschi, P.F. Laghi, P.E. Lazzerini, A.B. Maffione, L. Micheli, G. Bruni, C. Nencini, G. Giorgi, S. D'Errico, C. Fiore, C. Pomara, I. Riezzo, E. Turillazzi, and V. Fineschi, *J. Cell. Mol. Med.* **11**, 156 (2007).
- [16] S. Roychowdhury, G. Wolf, G. Keilhoff, and T.F. Horn, *Nitric Oxide* **8**, 39 (2003).
- [17] J.D. Port and M.R. Bristow, *J. Mol. Cell. Cardiol.* **33**, 887 (2001).
- [18] J. Sadoshima and S. Izumo, *Circ. Res.* **73**, 413 (1993).
- [19] S. Yang, H. Zhu, Y. Li, H. Lin, G. Gabrielson, M.A. Trush, and A.M. Diehl, *Arch. Biochem. Biophys.* **378**, 259 (2000).
- [20] J.J. Sedmark and S.E. Grossberg, *Anal. Biochem.* **79**, 544 (1977).
- [21] B.S. Kristal, J. Chen, and B.P. Yu, *Free Radic. Biol. Med.* **16**, 323 (1994).
- [22] D.B. Sawyer, R. Fukazawa, M.A. Arstall, and R.A. Kelly, *Circ. Res.* **84**, 257 (1999).
- [23] H. Hong and G.Q. Liu, *Life Sci.* **74**, 2959 (2004).
- [24] T. Satoh, Y. Enokido, H. Aoshima, Y. Uchiyama, and H. Hatanaka, *J. Neurosci. Res.* **50**, 413 (1997).
- [25] W.B. Luo and Y.P. Wang, *Acta Pharm. Sin.* **22**, 1135 (2001).