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Effects of crocetin on antioxidant enzymatic activities in cardiac hypertrophy induced by norepinephrine in rats

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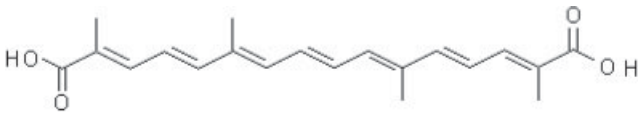
Crocetin, a carotenoid isolated from the Chinese herbal medicine *Crocus sativus* L (Saffron), has been shown to have cardiovascular protective effects. The present study investigated the protective action of the antioxidant crocetin against cardiac hypertrophy induced by norepinephrine (NE). This was evaluated by assaying for pathological histological changes with an optical microscope and cell image analysis system. Lipid peroxidation was quantified using thiobarbituric acid-reactive substances (TBARS). Myocardial superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and myocardial catalase (CAT) activities were assayed to evaluate the antioxidant capacity. After long term treatment with NE, antioxidant enzymatic activities were significantly decreased, while products of lipid peroxidation increased. Crocetin markedly reduced the content of lipid peroxidation (LPO), increased the GSH-Px and SOD activity in cardiac hypertrophy, and significantly improved the myocardial pathological histological changes induced by NE. These results suggest that the cardioprotective effects of crocetin are related to modulation of endogenous antioxidant enzymatic activities. Comparing crocetin with captopril, our results indicated that antioxidant activity is an important factor in the therapy of cardiac hypertrophy, but as an antioxidant only, its effects may be limited.

1. Introduction

The adrenergic agonist norepinephrine (NE) stimulates a variety of biological responses in cardiac myocytes including hypertrophic growth and the re-expression of fetal genes (Amin et al. 2001). Cardiac hypertrophy represents an initial physiological adaptive response to increase in blood pressure or afterload (Frohlich 1987). However, despite normalization of systemic blood pressure by medication, cardiac hypertrophy frequently decompensates into congestive heart failure (Vasan and Levy 1996). It is now apparent that reactive oxygen species (ROS) such as superoxide anion, hydroxyl radical, and hydrogen peroxide may act as intracellular signaling molecules for biological events including cell growth (Irani et al. 1997). Increasing evidence from experimental models of heart failure supports the concept that there is increased oxidative stress in the failing heart, and that this contributes to the pathogenesis of myocardial remodeling and failure (Kwon et al. 2003). Recent progress in understanding the mechanisms that mediate myocardial remodeling at the cellular level has led to evidence that reactive oxygen species (ROS) and oxidative stress play a central role in regulating the phenotype of cardiac myocytes and fibroblasts (Sawyer et al. 2002).

Those reactive oxygen species may induce cardiac damage either directly though interacting with and destroying cellular proteins, lipids and DNA, or indirectly by affecting normal cellular signaling pathways and gene regulations, resulting in injury of the cardiac function (Griendling et al. 1997; Cerutti 1985; Breimer 1990). Reactive oxygen species can be scavenged by endogenous antioxidants, including superoxide dismutase (SOD) that catalyses the dismutation of the superoxide anion, glutathione peroxidase (GSH-Px) and catalase (CAT) that mediates the breakdown of hydrogen peroxide (Sawyer et al. 2002). The importance treating cardiac hypertrophy has been shown recently (Takemoto et al. 2001). Therefore, it is beneficial for the treatment of ischemic diseases to maintain the activities of antioxidant enzymes.



Chemical structure of crocetin

Crocetin, a known carotenoid, is a primary active ingredient extracted from *Crocus sativus* L (Saffron) (Li et al. 1999). Saffron, the dried red stigmata of *Crocus sativus* L. flowers, and a traditional Chinese medicine (TCM), has long been used for treatment of coronary heart disease and hyperlipemia, and has the ability to promote blood circulation by preventing blood stasis. It has been proved that crocetin is effective for ischemic cardiovascular diseases (Gainer and Jones 1975; Gao and Zhu 1999). Many re-

ports and our previous research have indicated that crocetin exhibits a strong anti-oxidative effect (Liu et al. 2002; Wilkins and Wilkins 1978), the ability of anti-myocardial ischemia induced by deligating coronary artery in rats and dogs. In a recent study, crocetin has been shown to attenuate the development of pressure-overload cardiac hypertrophy in rats after chronic coarctation of the abdominal aorta *in vivo*, to improve a cardiac myocyte hypertrophy induced by NE *in vitro*, and to decrease the activities of MMP-2 and MMP-9 (Shen and Qian 2004; Shen et al. 2004). In this study, we mainly investigated the antioxidant enzymes of crocetin on the cardiac hypertrophy induced by NE. The SOD, GSH-Px and CAT activities, products of lipid peroxidation, were determined regarding the effects of crocetin on NE-induced cardiac hypertrophy.

2. Investigations and results

2.1. Ventricular mass

After 14 d treatment of rats with NE, the animals were killed. Hearts were used for the measurement of regional weight and for pathological histological section analysis. In our results, NE induced cardiac hypertrophy was evaluated by left ventricular index and right ventricular index, which refer to the ratios of left or right ventricular weight to body weight. The left ventricular index in the model groups was significantly increased compared with the control, but the right ventricular index was similar in all five groups. Both crocetin and captopril significantly attenuated the increase in left ventricular index induced by long term treatment with NE (Table).

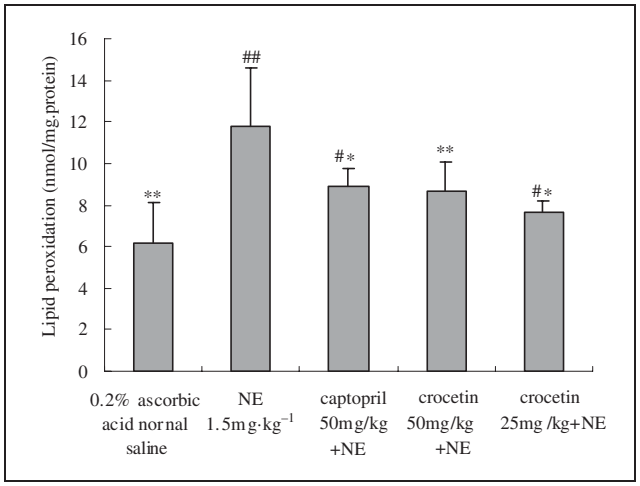


Fig. 1: Effect of crocetin on products of lipid peroxidation in cardiac hypertrophy induced by NE in rats. Products of lipid peroxidation was measured by TBARS assay. The data are presented as means ± SD with n = 7. $^{\#\#}p < 0.01$, $^{\#}p < 0.05$ vs. 0.2% ascorbic acid nornal saline; $^{**}p < 0.01$, $^{*}p < 0.05$ vs. NE 1.5 mg/kg

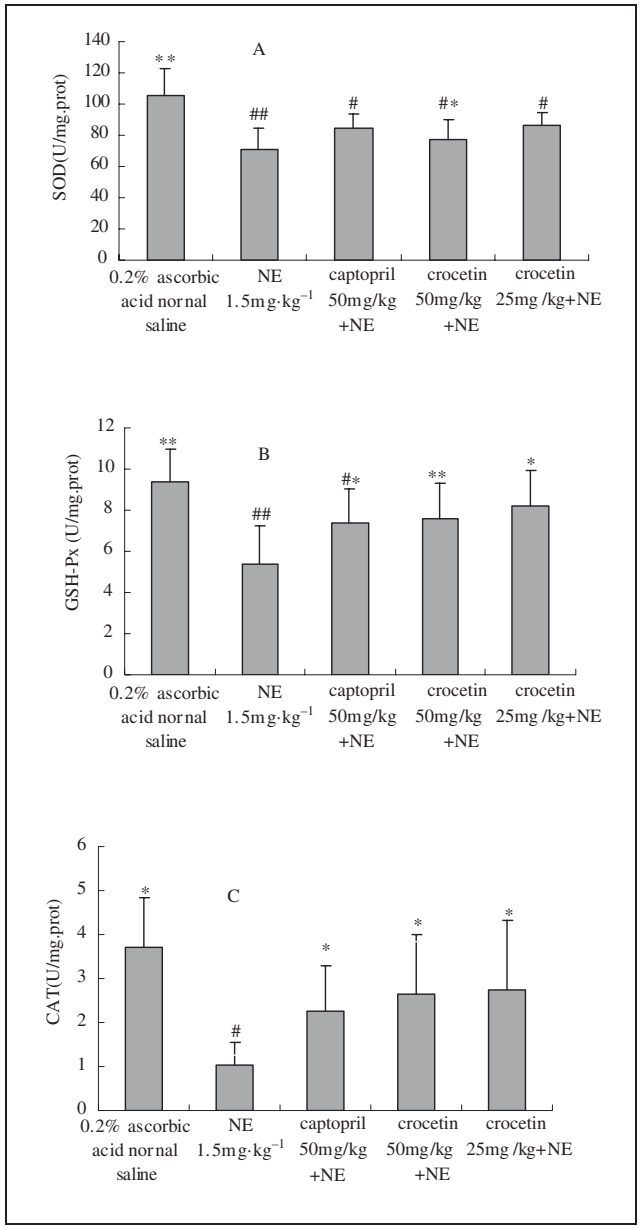


Fig. 2: Effect of crocetin on activities of antioxidant enzymes in left ventricular tissue in cardiac hypertrophy induced by NE in rats. Data represent mean ± SD, n = 7. $^{\#\#}p < 0.01$, $^{\#}p < 0.05$ vs. 0.2% ascorbic acid normal saline; $^{**}p < 0.01$, $^{*}p < 0.05$ vs. NE 1.5 mg/kg

2.2. Crocetin inhibited products of lipid peroxidation

Many cellular compounds are easily oxidized, and lipid peroxides have been used as a marker for oxidative stress (Kruman et al. 1998; Janero 1990). The resultant oxidative stress was determined by measuring the level of lipid per-

Table: Effects of crocetin on cardiac weight index

Group	BW (g)	LVW (mg)	RVW (mg)	LVW/BW	RVW/BW
0.2% Ascorbic acid nornal saline	254.9 ± 7.6	550.95 ± 44.5	127.58 ± 21.10	2.16 ± 0.16	0.50 ± 0.08
NE 1.5 mg/kg/d × 15 d	242.7 ± 8.6$^{\#\#}$	567.03 ± 53.45	120.56 ± 22.14	2.40 ± 0.16$^{\#\#}$	0.52 ± 0.08
Captopril 50 mg/kg/d + NE × 15 d	248.8 ± 10.8	515.93 ± 41.56*	113.07 ± 15.34	2.07 ± 0.15**	0.45 ± 0.06
Crocetin 50 mg/kg/d + NE × 15 d	234.1 ± 17.59$^{\#}$	516.44 ± 59.79	117.72 ± 25.36	2.18 ± 0.20*	0.50 ± 0.11
Crocetin 25 mg/kg/d + NE × 15 d	239.7 ± 6.0$^{\#\#}$	543.79 ± 29.19	114.63 ± 13.37	2.24 ± 0.11	0.46 ± 0.05

BW: body weight; RVW: right ventricular weight; LVW: left ventricular weight; LVW/BW: left ventricular weight/body weight ratio; RVW/BW: right ventricular weight/body weight ratio. Data are means ± SD (n = 7)
$^{\#}p < 0.05$; $^{\#}p < 0.01$ vs. 0.2% ascorbic acid nornal saline; $^{*}p < 0.05$; $^{*}p < 0.01$ vs. NE 1.5mg/kg/d × 15 d

oxidation. Lipid peroxidation of cardiac hypertrophy tissue induced by NE was assessed in a TBARS assay. Lipid peroxidation was markedly increased in left ventricular tissue of cardiac hypertrophy induced by NE. Captopril, an angiotensin I converting enzyme inhibitor widely used in cardiac hypertrophy, also significantly inhibited the products of lipid peroxidation at 50 mg/kg. Both doses of crocetin also significantly prevented the products of lipid peroxidation. In our results, crcoetin had clear dose-dependent effects, as shown in Fig. 1.

2.3. Crocetin protected free radical scavengers

As shown in Fig. 2 (A, B, C) in long term treatment with NE, the activity of CAT, SOD and GSH-Px was drastically decreased ($p < 0.05$, $p < 0.01$ compared with the control group). Both doses of crocetin significantly increased the activities of GSH-Px and CAT in left ventricular tissue of cardiac hypertrophy induced by NE in rats, but only the maximum dose of crocetin elevated SOD activity to a statistically significant extent. Comparison of our results for crocetin and nimodipine indicated that crocetin, presumably having higher catalytic activities or greater membrane permeability, exhibited higher potency against oxidative stress in left ventricular tissue of cardiac hypertrophy induced by NE in rats.

2.4. Histopathological study

Fig. 3 shows the HE micrograph of a control heart with normal architecture. On treatment with NE, augmentation and loose arrangement of the myocardial fibres, staining asymmetry, monstrosity of partly nucleolus, and marked edema were observed. Crocetin and captopril markedly improved the pathological changes. Changes were studied using a CMIS type cellular medicine image assay system

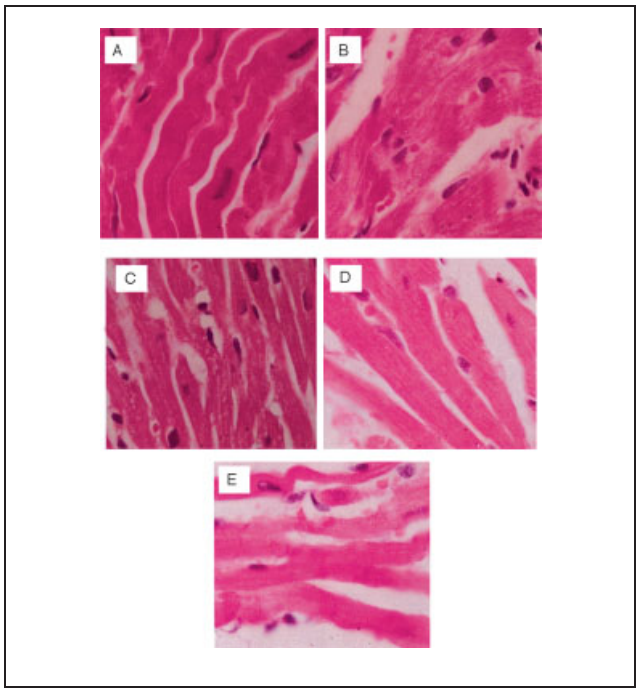


Fig. 3: Light micrograph demonstrating the effect of crocetin on cardiac hypertrophy induced by NE. × 200.
A: 0.2% ascorbic acid nornal saline; B: NE 1.5 mg/kg; C: captopril 50 mg/kg + NE; D: crocetin 50 mg/kg + NE; E: crocetin 25 mg/kg + NE

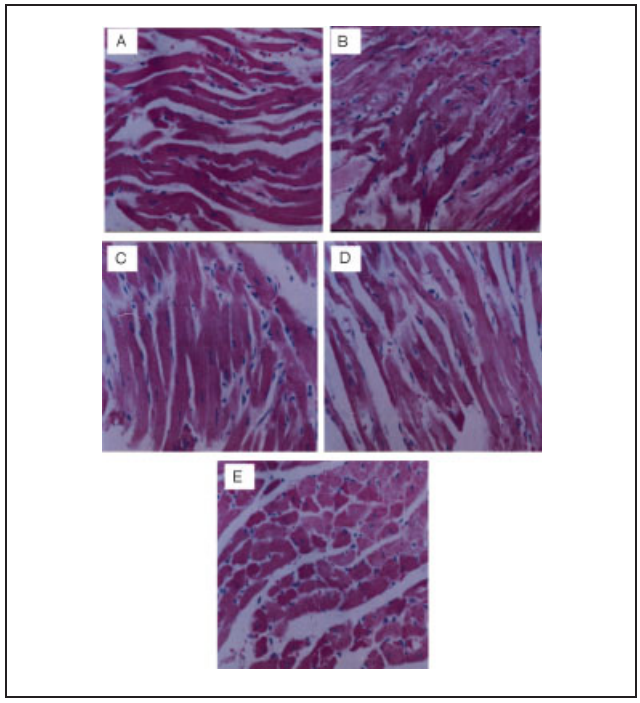


Fig. 4: The image of left ventricular tissue section by CMIS type cellular medicine image assay system.
A: 0.2% ascorbic acid nornal saline; B: model NE 1.5 mg/kg; C: captopril 50 mg/kg + NE; D: crocetin 50 mg/kg + NE; E: crocetin 25 mg/kg + NE

(ChongQing TanHai Ltd. China). At the field of vision, the cardiac myocytes drastically decreased and the interstitial of cardiac myoctytes increased under treatment with NE, crocetin and captopril improved (Fig. 4).

3. Discussion

Cardiac hypertrophy is a major cause of morbidity and mortality worldwide. Indeed, cardiac hypertrophy is an independent risk factor for cardiovascular disease, and increases cardiovascular mortality more than two-fold (Takemoto et al. 2001). It is important to find good new drugs for therapy of cardiac hypertrophy. Traditional Chinese Medicine (TCM) has been widely adopted for clinical use in eastern Asian countries for over 2000 years. In the long history of its development, TCM has demonstrated its great vitality because of its firm clinical foundation, significant therapeutic effects, and specific system of theory based on clinical practice. Nowadays, more and more countries pay attention to TCM, especially for dubious and complicated cases, such as cancer and cardiovascular diseases. Even though natural products have been screened for effectiveness, only limited scientific evidence has been reported for their traditional use and their mechanism of action.

In this paper, we investigate the antioxidant effects of crocetin on left ventricular tissue of cardiac hypertrophy induced by NE. A significant increase in the myocardial activities of SOD and GSH-Px was observed with crocetin, however no obvious effects on CAT were observed. The products of lipid peroxidation were significantly suppressed by crocetin in cardiac hypertrophy tissue induced by NE. To a certain degree, a dose dependent effect was observed. Captopril was used as a positive control drug, as an angiotensin I converting enzyme inhibitor, with a clinical indication for therapy of cardiac hypertrophy and

a previously reported antioxidative effect (Hayek et al. 1998; Jevtović-Stoimenov et al. 2003). Our results confirmed the antioxidative effects.

Lipid peroxides were assessed for levels of TBARS, including MDA, a marker of lipid peroxidation. The present study demonstrated that crocetin attenuated the increase in the products of lipid peroxidation in the left ventricular tissue of cardiac hypertrophy induced by NE, suggesting that crocetin has the potential to protect the membranes of cardiac myocytes from lipid peroxidative damage. This finding was consistent with our previous report showing its cardiovascular protective effects in cardiac ischemia (Liu and Qian 2003).

Reactive oxygen species are believed to play a central role in cardiac hypertrophy (Clerk et al. 2003). In oxidative stress, superoxide anion and hydrogen peroxide formed during pathological changes of cardiac hypertrophy cannot be readily scavenged because of the low activities of CAT, SOD and GSH-Px present in the cardiac tissue. Moreover, membranes of bioplasm are very rich in polyunsaturated fatty acids, which are especially sensitive to free radical-induced lipid peroxidation. Augmentation of endogenous antioxidants (SOD, CAT, GSHPx) has been recognized as an important pharmacological property, present in natural as well as many synthetic compounds (Gauthman et al. 2001). This constitutes a major mechanism for the protection against oxidative stress which they offer by them (Bhattacharya et al. 2002; Rajak et al. 2004). The most abundant reactive oxygen species generated in living systems is the superoxide radical which is acted upon by SOD to produce hydrogen peroxide which in turn is inactivated by catalase and/or GSHPx into water and oxygen. Thus an increase in both SOD and catalase along with GPx activity is considered to be particularly beneficial in the event of oxidative stress (Harman 1991). Our study showed that the activities of SOD, CAT, and GSH-Px in cardiac tissue tended to decrease after 14 d treatment with NE. Crocetin, when administered to rats, significantly elevated the activities of SOD, CAT, and GSH-Px, which was different from the effect of captopril which increased GSH-Px and CAT activity only. Crocetin clearly scavenged hydrogen peroxide and superoxide anion; thus, further decreasing the formation of hydroxyl radical and attenuating lipid peroxidative damage after NE treatment. However, it is not clear whether crocetin induced the expression of the endogenous antioxidant enzymes or directly protected them.

Increase in myocardial lipid peroxidation and depletion of myocardial endogenous antioxidants support the occurrence of oxidative stress in the model hearts following 14 d treatment with NE in the present study. It was also accompanied by tissue injury with marked edema and focal loss of myocardial fibres. Hearts from rats treated crocetin at both doses were protected against oxidative stress, as evidenced by inhibition of increase in TBARS, depletion of CAT, SOD, and GSH-Px and tissue injury after treatment with NE. The mechanism of such protection can be attributed to the augmented endogenous antioxidant reserve of the heart and to a direct antioxidant effect.

Our research results indicated that the antioxidative effects of crocetin were stronger than those of captopril, however comparing the effects of crocetin and captopril in improving cardiac hypertrophy particularly the left ventricular index, the order was reversed. Thus, antioxidant properties are important in the therapy of cardiac hypertrophy, but where there is only an antioxidant effect, our results indicate that its effect may be limited.

4. Experimental

4.1. Drugs and reagents

Crocetin ($C_{20}H_{24}O_4$, it purity $>90\%$, determined by HPLC) was isolated and purified in Professor Zhi-yu Qian's laboratory. NE was purchased from Shanghai Hefen Pharmaceutical Factory. Thiobarbituric acid-reactive substances (TBARS) were purchased from Fluka. Bovine serum albumin (BSA), and tetraethoxy propane were purchased from Sigma. Captopril was purchased from Weifang Pharmaceutical Ltd. Other reagents were all of AR grade.

4.2. Animals

Female Sprague-Dawley rats weighing 180–220 g were provided by the Experimental Animal Center of China Pharmaceutical University and raised under constant temperature and humidity conditions, and were given free access to a standard chow and tap water before and during the experimental period.

4.3. Cardiac hypertrophy model

NE 1.5 mg/kg in 0.2% ascorbic acid in normal saline was injected intraperitoneally twice daily for 15 d: this treatment schedule produces hypertrophy without necrosis (Xiang and Huang 2000; Tsoporis et al. 1998). The animals were divided into five groups: (1) control (0.2% ascorbic acid in normal saline), (2) NE 1.5 mg/kg/d, (3) NE + crocetin 50 mg/kg/d, (4) NE + crocetin 25mg/kg/d, (5) NE + captopril 50 mg/kg/d. Crocetin and captopril were administered by i.g. for 15 d. After the rats were sacrificed, their hearts were removed, and the free wall of the right ventricle was separated from the intra-ventricular septum which remained as a part of the left ventricle (LV). The left ventricles were weighed after freezing in liquid nitrogen and stored at $-70\,^{\circ}C$. The left and right ventricular index was calculated by the ratio between left ventricular weight or heart weight and body weight.

4.4. Homogenization of LV tissue

The LV tissues were sliced into small pieces and thoroughly washed with potassium phosphate buffer ($50\ mmol \cdot L^{-1}$) to avoid contamination from blood. Homogenization of the LV tissue was performed following the method described by Lee et al. (1996). Protein concentration was measured using the Coomassie blue method, and BSA was used as a reference standard (Sedmark and Grossberg 1977).

4.5. Thiobarbituric acid-reactive substance (TBARS) assay

The content of LPO in the supernatant was measured with thiobarbituric acid as described previously (Kristal et al. 1994; Janero 1990). The homogenate was mixed with 3 ml of 1% phosphoric acid, 1 ml of 0.67% thiobarbituric acid and 0.04% butylated hydroxytoluene (BHT) in glass test tubes, and the mixtures were incubated in a boiling water bath for 60 min. Marbles were placed on the tops of tubes during the incubation period to avoid excessive loss of reaction mixture. After cooling the tubes in ice, 1.5 ml of n-butanol was added and the reaction mixture was centrifuged at $1000 \times g$ for 10 min. The absorbance of the supernatant was read at 535 nm. The concentrations of TBARS were calculated using tetraethoxypropane as a reference standard. Results were expressed in nmol/mg · protein.

4.6. Measurement of SOD activity

SOD activity was measured by xanthine/xanthine oxidase mediated ferricytochrome c reduction assay (Yang et al. 2000). One unit of SOD activity was defined as the amount that reduced the absorbance at 550 nm by 50%. Fifty microliter of LV tissue homogenate were added to 2.9 ml of reaction buffer (0.5 μmol of xanthine, 0.1 mM NaOH, and 2 μmol of cytochrome c in 50 mM K_2HPO_4-Na_2HPO_4/0.1 mM EDTA, pH 7.8). The reaction was initiated by adding 50 μL of xanthine oxidase solution (0.2 U/mL in 0.1 mM EDTA). The change of absorbance was monitored for 3 min at $25\,^{\circ}C$. Activities were calculated using a concurrently run standard curve and expressed per milligram of protein. The results of SOD activity were expressed as U/mg · protein.

4.7. Estimation of GSH-Px activity

GSH-Px activity was determined by quantifying the rate of oxidation of reduced glutathione (GSH) to oxidized glutathione (GSSG) by H_2O_2 catalyzed by GSH-Px. One unit of GSH-Px was defined as the amount that reduced the level of GSH by $1\ \mu mol \cdot l^{-1}$ in 1 min per mg protein. GSH-Px activity was measured in a 1.0 ml cubette containing 400 μl of 0.25 M potassium phosphate buffer (pH 7.0), 200 μl of sample, 100 μl of 10 mM GSH, 100 μl of 2.5 mM NADPH and 100 μl of glutathione reductase (6 U/ml). Hydrogen peroxide (100 μl of 12 mM) was then added and change in absorbance was measured at 1 min intervals for 5 min at 366 nm (Diehl et al. 1988). GSH-Px activity is expressed as units/mg protein as compared to the standard.

4.8. Myocardial CAT

The activity of CAT was detected by methods developed in our laboratory. Briefly, 10 mmol · L^{-1} H_2O_2 (2.8 ml) was added to a 5.0 ml cubette that contained 0.2 ml 1 mol · L^{-1} Tris-HCl (pH 8.0) in an ice-water bath. Absorbance was immediately measured at 240 nm, and was called OD_0. Then supernatant of left ventricular tissue (10 μl) was added to the cubette. The mixture was completely reacted at 25 °C for 4 min and then the absorbance was detected at 240 nm, this being OD_4. For the nonenzymatic reaction cubette, the procedure was the same as for the detected cubette, the only difference being that distilled water was used instead of the supernatant of left ventricular tissue, and the absorbance was recorded as OD_n. The results were expressed as catalytic activity following the equation (units/mg · protein) = $(OD_0 - OD_4 - OD_n) \times C_{H_2O_2}/C_{protein} \times 4$.

4.9. Pathological histological changes

Left ventricular tissue was fixed in 4% buffered formaldehyde, embedded in paraffin, and the 5 μm sections obtained from the material were stained with hematoxylin-eosin. The sections were quantified morphometrically with an optical microscope and a computer assisted CMIS type cellular-body fluid medicine image assay system (ChongQingTan Hai Ltd. China.).

4.10. Statistical analysis

Throughout the test, data was expressed as means ± SD. The evaluation of statistical significance was determined by one-way ANOVA.

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