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Mari Kannan, M., and Darlin Quine, S. (2011) *Mechanistic Clues in the Protective Effect of Ellagic Acid Against Apoptosis and Decreased Mitochondrial Respiratory Enzyme Activities in Myocardial Infarcted Rats*. Cardiovascular Toxicology, 12 pp. 56-63.

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<http://dx.doi.org/10.1007/S12012%2D011%2D9138%2D7>

Mechanistic clues in the protective effect of ellagic acid against apoptosis and decreased mitochondrial respiratory enzymes in rats

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Running title: Mechanistic clues of effect of ellagic acid

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ABSTRACT

Introduction: Our previous study described the cardioprotective effects of ellagic acid in an isoproterenol-induced myocardial infarction model. In this study, we are reporting the mechanism of protective action of ellagic acid with respect to apoptosis and mitochondrial respiratory enzymes.

Methods: Ellagic acid (7.5 and 15 mg/kg) was administered orally as a pretreatment for 10 days. After that, isoproterenol (100mg/kg) was injected subcutaneously in rats at an interval of 24 h for 2 days. Myocardial infarction was quantified by planimetry. Apoptosis was measured by apoptotic gene expressions. The levels of mitochondrial respiratory enzymes were also measured.

Results: Isoproterenol-induced myocardial infarcted rats showed increased infarct size, a decrease in myocardial expression of the Bcl-2 gene and an increase in myocardial expression of the BAX gene. Fas ligand and caspases were markedly elevated along with compromised respiratory marker enzymes in isoproterenol-induced rats. Ellagic acid pretreatment reduced the infarct size, regulated apoptotic gene expressions and enhanced the levels of mitochondrial respiratory marker enzymes and cell viability.

Discussion: The decreased infarct size associated with inhibited apoptosis and increased respiratory marker enzymes explain the role of ellagic acid in anti-apoptotic mechanism and it may be the reason for its cardioprotective activity.

Key words: Myocardial infarction, apoptosis, Respiratory enzymes, Infarct Size.

1. Introduction

Myocardial infarction (MI) is an important pathological feature resulting in high levels of mortality and morbidity. It therefore attracts continuing attention from basic and clinical researchers, epidemiologists, and practising physicians. Pathogenesis of MI is a complex, multifactorial process involving progressive cardiovascular alterations at the molecular, cellular and organ level [1]. There is now increasing evidence from both animal and human models that apoptosis contributes to cardiomyocyte cell loss during acute myocardial injury and chronic heart failure [2], and therefore, a number of therapies are directed towards preventing apoptosis during pathological conditions. Evidence has accumulated that oxidative stress activates multiple cell signalling pathways, including the mitochondrial apoptotic pathways [3]. The mitochondrion is a key organelle for cardiac injury and mitochondrial damage generated by ischaemia results in the release of cytochrome c from mitochondria as a trigger of apoptosis [4]. Since apoptosis is implicated in the pathogenesis of MI, the inhibition of apoptosis promises to be an extremely important target for therapeutic intervention. During reperfusion, cardiomyocyte apoptosis occurs at a high rate during a defined time period. Thus, a short-term pretreatment may be highly effective. The concept of early therapeutic interference with acute pretreatment to prevent heart failure and protect mitochondrial respiratory enzymes is extremely attractive.

The β -adrenergic receptor signal transduction pathway is critical for rapid adjustments to increased cardiovascular demand [5]. The toxicity from β -adrenergic stimulation is in part associated with myocardial oxidative stress because treatment with β -adrenoceptor blockers is also known to reduce myocardial oxidative stress [6]. Isoproterenol (ISO), a synthetic catecholamine and a β -adrenoceptor agonist, has been reported to induce MI and to show

many metabolic and morphologic aberrations in the heart tissue of experimental animals similar to those observed in human MI [7].

A growing number of reports support the theory that bioactive compounds like polyphenols have a role in cardiovascular disease prevention. Ellagic acid, a dilactone of hexahydroxydiphenic acid, is found in nature in a wide variety of nuts and fruit such as raspberries, pomegranates, grapes and blackcurrants. It has been receiving significant attention because of its wide array of biological properties, such as radical scavenging, and chemopreventive properties [8,9]. Amongst suggested molecular targets for ellagic acid's effects are NF- κ B, cyclin D1, p21^{cip1}/waf1 and p53 [10], all of which are involved in the regulation of cell cycle and apoptosis. Previous reports from our laboratory confirmed the protective action of ellagic acid against ISO-induced MI and its antioxidant potential [11]. Despite these pharmacological benefits, the molecular mechanisms by which ellagic acid elicits cardioprotective effects are still unclear. We hypothesize that ellagic acid, a phenolic antioxidant can attenuate apoptosis in infarcted heart, especially in ischaemic conditions. Thus, the aim of the present study is to evaluate the anti apoptotic potential of ellagic acid and its impact on mitochondrial respiratory enzymes in an experimental model of MI.

2. Materials and methods

2.1. Drug and chemicals

Ellagic acid and isoproterenol hydrochloride were purchased from Sigma Chemical Co., St. Louis, MO, USA. Polymerase chain reaction (PCR) master mix, Easy Spin column DNA isolation kit, mRNA purification kit, oligo(dt), DNase and lambda DNA/Hind III were purchased from Medox Biotech India Private Limited, Chennai, India. Primers for reverse

transcriptase polymerase chain reactions (RT-PCR) were purchased from Ocimum Bio solutions Limited, Hyderabad, India. Trypan blue, acridine orange, ethidium bromide, potassium ferricyanide, N-phenyl-p-phenylenediamine, cytochrome-c and 2,3,5 triphenyl tetrazolium chloride were purchased from Himedia, Mumbai, India. All other chemicals used in this study were of analytical grade.

2.2. Experimental animals

All the experiments were carried out with male albino Wistar rats weighing 180-200 g, purchased from Mahaveer Enterprises, Hyderabad, India. They were housed in polypropylene cages ($47 \times 34 \times 20$ cm) lined with husk, renewed every 24 h under a 12 h light/dark cycle at around 22 °C with 50% humidity. The rats had free access to water and fed on a standard pellet diet (Pranav Agro Industries Ltd., Pune, Maharashtra, India) once daily. The experiment was carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, New Delhi, India and approved by the Institutional Animal Ethical Committee (Approval No: 20/IAE/J; Dated 05-04-2010).

2.3. Induction of experimental myocardial infarction

ISO (100 mg/kg) was dissolved in saline and subcutaneously injected in to rats twice at an interval of 24 h [12]. Animals were sacrificed 48 h after the first dose of ISO.

2.4. Experimental protocols

The rats were divided into six groups of six rats each. Group I: normal control rats were given 2 ml of saline orally by gastric intubation daily for a period of 10 days; Group II: normal rats were treated with ellagic acid (7.5 mg/kg) in 2 ml of saline orally by gastric intubation daily for a period of 10 days; Group III: normal rats were treated with ellagic acid (15 mg/kg) in 2 ml of saline orally by gastric intubation daily for a period of 10 days, Group IV: rats were subcutaneously injected with ISO (100 mg/kg) in 2 ml of saline twice at an interval of 24 h (on the 11th and 12th days); Group V: rats were pretreated with ellagic acid (7.5 mg/kg) in 2 ml of saline orally by gastric intubation daily for a period of 10 days and then subcutaneously injected with ISO (100 mg/kg) twice at an interval of 24 h (on the 11th and 12th days); Group VI: rats were pretreated with ellagic acid (15 mg/kg) in 2 ml of saline orally by gastric intubation daily for a period of 10 days and then subcutaneously injected with ISO (100 mg/kg) twice at an interval of 24 h (on the 11th and 12th days).

2.5. Infarct size assessment by cumulative planimetry

2, 3, 5-Triphenyltetrazolium chloride (TTC) staining of heart slices were done by the method of Ojha et al. [13]. The infarct size percentage (percent necrosis) was calculated in each individual slice by planimetry using Image tool for Windows (Version 2.0) software.

2.6. RNA Isolation

Total cellular ribonucleic acid (RNA) from the heart tissue was extracted using Easy spin column kit (Medox Biotech India Private Limited, Chennai, India) according to the manufacturer's suggested protocol. The isolated RNA was treated with RNase-free DNase I at

37 °C for 30 min for the removal of DNA. Samples were incubated at 30 °C for 60 min. Reactions were stopped by heating at 95 °C for 10 min.

2.7. Reverse transcription

Reverse transcription was carried out using 2 µg of the total RNA and 200 units of M-MuLV reverse transcriptase. Amplification was performed by the Medox PCR Master Mix in a volume of 25 µl. PCR was carried out, using a hot start method by adding 2 µl of cDNA product to 18 µl of PCR buffer containing 67 mM Tris (pH8.8), 1.5 mM MgCl₂, 16.6 mM (NH₄)₂SO₄, mixed dNTPs at 200 µM, 125 U ml⁻¹ Taq polymerase (Gibco), and 0.3 µM of sense and anti-sense primers. The primers were used as rat sense and anti-sense primers, respectively. GAPDH: 5' TTC TTG TGC AGT GCC AGC CTC GTC-3' and 5' TAG GAA CAC GGA AGG CCA TGC CAG -3', Bcl-xL: AGG CTG GCGATG AGT TTG AA and TGA AAC GCT CCT GGC CTT TC; Bcl-2: CTG GTG GAC AAC ATC GCT CTG and GGT CTG CTG ACC TCA CTT GTG. Bax: TTC ATC CAG GAT CGA GCA GA and GCA AAG TAG AAG GCA ACG; Fas: GCAATGCTTCTCTCTGTGACCACT and GCTGTTGTGCTCGATCTCATCG; Caspase 8: CTG GGA AGG ATC GAC GAT TA and CAT GTC CTG CAT TTT GAT GG; Caspase 9: AGC CAG ATG CTG TCC CAT AC and CAG GAG ACA AAA CCT GGG AA. The RT-PCR products were electrophoresed on 3% agarose gel and visualized by staining with ethidium bromide.

2.8. Analysis of heart mitochondrial respiratory enzymes

Heart mitochondria were isolated by the method of Takasawa et al. [14]. The activity of NADH-dehydrogenase estimated according to the standard procedures of Minakami et al. [15]. The activity of cytochrome-c-oxidase was estimated by Pearl et al. [16].

2.9. Analysis of apoptotic morphological changes

Apoptotic morphological changes in cardiomyocytes were analysed by acridine orange-ethidium bromide dual staining method [17].

2.10. Trypan blue exclusion assay

Trypan blue exclusion is a cell viability assay based on the ability of live cells to exclude the vital dye trypan blue [18]. Cell viability (%) = $N_1/(N_1+N_2) \times 100$ where N_1 and N_2 are the number of non-stained and stained cells, respectively.

2.11. Statistical analysis

Statistical analyses were performed by One-way Analysis of Variance followed by Duncan's multiple range tests using a Statistical Package for the Social Science software package version 16.00. The results were expressed as mean $\pm$ standard deviation for six rats in each group. The P values <0.05 were considered significant.

3. Results

In preclinical studies, the histological analysis is still considered to be the gold standard for measuring infarct size. Thus, slices of heart tissue of normal and experimental animals were analyzed for infarct size. The heart slices of normal and ellagic acid (7.5 and 15 mg/kg) treated rats showed completely viable tissue (with red colour/dark colour), indicates the presence of dehydrogenase and intact myocardial tissue (Fig. 1a-c). The heart slices of ISO-induced rats showed significant change in colour. The infarcted region of tissue is clearly visible as a bright spot and indicates the absence of dehydrogenase (Fig. 1d). Pretreatment with ellagic acid (7.5 mg/kg) in ISO-induced rats showed significant improvement in infarct size (Fig. 1e). A major portion of the heart tissue stained positively with TTC, and showed considerably reduced infarct size, compared to the heart slices of rats induced with ISO alone. The heart slices of rats that received ellagic acid (15 mg/kg) had the most portions stained positively, and infarct size was reduced more extensively (Fig. 1f), than in ISO-induced rats and ellagic acid (7.5mg/kg) pretreated ISO-induced rats. Fig. 2 further explains the infarct size measured by the quantitative planimetry method and confirms the infarct size reducing capacity of ellagic acid. The ISO-induced rats showed greater infarct size in planimetry analysis while pretreatment with ellagic acid (7.5 and 15 mg/kg) significantly ($p<0.05$) reduced infarct size.

The expressions of Bcl-X1 and Bcl-2 in myocardium were visualized by agarose gel electrophoresis. Compared with the normal control group, expressions of Bcl-xL and Bcl-2 in the ISO-induced rats decreased. Compared with the ISO-induced myocardial infarcted rat group, the expressions of Bcl-xL and Bcl-2 in the ellagic acid pretreated groups increased. (Fig.3).

We examined the involvement of BAX, Fas, Caspase-8 and Caspase-9 activation in the heart tissue of normal and experimental animals. The expression of these genes in the rat

heart were higher in the ISO-induced group than in the normal group, and lower in the ellagic acid pretreated group than in ISO-induced group (Fig.3).

The activities of NADH-dehydrogenase in the ISO-induced rats decreased compared to normal rats. Pretreatment with ellagic acid significantly increased the level of NADH-dehydrogenase of enzymes in the ISO-induced myocardial infarcted rats (Fig.4).

The activities of cytochrome-c-oxidase in the ISO-induced rats decreased compared to normal rats. Pretreatment with ellagic acid significantly increased the level of cytochrome-c-oxidase in the ISO-induced myocardial infarcted rats (Fig.5).

The number of cells showing features of apoptosis was counted as a function of the total number of cells present in the field. The staining pattern of individual cardiomyocytes will be described successively for each group. Intact, freshly isolated cells from the groups of normal and ellagic acid treated rats displayed intense green fluorescence. No red fluorescence from ethidium bromide could be detected in any cell indicating that in normal and ellagic acid treated rats it did not disrupt membrane integrity. This shows that the presence of normal live cells in the above mentioned groups. The cells isolated from ISO-induced rats showed more bright red fluorescence confirming the presence of dead cells. The cells isolated from the ellagic acid (7.5 and 15 mg/kg) pretreated groups showed significantly reduced numbers of red fluorescence dose dependently that evidenced the capacity of ellagic acid to protect the cells (Fig.6).

Figure 7 shows the viability of isolated cardiomyocytes of normal control and experimental rats judged by trypan blue exclusion. Compared to normal control, cardiomyocytes of ISO-induced myocardial infarcted rats showed a decrease in cell viability and cardiomyocytes from ellagic acid pretreated rats showed improved viability. Both concentrations of ellagic acid (7.5 and 15 mg/kg) significantly reduced the percentage of trypan blue stained non-viable cardiomyocytes in ISO-induced rats.

4. Discussion

Based on proven cardioprotective effects and antioxidant activities of ellagic acid on β -adrenergic agonist induced MI [11], we proposed that prophylactic treatment with ellagic acid might have the potential to act against pathological pathways involved in MI. The present data indicate that cell death associated with MI was lessened by EA.

Left ventricular dysfunction can be predictable according to myocardial infarct size. Baks et al. [19] and Miura and Miki [20] have reported a direct relationship between the extent of infarct size and mortality, since the myocardium does not have the ability to regenerate during infarction. We thought it would be interesting to see whether pretreatment with ellagic acid reduces myocardial infarct size, and whether or not the effect of ellagic acid on MI is associated with apoptosis in ischaemic heart tissue. We used the TTC staining method to quantify myocardial infarct size. The white crystalline powder of TTC that forms a colourless solution in water is enzymatically reduced to brick-red precipitations of formazan dye 1,3,5-triphenylformazan in viable tissues with intact mitochondrial respiration due to the activity of various dehydrogenases (i.e. succinate dehydrogenase) in the presence of electron donor NADH [21-23]. We observed significantly increased infarct size and an absence of the normal red colour/bright spot in ISO-induced rats. The metabolites of ISO were involved in free radical production and damaged the cardiomyocytes [24]. Oral pretreatment with ellagic acid significantly reduced the infarct size in ISO-induced rats. Ellagic acid contains four hydroxyl groups and two lactone groups and the hydroxyl groups are known to increase antioxidant activity in lipid peroxidation and to protect cells from oxidative damage [25]. The decreased infarct size in ellagic acid pretreated ISO-induced rats showed the beneficial effect of pretreatment with ellagic acid against ISO-induced MI.

A number of studies have shown that the progress of apoptosis is regulated by the expression of members of the Bcl-2 family [26]. Bcl-xL has strong anti apoptotic effects, and it has been reported that Bcl-xL expression is modulated by a distinct mechanism with Bcl-2 in the context of ischaemia [4]. Bcl-2, an anti-apoptotic gene, is known for regulating the apoptotic pathways and protecting against cell death, while BAX, a pro-apoptotic gene of the family, is expressed abundantly and selectively during apoptosis and promotes cell death [27]. Bcl-2 is located on the outer mitochondrial membrane, the nuclear envelopes and the membrane of smooth endoplasmic reticulum. It can form homodimers or it may dimerise with other members of the Bcl-2 family, including Bcl-x and BAX. In the presence of Bcl-2, Bcl-2-BAX heterodimers predominate and promoting cell survival. However, when Bcl-2 levels are reduced, BAX can form homodimers, which promote cell death [28]. Our results showed decreased levels of Bcl-2 and increased levels of BAX in ISO-induced rats. In an earlier study Prabhu et al. [29] demonstrated that the activation of the β_1 -adrenergic receptor in cardiomyocytes increases the expressions of pro-apoptotic genes and our results are consistent with that report. Ellagic acid pretreatment increased the expression of Bcl-2 and decreased the expression of BAX. Stimulation of β -receptors and increased contractility of myocardium consequently produce hypoxia followed by ischaemia, which may lead to excessive apoptosis. These results show that ellagic acid may control apoptosis by efficiently modulating the expressions of BAX and Bcl-2 genes. Antioxidants involved in the reduction of myocardial apoptosis and cardioprotective action have been reported previously [30].

Caspase-8 and caspase-9 are essential apoptotic caspases. They are activated in a sequential cascade of cleavage by other caspase family members [31]. Caspase-8 mediates signal transduction downstream of death receptors located on the plasma membrane. Caspase-9 mediates apoptotic signals after mitochondrial damage. Caspase-9 is activated by interaction with Apaf-1 associated with cytochrome c release from mitochondria, and it

activates caspase-3 [32, 33]. We examined the involvement of caspase-8 and caspase-9 activation in normal and experimental rats. Our results clearly show increased expression of caspases in ISO-induced rats compared to normal control rats. The involvement of caspase-8 and caspase-9 in the DNA fragmentation induced by oxidative stress is well documented. Two explanations are possible for these reactions. One is that oxidative stress produced by metabolites of ISO may increase the accumulation of unfolded death receptors with the death domain in the myocardial cellular membrane and induce Fas associated protein with death domain (FADD) oligomerization, activating caspase-8. The other possibility is that caspase-8 could be activated independently of death receptor complexes, through a direct interaction with myocardial cellular membrane proteins. Though the mechanism of activation of caspase-9 during hypoxia that leads to the initiation of programmed cell death in cardiomyocytes is not known exactly, there are several potential mechanisms for caspase-9 activation during hypoxia. Increased generation of nitric oxide free radicals and nitration of several proteins including the N-methyl-d-aspartate receptor, are among those that are important. In our study, cardiomyocytes from ellagic acid pretreated rats showed decreased expression of caspases compared to ISO-induced myocardial infarcted rats. The exact mechanism of this action of ellagic acid against caspase expression is not clear. Hwang et al. recently reported that ellagic acid can prevent physiological and morphological signs of experimentally induced cell necrosis and apoptosis and the protection afforded by ellagic acid was consistent with its antioxidant activity [34].

Previous *in vitro* studies have indicated that the apoptotic caspase cascade is activated by cytochrome c and adenosine triphosphate. A large body of evidence suggests that mitochondria play crucial roles in hypoxic cell death, either passively due to their inability to synthesize ATP, or actively by releasing proapoptotic molecules such as cytochrome c into the cytoplasm [35, 36]. In addition, the mitochondrial respiratory chain is a major source of

ROS under pathological conditions, including myocardial ischaemia. NADH dehydrogenase and cytochrome c oxidase are the enzymes playing important role in the mitochondrial respiratory chain [37]. Disturbances in the enzyme level affect the regular respiratory functions of mitochondria and their energy production process. We thought it would be interesting to see whether pretreatment with ellagic acid enhances the mitochondrial respiratory enzymes in the mitochondria of the heart, and whether or not the effect of these changes in mitochondrial respiratory enzymes is associated with apoptosis. We analysed the activities of NADH dehydrogenase and cytochrome c oxidase in the mitochondrial fraction of normal and experimental rat heart. Our results show a decreased activity of respiratory marker enzymes in the mitochondrial fractions of ISO-induced myocardial infarcted rats compared to normal rats. A previous study by Vijayapadma and Shyamala Devi [38] reported that the non availability of cardiolipin due to enhanced phospholipid degradation in ISO-induced rats may be the reason for decreased activities of respiratory marker enzymes. Pretreatment with ellagic acid significantly increased the levels of these respiratory marker enzymes in ISO-induced rats. The ability of ellagic acid to stabilize mitochondrial membranes and prevent the release of cytochrome c oxidase was reported by Ou et al. [39].

Acridine orange and ethidium bromide staining with DNA allowed visualization of the condensed chromatin of dead apoptotic cells. Stained cells were viewed under a fluorescence microscope. During apoptosis DNA becomes condensed and fragmented. This clearly confirms the excellent viability of the cardiomyocytes of the normal and ellagic acid treated rats. It is important to note that during the incubation period, very few cells died in the normal control group and these cells were characterized by their orange colour due to the uptake of ethidium bromide caused by a loss of membrane integrity. The number of apoptotic death cells is greater in ISO-induced myocardial infarcted rats. Oral pretreatment with ellagic

acid reduced the number of cell deaths in the ISO-induced rats and proved the protective effect of ellagic acid against apoptosis.

In the trypan blue execution assay, cardiomyocytes from ISO-induced rats manifested cell death under conditions of apoptosis, hypoxia and oxidative stress. Furthermore, inhibition in mitochondrial respiratory marker enzymes by ISO may induce cell death. Pretreatment with ellagic acid showed its capacity to minimize cell death. The protective effect of pretreatment with ellagic acid may depend on its anti apoptotic effect.

In conclusion, the present study revealed a novel function of ellagic acid in alleviating heart damage in β -adrenergic receptor induced MI as demonstrated by decreasing infarct size and improving cell viability. This cardioprotective effect may be associated with anti-apoptotic mechanism through maintaining mitochondrial respiratory enzymes. Understanding the mechanisms involved in the apoptotic cascade may prove useful in better understanding of heart failure and its management. While *in vivo* detection of apoptosis may prove useful in the diagnosis and risk stratification of patients with ischaemic heart disease, anti-apoptotic treatments may be effective in the prevention and treatment of post-infarction remodelling and heart failure. The results of the present study may provide the basis for a new therapeutic application of ellagic acid to prevent or treat myocardial cell injury caused by oxidative stress in MI.

References

1. Sparagna, G.C., Chicco, A.J., Murphy, R.C., Bristow, M.R., Johnson, C.A., Rees, M.L., Maxey, M.L., McCune, S.A., Moore, R.L. (2007). Loss of cardiac tetralinoleoyl cardiolipin in human and experimental heart failure. *J. Lipid Res.* 48:1559-1570.

2. Krijnen, P.A, Nijmeijer, R., Meijer, C.J., Visser, C.A., Hack, C.E., Niessen, H.W., (2002). Apoptosis in myocardial ischaemia and infarction. *J. Clin. Pathol.* 55:801-811.
3. Hare, J.M., (2001). Oxidative stress and apoptosis in heart failure progression. *Circ. Res.* 89:198-200.
4. Huang, J., Ito, Y., Morikawa, M., Kobune, M., Sasaki, K., Abe, T., Hamada, H. (2003). Bcl-xL gene transfer protects the heart against ischemia/reperfusion injury. *Biochem. Biophys. Res. Commun.* 311:64-70.
5. Vatner, D.E., Asai, K., Iwase, M., Ishikawa, Y., Shannon, R.P., Homcy, C.J., Vatner, S.F. (1999). Beta-adrenergic receptor-G protein-adenylyl cyclase signal transduction in the failing heart. *Am. J. Cardiol.* 83:80H-85H.
6. Kukin, M.L, Kalman, J., Charney, R.H., Levy, D.K., Buchholz-Varley, C., Ocampo, O.N., Eng, C. (1999). Prospective, randomized comparison of effect of long-term treatment with metoprolol or carvedilol on symptoms, exercise, ejection fraction, and oxidative stress in heart failure. *Circulation.* 99:2645-2651.
7. Nirmala, C., Puvanakrishnan, R. (1996). Protective role of curcumin against isoproterenol induced myocardial infarction in rats. *Mol. Cell. Biochem.* 159:85-93.
8. Yüce, A., Ateşşahin, A., Çeribaşı, A.O., Aksakal, M. (2007). Ellagic acid prevents cisplatin induced oxidative stress in liver and heart tissue of rats. *Basic. Clin. Pharmacol. Toxicol.* 101:345-349.
9. Türk, G., Ateşşahin, A., Sönmez, M., Çeribaşı, A.O., Yüce, A. (2008). Improvement of cisplatin induced injuries to sperm quality, the oxidant-antioxidant system, and the histologic structure of the rat testis by ellagic acid. *Fertil. Steril.* 89:1474-1481.
10. Aggarwal, B.B., Shishoida, S. (2006). Molecular targets of dietary agents for prevention and therapy of cancer. *Biochem. Pharmacol.* 71:1397-1421.

11. Mari Kannan, M., Darlin Quine S. (2011). Ellagic acid ameliorates isoproterenol induced oxidative stress: evidences from electrocardiological, biochemical and histological studies. *Eur. J. Pharmacol.* 659: 45-52
12. Punithavathi, V.R., Prince, P.S.M. (2010). Pretreatment with a combination of quercetin and alpha-tocopherol ameliorates adenosine triphosphatases and lysosomal enzymes in myocardial infarcted rats. *Life. Sci.* 86:178-184.
13. Ojha, N., Roy, S., Radtke, J., Simonetti, O., Gnyawali, S., Zweier, J.L., Kuppusamy, P., Sen, C.K. (2008). Characterization of the structural and functional changes in the myocardium following focal ischemia–reperfusion injury. *Am. J. Physiol. Heart. Circ. Physiol.* 294:H2435-H2443.
14. Takasawa, M., Hayakawa, M., Sugiyama, S., Hattori, K., Ito, T., Ozawa, T. (1993). Age-associated damage in mitochondrial function in rat hearts. *Exp. Gerontol.* 28:269-280.
15. Minakami, S., Ringler, R.L, Singer, T.P. (1962). Studies on the respiratory chain-linked dihydrodiphosphopyridine nucleotide dehydrogenase: assay of the enzyme in particulate and in soluble preparations. *J. Biol.Chem.* 237:569-576.
16. Pearl, W., Cascarano, J., Zweifach, B.W. (1963). Microdetermination of cytochrome oxidase in rat tissues by oxidation on N-phenyl-p-phenylene diamine or ascorbic acid. *J. Histochem. Cytochem.* 11:102-104.
17. Kato, F., Tanaka, M., Nakamura, K. (1999). Rapid fluorometric assay for cell viability and cell growth using nucleic acid staining and cell lysis agents. *Toxicol. In Vitro.* 13:923-929.
18. Strober, W. Trypan blue exclusion test of cell viability. In: Coico R, editor. *Current protocols in immunology.* John Wiley & Sons, (1997); A.3B.1-A.3B.

19. Baks, T., van Geuns, R.J., Biagini, E., Wielopolski, P., Mollet, N.R., Cademartiri, F., Boersma, E., van der Giessen, W.J., Krestin, G.P., Duncker, D.J., Serruys, P.W., de Feyter, P.J. (2005). Recovery of left ventricular function after primary angioplasty for acute myocardial infarction. *Eur. Heart. J.* 26:1070-1077.
20. Miura, T., Miki, T. (2008). Limitation of myocardial infarct size in the clinical setting: current status and challenges in translating animal experiments into clinical therapy. *Basic. Res. Cardiol.* 103:501-513.
21. Altman, F.P. (1976). Tetrazolium salts and formazans. *Prog. Histochem. Cytochem.* 9:1-56.
22. Nachlas, M.M., Shnitka, T.K. (1963). Macroscopic identification of early myocardial infarcts by alterations in dehydrogenase activity. *Am. J. Pathol.* 42:379-405.
23. Ramkissoon, R.A. (1966). Macroscopic identification of early myocardial infarction by dehydrogenase alterations. *J. Clin. Pathol.* 19:479-481.
24. Stanely Mainzen Prince, P., Priya, S. (2010). Preventive effects of rutin on lysosomal enzymes in isoproterenol induced cardio toxic rats: biochemical, histological and in vitro evidences. *Eur. J. Pharmacol.* 649:229-235.
25. Pari , L., Sivasankari, R. (2008). Effect of ellagic acid on cyclosporine A-induced oxidative damage in the liver of rats. *Fundam. Clin. Pharmacol.* 22:395-401.
26. Nihal, M., Ahmad, N., Mukhtar, H., Wood, G.S. (2005). Anti-proliferative and proapoptotic effects of (–)-epigallocatechin-3-gallate on human melanoma: possible implications for the chemoprevention of melanoma. *Int. J. Cancer.* 114:513-521.
27. Oltvai, Z.N., Milliman, C.L., Korsmeyer, S.J. (1993). Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. *Cell.* 74:609-619.

28. Ling, H., Lou, Y. (2005). Total flavones from *Elsholtzia blanda* reduce infarct size during acute myocardial ischemia by inhibiting myocardial apoptosis in rats. *J. Ethnopharmacol.* 101:169-175.
29. Prabhu, S.D., Wang, G., Luo, J., Gu, Y., Ping P., Chandrasekar B. (2003). Beta-adrenergic receptor blockade modulates Bcl-X(S) expression and reduces apoptosis in failing myocardium. *J. Mol. Cell. Cardiol.* 35:483-493.
30. Kawai, K., Qin, F., Shite, J., Mao W., Fukuoka S., Liang C.S. (2004). Importance of antioxidant and antiapoptotic effects of beta-receptor blockers in heart failure therapy. *Am. J. Physiol. Heart. Circ. Physiol.* 287: H1003–H1012.
31. Thomberry, N.A., Lazebnik, Y. Caspases: enemies within. *Science* (1998); 281:1312-1316.
32. Zou, H., Henzel, W.J., Liu, X., Lutschg, A., Wang, X. (1997) Apaf-1, a human protein homologous to *C. elegans* CED-4, participates in cytochrome c-dependent activation of caspase-3. *Cell.* 90:405-413.
33. Zou, H., Li, Y., Liu X., Wang, X. (1994). An APAF-1 cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9. *J. Biol. Chem.* 274:11549-11556.
34. Hwang, J.M., Cho, J.S., Kim, T.H., Lee, Y.I. (2010). Ellagic acid protects hepatocytes from damage by inhibiting mitochondrial production of reactive oxygen species. *Biomed. Pharmacother.* 64:264-270.
35. Malhotra, R., Lin, Z., Vincenz, C., Brosius F.C. 3rd. (2001). Hypoxia induces apoptosis via two independent pathways in Jurkat cells: differential regulation by glucose. *Am. J. Physiol. Cell. Physiol.* 281:C1596-C1603.
36. Susin, S.A., Lorenzo, H.K., Zamzami N., Marzo, I., Snow, B.E., Brothers, G.M., Mangion, J., Jacotot, E., Costantini, P., Loeffler, M., Larochette, N., Goodlett, D.R.,

- Aebersold, R., Siderovski, D.P., Penninger, J.M., Kroemer, G. (1999). Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature*. 397:441-446.
37. Senthilnathan, P., Padmavathi, R., Magesh, V., Sakthisekaran, D. (2006). Modulation of TCA cycle enzymes and electron transport chain systems in experimental lung cancer. *Life Sci*. 78:1010-1014.
 38. Vijayapadma, V., Shyamaladevi, C.S. (2001). Effect of fish oil on mitochondrial respiration in isoproterenol induced myocardial infarction in rats. *Ind. J. Exp. Biol*. 40:268-272.
 39. Ou, H.C., Lee, W.J., Lee, S.D., Huang C.Y., Chiu T.H., Tsai K.L., Hsu W.C., Sheu W.H. (2010). Ellagic acid protects endothelial cells from oxidized low-density lipoprotein-induced apoptosis by modulating the PI3K/Akt/eNOS pathway. *Toxicol. Appl. Pharmacol*. 248:134-143.

Figure legends

Fig.1. Images of a rat heart slices after 2, 3, 5 triphenyltetrazolium chloride staining.

Group I: Normal control rat with completely viable tissue without any infarction

Group II: Ellagic acid (7.5mg/kg) treated group rats with completely viable tissue without any infarction

Group III: Ellagic acid (15mg/kg) treated group rats with completely viable tissue without any infarction

Group IV: ISO (100 mg/kg) induced myocardial infarcted rat with infarcted region.

Group V: **Pretreated** with EA (7.5 mg/kg) and induced with ISO (100 mg/kg) showing reduced infarction when compared to Group IV.

Group VI: Pretreated with EA (15 mg/kg) and induced with ISO (100 mg/kg) showing much reduced infarction when compared to Group IV and V.

Fig.2. Myocardial infarction size measured by planimetry. Each value is mean $\pm$ standard deviation for six rats in each group, values not sharing a common superscript (a,b,c) differ significantly with each other ($p < 0.05$, duncan's multiple range test).

Fig.3. Effect of ellagic acid on apoptosis related genes in the heart tissue of normal and experimental rats.

Fig.4. Effect of ellagic acid on NADH-dehydrogenase. Each value is mean $\pm$ standard deviation for six rats in each group, values not sharing a common superscript (a,b,c,d) differ significantly with each other ($p < 0.05$, duncan's multiple range test).

Fig.5. Effect of ellagic acid on Cytochrome c- oxidase. Each value is mean $\pm$ standard deviation for six rats in each group, values not sharing a common superscript (a,b,c,d) differ significantly with each other ($p < 0.05$, duncan's multiple range test).

Fig.6. Effect of ellagic acid on apoptotic morphological changes.

Fig.7. Effect of ellagic acid on cell viability. Each value is mean $\pm$ standard deviation for six rats in each group, values not sharing a common superscript (a,b,c,d) differ significantly with each other ($p < 0.05$, duncan's multiple range test).

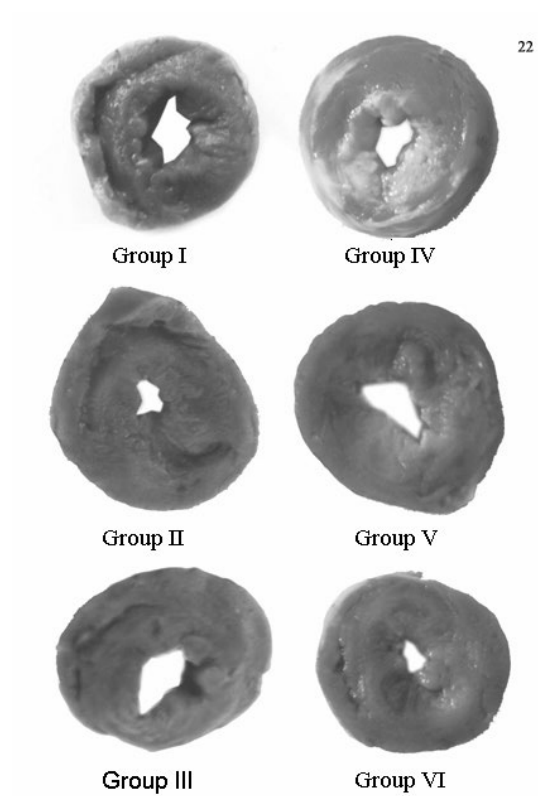


Figure 1

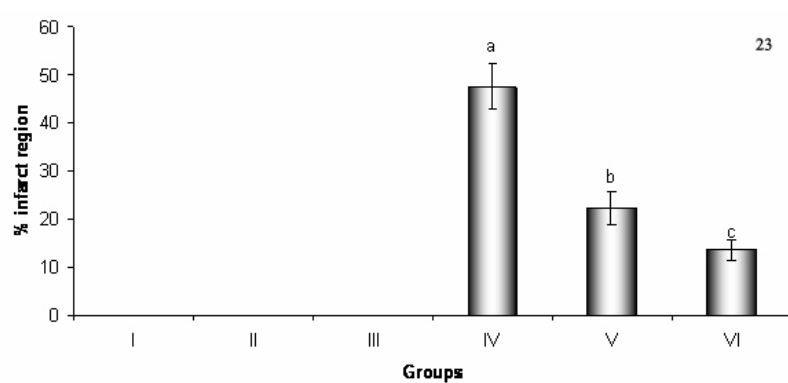


Figure 2

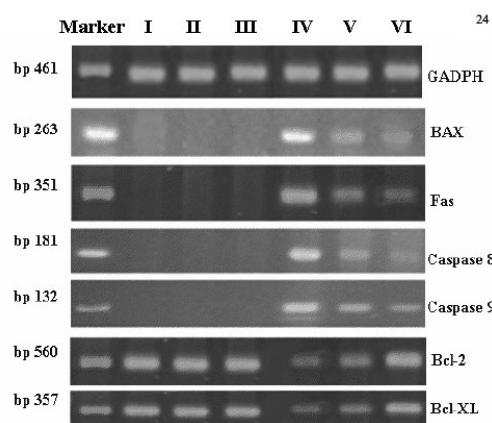


Figure 3

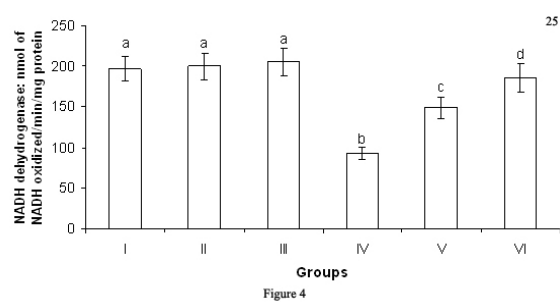


Figure 4

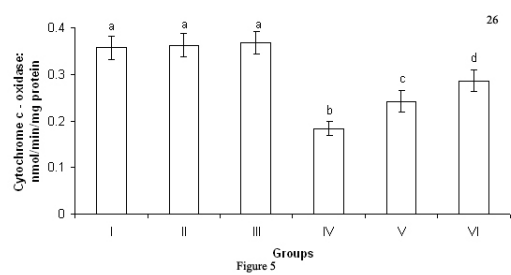


Figure 5

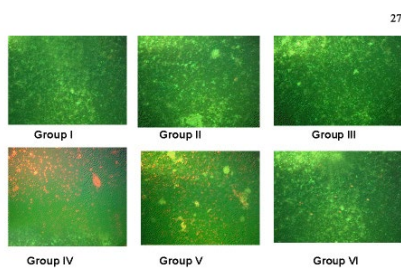


Figure 6

