Protective Effect of Natural Antioxidants on Heart Against Ischemia-Reperfusion Damage

Yan Zhao[1] and Baolu Zhao[2,*]

[1]*Department of Food Sciences and Technology, Harbin Institute of Technology at Weihai, 264209, China;* [2]*State Key Laboratory of Brain and Cognitive Science, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China*

Abstract: The incidence and mortality of heart disease are the highest among all diseases all over the world, and are still increasing with a world wide rise in living standards. To find effective treatments for prevention and curing heart disease, it is important to understand the mechanisms behind the cause and the development of the disease. Increasing evidences have shown that reactive oxygen species (ROS) and reactive nitrogen species (RNS) play important roles in the initiation and progression of heart disease. The potential of using antioxidants, especially the natural antioxidants, in preventing and curing the disease has attracted enormous interest. In this paper we reviewed the progress made in understanding the oxidative stress caused by myocardial ischemia-reperfusion and the cardioprotective effect of natural antioxidants against ischemia-reperfusion injury.

Keywords: Heart disease, reactive oxygen species (ROS), reactive nitrogen species (RNS), oxidative stress, ischemia-reperfusion, free radicals, natural antioxidants.

1. INTRODUCTION

The incidence and mortality of heart disease are the highest among all disease in China and all over the world, and are still increasing with the rising living standards. To find effective treatment and prevention of the heart disease, it is important to thoroughly understand the pathobiology of the disease. Increasing evidences have shown that reactive oxygen species (ROS) and reactive nitrogen species (RNS) play important roles in the development of heart disease [1-12]. Generation of ROS and RNS are normally tightly regulated. At low and moderate concentrations, ROS/RNS mediate signal transduction cascades involved in a variety of cellular and physiological functions, and are important for defense against infectious agents. In contrast, overproduction of ROS/RNS results in oxidative stress, a deleterious process that causes damages to cell structures including lipids, proteins, and DNA, which can eventually lead to cellular senescence and apoptosis [13, 14]. Oxidative stress has been detected during myocardial ischemia-reperfusion while antioxidants protect heart from ischemia-reperfusion injury. Therefore, the potential of using antioxidants, especially natural antioxidants, in prevention and treatment of heart disease has attracted enormous interest [15-20]. In this paper we reviewed the progress made in understanding the oxidative stress caused by myocardial ischemia-reperfusion and the cardioprotective effect of natural antioxidants against ischemia-reperfusion injury.

*Address correspondence to this author at the State Key Laboratory of Brain and Cognitive Science, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China; Tel: 861064888569, Fax: 861064871293; E-mails: zhaobl@sun5.ibp.ac.cn

2. OXIDATIVE STRESS IN ISCHEMIA-REPERFUSED HEART

2.1. ROS Generated During Myocardial Ischemia-Reperfusion

ROS include hydroxyl radical (•OH), superoxide free radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), singlet oxygen, and other reactive molecules containing oxygen atoms. Due to the high rate of oxidative metabolic activity, the heart is extremely susceptible to the damage caused by oxygen radicals. During myocardial ischemia/reperfusion (IR), ROS can be generated from mitochondrial respiratory chain, ischemia-activated xanthine/hypoxanthine oxidase and lipid metabolism [1].

Electron paramagnetic resonance (EPR) spectroscopy can be applied to measure free radical directly. To determine the free radical generation during ischemia-reperfusion, Zweier *et al.* examined perfused rabbit hearts using EPR [2, 3]. Hearts were freeze-clamped at 77 degrees K during control perfusion after 10 min of normothermic global ischemia or following post-ischemic reperfusion with oxygenated perfusate. The EPR spectra of these hearts exhibited three different signals with different power saturation and temperature stability. Signal 1 was identical to those of a carbon-centered semiquinone, whereas those of signal 2 were similar to alkyl peroxyl or superoxide oxygen-centered free radicals; signal 3 was most likely a nitrogen-centered free radicals. In the control heart samples, signal 1 predominated, whereas in ischemic hearts signal 1 decreased in intensity, and signals 2 and 3 became more intense; with reperfusion all three signals markedly increased. Free radical concentrations derived from the intensities of the 2 and 3 signals peaked at 10 sec after initiation of reflow. At this time the oxygen-centered free radical concentration derived from the intensity of signal 2

1389-2010/10 $55.00+.00

was increased six times over the concentration measured in control hearts and two times over the concentration measured in ischemic hearts. Hypoxic reperfusion did not increase any of the free radical signals over the levels observed during ischemia. These results demonstrated that ROS were produced in hearts during ischemia and that a burst of oxygen radical generation occurred within moments of reperfusion.

Similar results were obtained by our laboratory when we measured the free radical generation in ischemia and reperfused rabbit hearts using EPR spectroscopy. The hearts were freeze-clamped at 100 degrees K during control perfusion after 150 min of global ischemia or following post-ischemic reperfusion with oxygenated perfusate for 15 seconds. The spectra of the ischemia hearts exhibited two different signals with different power saturation and temperature stability. Signal 1 was identical to oxygen radicals, which were disappeared when superoxide dismutase (SOD) and catalase were added to the perfusion solution, indicating that they were the oxygen free radicals generated from ischemia-reperfusion heart [4-7].

Using EPR spectroscopy and the spin trap 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), Sanders *et al*. determined the species and sources of the free radicals generated in a hyperoxic endothelial cell model. They found that sheep pulmonary microvascular endothelial cells exposed to 100% O_2 for 30 min exhibited a prominent signal of trapped hydroxyl radical, DMPO-OH and, occasionally, additional smaller DMPO-R signals thought to arise from the trapping of superoxide anion ($O_2^{\cdot-}$), hydroxyl (•OH), and alkyl (•R) radicals. SOD quenched both signals, suggesting that these observed radicals were derived from $O_2^{\cdot-}$. The generation of •R occurred secondary to the formation of •OH from $O_2^{\cdot-}$ via an iron-mediated Fenton reaction since removing iron with deferoxamine decreased •R signal. Blocking mitochondrial electron transport with rotenone (20mM) markedly decreased radical generation, suggesting that endothelial cells exposed to hyperoxia produced free radicals via mitochondrial electron transport. Cell mortality increased slightly in these oxygen-exposed cells, which was not altered by SOD or deferoxamine, or different from the mortality observed in air-exposed cells. Therefore, the radical generation did not appear to cause cell death under the experimental conditions [9].

Altough the EPR spectroscopy can be applied to directly measure free radicals, the conventional spectrometer designs are not suitable for performing measurements on large aqueous structures such as whole organs or tissues. In an effort to obtain optimum performance in measuring free radicals in intact biologic organs or tissues, Zweier and Kuppusamy developed a spectrometer that consisted of a 1- to 2-GHz microwave bridge with the source locked to the resonant frequency of a specially designed loop-gap resonator. Real time measurements of free radicals and cardiac contractile function can be performed simultaneously on isolated hearts using this spectrometer, enabling the evaluation of both free radical generation and organ function [10]. Furthermore, EPR imaging instrumentation suitable for performing three-dimensional spectral-spatial EPR imaging experiments on large biologic samples made it possible to measure free radicals in spatially defined tissue structure. In the isolated rat heart, imaging experiments using this instrumentation demonstrated a transmural gradient in the rate of myocardial radical clearance with a slower clearance in the endocardium, suggesting that endocardium is more susceptible to ischemia injury [11]. Alongside, the study also demonstrates that the spectral-spatial EPR imaging is a powerful tool in providing spatial information regarding the free radical distribution, metabolism, and tissue oxygenation in living biological organs and tissues.

2.2. NO Free Radicals Generated During Myocardium Ischemia-Reperfusion

Nitric oxide (NO) is a simple gas with free radical properties. It appears as a major signaling molecule in cardiovascular, immune and nervous systems. NO is generated by three isoforms of NO synthase (NOS) in the body, endothelial NO synthase (eNOS), neuronal NO synthase (nNOS) and inducible NO synthase (iNOS), and is important for regulating many physiological processes such as blood pressure and vascular tone [21, 22]. However, excessive NO produced by iNOS results in inhibition of cardiac contractility, impairment of mitochondrial respiration and apoptosis, thus contributing to ischemia-reperfusion injury [23].

The binding of NO to hemoglobin (Hb) subunits specifically at low temperature allows the measurement of NO using EPR spectroscopy. In EPR spectrum, NO bound to α-subunit of Hb exhibits peaks at g=2.078 and g=2.01 with a splitting of 17.5G, while the signal with a peak at g=2.04 and a valley at g=2.015 is the characteristic of NO bound to β-subunit of Hb [24, 25]. We found that the EPR spectrum of normal myocardium showed semiquinone free radical and transition metal cation in the myocardium. Two additional peaks at g=2.04 and g=2.03 were detected from the ischemia-reperfused myocardium: the peak at g=2.04 was similar to that of the NO bound to β-subunit of Hb and the peak at g=2.03 was tentatively attributed to oxygen and alkyl peroxide radicals. The signal at g=2.04 could be decreased by the addition of N^G-nitro-L-arginine methyl ester (NAME, the inhibitor of NO synthase) in the reperfusion solution, while increased by L-arginine (substrate of NOS), indicating that it might be associated with NO generation. Both the signals at g=2.04 and g=2.03 were decreased by SOD/catalase but increased by the addition of Fe/H_2O_2 or xanthine/xanthine oxidase in the reperfusion solution, indicating that they were both dependent on ROS production [12].

NO has a high affinity to ferrous-containing protein. It can form a stable paramagnetic mononitrosyl iron complex-($NOFe^{2+}DETC$) with diethyldithiocarbamate (DETC) and iron. This complex shows characteristic peaks at g=2.035 and g=2.020 with triplet hyperfine structure in EPR spectrum , therefore $Fe^{2+}DETC$ has been used as NO trapping reagents to detect NO specifically in tissues [26, 27]. Using DETC/Fe^{2+}, we studied the NO free radical generation in rat heart during ischemia-reperfusion. It was shown that in normal heart, there was a baseline of the EPR signals with characteristic of $NOFe^{2+}DETC$ complex, perhaps represented the physiological level of NO. The EPR signal intensity of $NOFe^{2+}$ DETC complex in rat myocardium was increased remarkably after 30 min of ischemia. L-arginine increased while N^G-nitro-L-arginine (NNA, inhibitor of NOS) decreased the sig-

nal intensity, indicating that the signal was originated from NO. After 10 min of reperfusion followed 30 min of ischemia, the signal intensity of $NOFe^{2+}DETC$ in rat hearts was significantly reduced than that in ischemia only myocardium. It appears that the NO production increases during ischemia and decreases during reperfusion. Interestingly, the signal intensity of $NOFe^{2+}DETC$ complex was increased significantly by SOD treatment, suggesting that superoxide anions might contribute to the decrease of NO level in the ischemia-reperfusion myocardium, because SOD scavenged the superoxide free radicals and saved NO [18, 19].

It has also been shown that iNOS expression plays a role in myocardial chamber dilation and hypertrophy induced by pressure overload [28]. In wild type mice, chronic transverse aortic constriction (TAC) resulted in myocardial iNOS expression, cardiac hypertrophy, ventricular dilation and dysfunction, and fibrosis, while mice deficient in iNOS displayed much less cardiac hypertrophy, dilation, fibrosis and dysfunction. Consistent with these findings, TAC resulted in marked increases of myocardial atrial natriuretic peptide (ANP), 4-hydroxy-2-nonenal (4HNE, a marker of lipid peroxidation) and nitrotyrosine (a marker for peroxynitrite) in wild type but not in iNOS-deficient mice. In response to TAC, there was an increase in ROS production in myocardium, which was accompanied by the expression of iNOS, suggesting that iNOS was a source for the increased oxidative stress. Furthermore, selective iNOS inhibition with 1400W significantly attenuated TAC induced myocardial hypertrophy and pulmonary congestion. These data implicated iNOS in the maladaptative response to systolic overload, and suggest that selective inhibition on iNOS activity might be effective for treatment of systolic overload-induced cardiac dysfunction [28].

2.3. Oxidative Stress Induced Myocardium Damage and Apoptosis in Ischemia-Reperfusion

Ischemia-reperfusion causes damages to myocardium, which is demonstrated by increased activities of lactate dehydrogenase (LDH) and creatine kinase (CK) [12,16,19]. It was also shown that ischemia caused a significant increase in serum thiobarbituric acid reaction substance (TBARS) concentration which was further increased during the reperfusion. The increased lipid peroxidation and enzyme leakage from the ischemia-reperfusion myocardium were significantly decreased by SOD/catalase treatment [12,16,19]. In contrast, addition of Fe^{2+}/H_2O_2 (or Fe^{2+} and H_2O_2 alone) or xanthine/xanthine oxidase into the reperfusion buffer significantly increased the elevated activities of LDH and CK in the coronary artery effluent [12]. Meanwhile, SOD treatment reduced the elevation of heart rate and the incidence of ventricular arrhythmias caused by ischemia-reperfusion [19]. These results indicated that ROS could contribute to the myocardium injury induced by ischemia-reperfusion and the reduction of ROS and lipid peroxidation might prevent myocardium damage and cardiac injury. The role of NO in ischemia-reperfusion-induced myocardium damage is more complicated. In isolated heart, high concentration of L-arginine increased the activities of LDH and CK in the coronary artery effluent while NAME decreased the activities of LDH and CK in the coronary artery effluent, suggesting high concentration of NO was associated with cardiac injury [12,16]. Administration of L-arginine (50mg/kg/wt) to rat hearts subjected to ischemia-reperfusion *in vivo* cause a reduction in lipid peroxidation and CK activity as well as a decrease in the elevated heart rate and the incidence of ventricular arrhythmias caused by ischemia-reperfusion [19]. Thus, moderated production of NO may be beneficial for protecting the heart from ischemia-reperfusion, while excess NO causes injury to mycardium [19]. In fact, the cardioprotective function of NO in myocardial ischemia-reperfusion has been well documented by Bolli in a review paper, cardioprotective function of inducible nitric oxide synthase and role of nitric oxide in myocardial ischemia and preconditioning: an overview of a decade of research [29].

Oxidative stress leads cells to the exposure of ROS, such as superoxide, hydroxyl radicals and NO, and triggers apoptosis [30]. In a cardiomyocyte model, we found that hypoxia caused a slight increase in lipid peroxidation and cell damage as manifested by increased LDH activity, while reoxygenation of the hypoxic cardiomyocytes induced oxygen burst and a much severe lipid peroxidation injury [31]. This is consistent with the observation that hypoxia induced apoptosis which was further increased by reoxygenation [19, 31]. SOD/catalase inhibited apoptosis in hypoxia-reoxygenated cardiomyocytes, suggesting that superoxide may be one of the important mediators in apoptotic cell death during reoxygenation injury. In this cell model, NO production was increased by hypoxia, but decreased by reoxygenation [31]. The augmentation of NO production during hypoxia may be caused by an up-regulation of NOS activity in response to hypoxia [31]. It is also possible that a direct disproportion or reduction of nitrite to NO under the acidic and highly reduced conditions, such as ischemia or hypoxia, leads to the accumulation of NO during hypoxia. In fact, the enzyme independent mechanism has been demonstrated to not only contribute to the postischemic myocardial injury, but also reverse the protective effects of NOS inhibitors.

To further elucidate the apoptotic mechanisms of hypoxia-reoxygenation injury, we examined the alteration in the expression levels of bcl-2 and p53 proteins associated with hypoxia and hypoxia-reoxygenation induced cell death. The changes in the protein levels of p53 and bcl-2 in response to hypoxia and hypoxia-reoxygenation showed that hypoxia induced the up-regulation of bcl-2 and p53 proteins in cardiomyocytes, while reoxygenation further up-regulated p53 protein, but down-regulated bcl-2 protein [31]. These results suggested that nitric oxide and oxygen radicals induced apoptosis via bcl-2 and p53 pathway in hypoxia-reoxygenated cardiomyocytes.

The reaction of superoxide with NO results in the formation of $ONOO^-$ [30]. Therefore, the decrease of detectable NO may be attributed to the generation of oxygen radicals in reoxygenated cardiomyocytes. Inhibition of NO production by L-NAME inhibited the apoptosis induced by hypoxia and reoxygenation, suggesting the involvement of endogenous NO in cardiomyocyte apoptosis during hypoxia and reoxygenation [31]. The cytotoxicity of NO has been shown to be associated with the formation of $ONOO^-$ [32]. Low concentration of $ONOO^-$ promotes apoptosis whereas large amounts of $ONOO^-$ rapidly lead to necrotic cell death, a primary mechanism responsible for cell death from ischemia. In cultured cardiomyocytes, hypoxia induced mainly cell apoptosis

while reoxygenation led to both apoptotic and necrotic cell death, which may be contributed by the formation of oxygen radicals and $ONOO^-$.

Increased O^-_2 and NO production is a key mechanism of mitochondrial dysfunction in myocardial ischemia/reperfusion injury. Succinate ubiquinone reductase (SQR or Complex II) is a crucial component of the mitochondrial electron transport chain. The intrinsic protein S-glutathionylation at cys(90) of the 70-kDa FAD-binding subunit of SQR makes the protein susceptible to redox change induced by oxidative stress [34]. IR of rat hearts *in vivo* or *in vitro* caused deglutathionylation of the 70-kDa FAD-binding subunit and significantly decreased the electron transfer activity of SQR [34]. IR also caused enhancement of tyrosine nitration of SQR. Site-specific nitration at the 70-kDa FAD-binding subunit with peroxynitrite impaired the interaction of SQR with Complex III and the electron transfer activity [34]. It was found that the *S*-glutathionylation protected the protein from oxidative modification and impairment mediated by peroxynitrite [34]. These studies on SQR give a hint on how ROS generated during IR may lead to mitochondrial dysfunction and IR injury.

AMP activated protein kinase (AMPK) is a key regulatory enzyme regulating myocardial metabolism and protein synthesis, which can be activated by cellular stresses that increase the AMP-to-ATP ratio, such as hypoxia/anoxia, glucose deprivation, and pressure overload induced hypertrophy [35]. The activation of AMPK has been shown to have protective effect against IR injury by promoting ischemic preconditioning [35]. A number of downstream signaling pathways have been implicated in the cardioprotective mechanisms of AMPK such as the activation of eNOS, p38 mitogen-activated protein kinase and the inactivation of eEF2 [36-38]. Activation of AMPK also attenuates hypertrophy in cultured cardiac myocytes. In mice deficient in AMPKα2 gene, TAC-induced ventricular hypertrophy and dysfunction were significantly exacerbated, suggesting that regulation of AMPKα2 may be a potential therapeutic approach to attenuate pressure overload induced ventricular hypertrophy [39].

3. PROTECTIVE EFFECTS OF NATURAL ANTIOXIDANTS ON HEART AGAINST ISCHEMIA-REPERFUSION DAMAGE

Abundant evidences have shown that antioxidants, especially the natural antioxidants, have great potential in the prevention and treatment of heart diseases. Here we will review the studies on several natural antioxidants including tanshinone from *salvia miltiorriza*, EGb 761 from *Ginkgo biloba* and chinonin from traditional Chinese medicine *rhizoma anemarhenea* in preventing the heart against ischemia-repperfusion injury.

3.1. Scavenging Effect of Salvia Miltiorriza (Tanshinone) on Free Radicals and its Protective Effect on Ischemia-Reperfusion Injury

Salvia miltiorriza is a traditional Chinese medicine that has been used to treat heart disease for a long time; however, its mechanism is not clear. The phenanthrenequinone derivative tanshinone is one of the effective components isolated from *salvia miltiorriza* [40]. Yang *et al.* found that tanshinone could effectively scavenge the superoxide free radicals generated from xanthin/xanthine oxidase and the respiratory burst of human polymorphonuclear leukocytes [41]. Tanshinone was also effective in scavenging hydroxyl radicals [42] and lipid free radicals induced by calcium overload in mitochondria and sarcoplasma [43, 44].

In ischemia-reperfusion heart, addition of tanshinone and SOD to the reperfusion solution significantly decreased the oxygen free radical originated EPR signals, and protected the heart from IR damage, indicating that tanshinone and SOD may protect the ischemia-reperfused heart through scavenging the free radicals [15, 44]. In addition, it was found that sodium tanshinone IIA sulphonate (STS) could stimulate mitochondrial NADH oxidation dose-dependently and partially restore NADH oxidation in the presence of respiratory inhibitor (rotenone or antimycin A or KCN) [45]. It was hypothesized that STS can accept electrons from complex I similar to ferricyanide, and is converted to its semiquinone form, which can then reduce oxygen molecule. STS was also shown to reduce cytochrome C in the presence of KCN. These results suggested that STS may inhibit ROS formation through electron transfer reaction in mitochondria and this may contribute to the protection of STS on ischemia-reperfusion injury [45].

Adriamycin (ADR) is an anthracycline antibiotic with a broad spectrum of activities against a variety of human cancers. However, it can cause a cumulative dose-related cardiotoxicity, which limits the dose of ADR that can be safely prescribed clinically. It has been proposed that the cardiotoxicity of ADR is a direct result of ADR-induced free radical formation [46], and possibly the lipid peroxidation initiated by the free radicals. *In vitro* experiments showed that STS inhibited adriamycin induced mitochondrial lipid peroxidation and swelling and scavenged adriamycin semiquinone free radical in heart homogenate dose-dependently. Administration of STS prevented rats against ADR-induced weight loss [47]. Myocardial lipid peroxidation was decreased by STS treatment while the activities of key antioxidant enzymes, such as SOD, glutathione peroxidase and catalase, were increased [48]. Thus, STS might protect heart from the toxicity of ADR through the reduction of ROS and increase of antioxidant enzyme activities [47, 48].

3.2. Effects of EGb 761 on Nitric Oxide and Oxygen Free Radicals, Myocardial Damage and Arrhythmia in Ischemia-Reperfusion Injury *In Vitro* and *In Vivo*

Ginkgo biloba leaf has been used in traditional Chinese medicine for thousands of years [49]. Currently, *Ginkgo biloba* extract is widely used in treating cardiovascular diseases and cerebral vascular diseases in many countries. Its main ingredients are ginkgo-flavone glycosides and terpenoids. Because the chemical ingredients of *Ginkgo biloba* extract are complex and the extraction methods are different with different investigators, it has been hard to evaluate the biological effects of *Ginkgo biloba* extract. EGb 761 is a standardized extract of *Ginkgo biloba* consisting of 24% ginkgo-flavone glycosides (kaempferol, quercetin and isorhamnetin) and 6% terpenoid including ginkgolide A, ginkgolide B, ginkgolide C, ginkgolide M, ginkgolide J, and bilobalide B. Many studies have shown that EGb 761 protect rat hearts

from ischemia-reperfusion-induced arrhythmias and functional damages and the cardioprotective effect of EGb 761 is at least partly attribute to its antioxidant properties [16, 49-52]. It was shown that EGb 761 could effectively scavenge the superoxide free radicals generated from xanthin/xanthine oxidase and hydroxyl free radicals generated from Fenton's reaction [52, 53]. In isolated hearts subjected to ischemia and reperfusion *in vitro*, EGb 761 decreased the productions of both NO and oxygen free radicals, similar to SOD/catalase and L-NAME [16]. The decrease in NO production was shown to be a result from the inhibition of iNOS expression by EGb 761 [54]. By lowering the ROS/RNS production, EGb 761 preserved the alpha-tocopherol storage in myocardium and reduced the prostaglandin biosynthesis during ischemia-reperfusion [55].

EGb 761 also significantly reduced the leakages of LDH and CK induced by ischemia-reperfusion and protected mitochondrial structure from ischemia-reperfusion induced damage [16, 52]. Consistently, the *in vivo* studies showed that EGb 761 inhibited lipid peroxidation and NO generation as well as CK release and mitigated the incidence of ventricular arrhythmias in a dose dependent way, thus, the reduction of NO and oxygen free radicals and inhibition of lipid peroxidation play an important role in the cardioprotective effect of EGb 761 on myocardial ischemia-reperfusion injury [16, 52]. Myocardial ischemia-reperfusion results in endothelial dysfunction characterized by a loss of endothelium-derived relaxing factor (EDRF) release in response to endothelium-dependent dilators, which is prevented by SOD. Since EGb 761 can scavenge superoxide anions produced in ischenia-reperfusion, it was hypothesized that the prolongation of the half-life of endothelium-derived relaxing factor (EDRF) and enhancement of the cellular action of EDRF might prevent the endothelial dysfunction and contribute to the cardioprotective mechanism of EGb 761 [56, 57].

FK506 is an immunosuppressant that blocks the activation of calcineurin that regulates genes involved in the development of cardiac hypertrophy and ultimately heart failure. Treatment of rats with FK506 resulted in improvement of postischemic cardiac function and decrease in arrhythmias. EGb 761 potentiated the cardioprotective effect of FK506 in the combined treatment and the actions of FK506 and EGb 761 appeared to be synergistic. Since adverse effects such as kidney damage and impaired immune response to infections were observed after prolonged use of the FK506, this finding raised the possibility to manage cardiac ischemia-reperfusion injury and prevent cardiac hypertrophy using FK506 at subtoxic doses in combined therapy by coadministration of EGb 761 [58].

3.3. Chinonin, a Novel Drug against Cardiomyocyte Apoptosis Induced by Ischemia Reperfusion Injury *In Vivo and In Vitro*

Chinonin is a flavonoid component isolated from Chinese herb *rhizoma anemarhenea*, which has been used for several thousand years for treatment of heart diseases. We studied the scavenging effects of chinonin on NO and oxygen free radicals generated from ischemia-reperfusion heart *in vitro* [12, 59]. It was found that the ESR signals of oxygen and NO free radicals decreased simultaneously after addition of chinonin (100μM) similar to the effect of SOD/catalase and L-NAME, indicating that chinonin could scavenge ROS and NO free radical generated from the ischemia-reperfusion heart. In addition, the levels of LDH and CK in the reperfusion solution were decreased significantly by chinonin treatment. Chinonin was also found to protect the structure of mitochondria from the ischemia-reperfusion induced damage. These results suggested that chinonin has protective effect on myocardial ischemia-reperfusion injury and its cardioprotective effects may be attributed to its antioxidant properties. *In vivo* study showed that chinonin inhibited the formation of TBARS, the release of CK, and mitigated the incidence of ventricular arrhythmias in a dose dependent way. The effect of chinonin on NO production varied at different doses. While administration of chinonin at 100 mg/kg increased the signal intensity of NO displayed on ESR spectrum, the administration of chinonin at 200 mg/kg decreased the signal intensity of NO, however, chinonin affected the changes of TBARS, the release of CK, and mitigated the incidence of ventricular arrhythmias in a dose dependent way. These results indicate that chinonin has cardiovascular protective effects by means of adjusting the level of NO and inhibiting oxygen free radicals induced lipid peroxidation in myocardial ischemia-reperfusion injury *in vivo* [12, 59].

In order to further elucidate the cardioprotective effects of chinonin, Shen *et al*. investigated the preventive effects of chinonin on the apoptotic and necrotic cell death of cardiomyocytes during hypoxia and reoxygenation process. Neonatal rat cardiomyocytes were subjected to 24-h hypoxia and 4-h reoxygenation and it was found that chinonin significantly decreased the apoptosis and necrosis induced by hypoxia and reoxygenation in cardiomyocytes. The decreased apoptosis might be caused by a down-regulation of p53 protein expression and an up-regulation of bcl-2 protein expression under the chinonin treatment. Meanwhile, chinonin treatment decreased the levels of NO^-_2/NO^-_3 and TBARS and inhibited the leakage of LDH in hypoxia-reoxygenated cardiomyocytes. These results suggest that chinonin may prevent apoptotic and necrotic cell death of cardiomyocytes during the hypoxia and reoxygenation process via reducing the production of NO and ROS and modulating the expression of bcl-2 and p53 proteins [18-20].

4. CONCLUSION AND PERSPECTIVE

In conclusion, it is evident that ROS and RNS play important roles in the ischemia-reperfusion induced cardiac injury. And natural antioxidants have great potentials for the prevention and treatment of the heart disease. In the future, more effort should be spent to evaluate the efficiency of the nature antioxidants on heart diseases using clinical trials. In addition, the combination therapy using natural antioxidants and other drugs should be explored. This may allow the use of drugs at lower dosages that eliminate the cytoxicity and lead to new and powerful approaches for management of cardiac ischemia-reperfusion injury. It is as well important to study the molecular mechanisms of the cardioprotective effect of nature antioxidants, so that more effective medicines can be developed to fight heart disease. One obstacle for understanding the action of natural antioxidants is their complex composition, therefore, the isolation and purification of

the natural antioxidants may provide more insights of the cardioprotective mechanisms of the natural antioxidants.

REFERENCES

[1] Ferrari, R.; Guardigli, G.; Mele, D.; Percoco, G. F.; Ceconi, C.; Curello, S. Oxidative stress during myocardial ischaemia and heart failure. *Curr. Pharm. Des.*, **2004**, *10*, 1699-1711.

[2] Zweier, J. L.; Flaherty, J. T.; Weisfeldt, M. L. Direct measurement of free radical generation following reperfusion of ischemic myocardium. *Proc. Natl. Acad. Sci. USA*, **1987**, *84*, 1404-1407.

[3] Zweier, J.L.; Kuppusamy, P.; Williams, R.; Rayburn, B.K.; Smith, D.; Weisfeld, M.L.; Flahery, J.T. Measurement and charactrization of postischemic free radical generation in the isolated perfused heart. *J. Biol. Chem.*, **1989**, *261*, 18890-18895.

[4] Zhao, B-L.; Shen, J-G.; Li, M.; Xin, W-J. Synergic effect of NO and oxygen free radicals in ischemia-reperfusion rabbit myocardium. *Sci. China*, **1996**, *26*, 331-338.

[5] Zhao, B-L.; Xin, W-J.; Yang, W-D.; Zhu, H-L. Direct measurement of active oxygen free radicals from ischemia-reperfusion rabbit myocardium. *Chin. Sci. Bull.*, **1989**, *34*, 780-787.

[6] Cheng, S.; Zhao, B-L.; Xin, W-J.; Tang, Z-S. Myocadium damage during ischemia-reperfusion of rat heart. *Chin. Circ.*, **1990**, *5*, 222-226.

[7] Huang, N.; Chen, Y.; Zhao,B-L.; Xin,W-J. Studies on free radicals generated during ischemia-reperfusion of rat heat. *J. Chin. Med.*, **1990**, *70*, 691-694.

[8] Zweier, J.L.; Rayburn, B.K.; Flaherty, J.T.; Weisfeldt, M.L. The effect of superoxide dismutatase on free radical concentration in post ischemic myocardium. *Circulation*, **1986**, *74*, 371-380.

[9] Sanders, S. P.; Zweier, J. L.; Kuppusamy, P.; Harrison, S. J.; Bassett, D. J.; Gabrielson, E. W.; Sylvester, J. T. Hyperoxic sheep pulmonary microvascular endothelial cells generate free radicals via mitochondrial electron transport. *J. Clin. Invest.*, **1993**, *91*, 46-52.

[10] Zweier, J. L.; Kuppusamy, P. *In vivo* EPR spectroscopy of free radicals in the heart. *Environ. Health Perspect.*, **1994**, *102*(Suppl 10), 45-51.

[11] Kuppusamy, P.; Chzhan, M.; Vij, K.; Shteynbuk, M.; Lefer, D. J.; Giannella, E.; Zweier, J. L. Three-dimensional spectral-spatial EPR imaging of free radicals in the heart: a technique for imaging tissue metabolism and oxygenation. *Proc. Natl. Acad. Sci. USA*, **1994**, *91*, 3388-3392.

[12] Zhao, B-L.; Shen, J-G..; Li, M.; Xin,W-J. Scavenging effect of Chinonin on NO and oxygen free radicals generated from ischemia reperfusion myocadium. *Biachem. Biophys. Acta*, **1996**, *1317*, 131-137.

[13] Afanas'ev, I.B. On mechanism of superoxide signaling under physiological and pathophysiological conditions. *Med. Hypotheses*, **2005**, *64*, 127-129.

[14] Linnane, A.W.; Kios, M.; Vitetta, L. The essential requirement for superoxide radical and nitric oxide formation for normal physiological function and healthy aging. *Mitochondrion*, **2007**, *7*, 1-5.

[15] Zhao, B-L.; Jiang, W.; Zhao,Y.; Hou,J-W.; Xin,W-J. Scavenging effect of salvia miltiorriza on free radicals and its protection for myocardial mitochondrial membrane from ischemia-reperfusion injury. *Biochem. Mol. Biol. Intern.*, **1996**, *38*, 1171-1182.

[16] Shen, J-G..; Wang, J.; Zhao, B-L.; Hou, J-W.; Gao, T-L.; Xin, W-J. Effects of EGb-761 on nitric oxide, oxygen free radicals, myocardial damage and arrhythmias in ischemia-reperfusion injury *in vivo*. *Biochim. Biophys. Acta*, **1998**, *1406*, 228-236.

[17] Zou, X-L.;Wan, Q.; Li, M-F.; Zhao, B-L.; Xin,W-J. Scavenging effect of green tea polyphenols on oxygen free radicals generated from ischemia-reperfused myocardium. *Chin. J. Magn. Reson.*, **1995**, *12*, 237-244.

[18] Shen, J-G.; Guo, X-S.; Jiang, B.; Li, M.; Xin, W-J.; Zhao, B-L. Chinonin, a novel drug against cardiomyocyte apoptosis induced by hypoxia and reoxygenation. *Biochim. Biophyscs. Acta*, **2000**, *1500*, 217-226.

[19] Shen, J-G.; Li M.; Xin, W-J.; Zhao, B-L. Effects of Chinonin on nitric oxide free radical, myocardial damage and arrhythmia in ischemia-reperfusion injury *in vivo*. *Appl. Magn. Reson.*, **2000**, *19*, 9-19.

[20] Zhao,B-L.; Zhou, W-A.; Ni,Y-C.; Hou, J-W.; Gao,T-L.; Xin,W-J. Kinetic scavenging effects of chinonin on NO and oxygen free radicals generated from ischemia reperfusion myocardium and its protection effects on the myocardium. *Res. Chem. Intermed.*, **2000**, *26*, 747-762.

[21] Bredt, D.S.; Hwang, P.M.; Snyder S.H. Localization of nitric oxide synthase indicating a neural role for nitric oxide. *Nature*, **1990**, *347* (6295), 768-770.

[22] Li, H-T.; Zhao, B-L.; Hou, J-W.; Xin W-J. Two peak kinetic curve of chemiluninencence in phorbol stimulated macrophage. *Biochem. Biophys.Res. Commn*., **1996**, *223*, 311-314.

[23] Ghafourifar, P.; Schenk, U.; Klein, S.D.; Richter C. Mitochondrial nitric-oxide synthase stimulation causes cytochrome c release from isolated mitochondria. Evidence for intramitochondrial peroxynitrite formation. *J. Biol. Chem.*, **1999**, *274*, 31185-31188.

[24] Zhao, B-L.; Shen, J-G.; Li, M.; Xin, W-J. Study on NO free radicals generated from ischemia-reperfused heart and macrophage. *Chinese J. Magn. Reson.*, **1997**, *14*, 99-106.

[25] Zhao, B-L.; Shen, J-G.; Tang C.; Hou, J-W.; Xin, W-J. Analysis of EPR spectrum about NO free radicals trapped by DETCFe^{2+}. *Chin. J. Magn. Reson.*, **1998**, *15*, 307,311.

[26] Zhang, D.-L.; Xiong, J.; Hu, J.; Li, Y.; Zhao, B.-L. improved method to detect nitric oxide in biological syste. *Appl. Magn. Reson.*, **2001**, *20*, 345-358.

[27] Zhou, G-Y.; Zhao, B-L.; Hou, J-W.; Li, M-F.; Chen, C.; Xin, W-J. Detection of nitric oxide in tissue by spin trapping EPR spectropy and triacetylglycerol extraction. *Biotech. Tech.*, **1999**, *13*, 507-511.

[28] Zhang, P.; Xu, X.; Hu, X.; Deel, E.; Zhu, G.; Chen,Y. iNOS deficiency protects the heart from systolic overload induced ventricular hypertrophy and congestive heart failure. *Circ. Res.*, **2007**, *100*, 1089-1098.

[29] Bolli, R. Cardioprotective function of inducible nitric oxide synthase and role of nitric oxide in myocardial ischemia and preconditioning: an overview of a decade of research. *J. Mol. Cell Cardiol.*, **2001**, *33*,1897-1918.

[30] Beckman J.S.; Koppenol W.H. Nitric oxide, superoxide and peroxynitrite: The good, the bad, and the ugly. *Am. J. Physiol.*, **1996**, *271*, C1424-C1437.

[31] Shen, J-G.; Qiu, X-S.; Jiang, B.; Zhang D-L.; Xin W-J.; Fung, P.C.W.; Zhao, B-L. Nitric oxide contributes to redistribution of phosphatidylserine and triggers apoptosis via peroxynitrite and p53 pathway in hypoxia-reoxygenated Cardiomyocytes. *Sci. China*, **2003**, *46*, 27-39.

[32] Troy, C.M.; Derossi, D.; Prochianz, A.; Greene, L.A.; Shelanski, M.L. Downregulation of Cu/Zn superoxide dismutase leads to cell death via the nitric oxide-peroxynitrite pathway. *J. Neurosci.*, **1996**, *16*, 253-261.

[33] Chen, Y. R.; Chen, C. L.; Pfeiffer, D. R.; Zweier, J. L. Mitochondrial complex II in the post-ischemic heart: oxidative injury and the role of protein S-glutathionylation. *J. Biol. Chem.*, **2007**, *282*, 32640-32654.

[34] Chen, C-L.; Chen, J.; Rawale, S.; Varadharaj, S.; Kaumaya, P. P. T.; Zweier, J. L.; Chen, Y-R. Protein tyrosine nitration of the flavin subunit is associated with oxidative modification of mitochondrial complex ii in the post-ischemic myocardium. *J. Biol. Chem.*, **2008**, *283*, 27991-28003.

[35] Young, L.H. AMP-activated protein kinase conducts the ischemic stress response orchestra. *Circulation*, **2008**, *117*, 832-840.

[36] Li, J.; Hu, X.; Selvakumar, P.; Russell, R.R.; Cushman, S.W.; Holman, G. D.; Young, L.H. Role of the nitric oxide pathway in AMPK-mediated glucose uptake and GLUT4 translocation in heart muscle. *Am. J. Physiol.*, **2004**, *287*, E834-E841.

[37] Terai, K.; Hiramoto, Y.; Masaki, M.; Sugiyama, S.; Kuroda, T.; Hori, M.; Kawase, I.; Hirota, H. AMP-activated protein kinase protects cardiomyocytes against hypoxic injury through attenuation of endoplasmic reticulum stress. *Mol. Cell Biol.*, **2005**, *25*, 9554-9575.

[38] Li, J.; Miller, E. J.; Ninomiya-Tsuji, J.; Russell, R.R. Young, L.H. AMP-activated protein kinase activates p38 mitogen-activated protein kinase by increasing recruitment of p38 MAPK to TAB1 in the ischemic heart. *Circ. Res.*, **2005**, *97*, 872-879.

[39] Zhang, P.; Hu, X.; Fassett, J. E.; Zhu, G.; Viollet, B.; Xu,W.; Wiczer, B.; Bernlohr, D.A.; Bache, R. J.; Chen, Y. AMPKα2 deficiency exacerbates pressure-overload induced left ventricular hypertrophy and dysfunction in mice. *Hypertension*, **2008**, *52*, 918-924.

[40] Chen, W.; Dong, Y.; Wang, C.; Ting, G. Pharmacological studies of sodium tanshinone IIA sulfonate. *Acta Pharmacol. Sin*., **1979**, *14*, 277-283.

[41] Yang, W.; Zhu, H.L.; Zhao, B.L. Scavenging effect of tanshinone on free radicals. *Bult. Chin. Pharmacol.*, **1990**, *6*, 118-123.

[42] Ma, Z.; Zhao, B-L.; Yuan, Z. Application of electrochemical and spin trapping techniques in the investigating the scavenging effect of tanshinone on hydroxyl radicals. *Anal. Chem. Acta*, **1999**, *389*, 213-218.

[43] Jiang, W.; Zhao, Y.; Wan, Q.; Zhao, B-L.; Xin, W-J. Scavenging effect of salviol (Tanshinone) on the lipid free radicals generated from lipid peroxidation in sarcoplasma. *Acta Biophys. Sin.*, **1994**, *10*, 685-689.

[44] Zhao, Y.; Jiang, W.; Hou, J-W.; Zhao, B-L.; Xin, W-J. Effect of calcium overload and salviol (Tanshinone) on the lipid free radicals generated from lipid peroxidation of mitochondrial membrane. *Chin. J. Biochem. Biophys.*, **1995**, *28*, 269-276.

[45] Zhou, G-Y.; Jiang, W.; Zhao, Y.; Ma, G-E.; Xin, W-J.; Yin, J.; Zhao, B-L. Sodium tanshinone IIA sulfonate mediates electron transfer reaction in rat heart mitochondria. *Biochem. Biopharm.*, **2003**, *7465*,1-7.

[46] Keizer, H.G.; Pinedo, H.M.; Schuurhuis, G.J.; Joenje, H. Doxorubin Adriamycin: A critical review of free radical-dependent mechanism of cyotoxicity. *Pharmacol. Ther.*, **1990**, *47*, 219-231.

[47] Zhou, G-Y.; Jiang W.; Zhao, Y.; Ma, G-E.; Li, S-G.; Xin, W-J.; Zhao, B-L. Interaction between sodium tanshinone IIA sulfonate and the adriamycin semiquinone free radical: A possible mechanism for antagonizing adriamycin-induced cardiotoxity. *Res. Chem. Interm.*, **2002**, *28*, 277-290.

[48] Yang, W.; Zhu, H.L.; Zhao, B-L. Studied on the free radicals generated during ischemia-reperfusion heart and scavenging effect of tanshinone on the free radicals. *Chin. Myovasc. J.*, **1989**, *17*, 178-194.

[49] Tredici, P. D. Ginkgos and people - A thousand years of interactions, *Arnoldia*, **1991**, *51*, 2-15.

[50] Tosaki, A.; Droy-Lefaix, M.T.; Pali, T.; Das, D.K. Effects of SOD, catalase, and a novel antiarrhythmic drug, EGB 761, on reperfusion-induced arrhythmias in isolated rat hearts. *Free Radic. Biol. Med.*, **1993**, *14*, 361-370.

[51] Haramaki, N.; Aggarwal, S.; Kawabata, T.; Droy-Lefaix, M.T.; Packer, L. Effects of natural antioxidant ginkgo biloba extract (EGB 761) on myocardial ischemia-reperfusion injury. *Free Radic. Biol. Med.*, **1994**, *16*, 789-794.

[52] Shen, J-G.; Zhao, B-L.; Li, M-F.; Wan, Q.; Xin,W-J. Inhibitory effects of Ginkgo biloba extract (EGB761) on oxygen free radicals, nitric oxide and myocardial injury in isolated ischemic-reperfusion hearts. In: *Proceedings of the International Symposium on Natural Antioxidants Molecular Mechanisms and Health Effects;* Packer, L.; Traber, M.G.; Xin, W.; Raber, M.G.; Xin, W., Eds.: AOCS Press: Champaign, Illinois, **1996**.

[53] DeFeudis, F.V. *Ginkgo biloba* extract (EGb 761): *pharmacological activities and clinical application.* Amsterdam, Elsevier, London, Paris, New Yourk, Tokyo, **1991**.

[54] Varga, E.; Bodi, A.; Ferdinandy, P.; Droy-Lefaix, M.T.; Blasig, I.E.; Tosaki, A. The protective effect of EGb 761 in isolated ischemic/reperfused rat hearts: a link between cardiac function and nitric oxide production. *J. Cardiovasc. Pharmacol.*, **1999**, *34*,711-717.

[55] Kusmic, C.; Basta, G.; Lazzerini, G.; Vesentini, N.; Barsacchi, R. The effect of Ginkgo biloba in isolated ischemic/reperfused rat heart: a link between vitamin E preservation and prostaglandin biosynthesis. *J. Cardiovasc. Pharmacol.*, **2004**, *44*, 356-362.

[56] Furchgott, R.; Zawadzki, J.V. The obligatory role of the endothelium in the relaxation of arterial smooth muscle by acetycholine. *Nature*, **1980**, *288*, 373-376.

[57] Ignarro, L.J.; Byrns, R.E.; Wood, K.S. Pharmacological and biological properties of endothelium-derived relaxing factor(EDRF): Evidence that EDRF is closely related to nitric oxide radical. *Circulation*, **1986**, *74*(2), 287.

[58] Haines, D.D.; Bak, I.; Ferdinandy, P.; Mahmoud, F.F.; Al-Harbi, S.A.; Blasig, I.E; Tosaki, A. Cardioprotective effects of the calcineurin inhibitor FK506 and the PAF receptor antagonist and free radical scavenger, EGb 761, in isolated ischemic/reperfused rat hearts. *J. Cardiovasc. Pharmacol.*, **2000**, *35*, 37-44.

[59] Zhao, B-L.; Shen, J-G.; Li, M.; Wan, Q.; Li, M-F.; Xin, W-J. In: *Chinonin Can Scavenging No Free Radicals and Protect the Myocardium Against Ischemia-Reperfusion Injury*. Proceedings of the international symposium on natural antioxidants Molecular mechanisms and health effects. Packer, L.; Ttaber, M.G.; Xin, W. Eds.; AOCS Press: Champaign, Illinois, **1996**.

Received: May 02, 2010 **Accepted: May 28, 2010**