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Research paper

Effect of ursolic acid treatment on apoptosis and DNA damage in isoproterenol-induced myocardial infarction

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ABSTRACT

The present study was designed to evaluate the protective effect of ursolic acid (UA) against isoproterenol-induced myocardial infarction. Myocardial infarction was induced by subcutaneous injection of isoproterenol hydrochloride (ISO) (85 mg/kg BW), for two consecutive days. ISO-induced rats showed elevated levels of cardiac troponins T (cTn T) and I (cTn I) and increased activity of creatine kinase-MB (CK-MB) in serum. Lipid peroxidative markers (thiobarbituric acid reactive substances (TBARS), conjugated dienes (CD) and lipid hydroperoxides (HP)) elevated in the plasma and heart tissue whereas decreased activities of enzymatic antioxidants (superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione-S-transferase (GST) and glutathione reductase (GR)) in erythrocytes and heart tissue of ISO-induced rats. Non-enzymatic antioxidants (vitamin C, vitamin E and reduced glutathione (GSH)) levels were decreased significantly in the plasma and heart tissue of ISO-induced rats. Furthermore, ISO-induced rats showed increased DNA fragmentation, upregulations of myocardial pro-apoptotic B-cell lymphoma-2 associated-x (Bax), caspase-3, -8 and -9, cytochrome c, tumor necrosis factor-α (TNF-α), Fas and down-regulated expressions of anti-apoptotic B-cell lymphoma-2 (Bcl-2) and B-cell lymphoma-extra large (Bcl-xL). UA-administered rats showed decreased levels/activity of cardiac markers, DNA fragmentation and the levels of lipid peroxidative markers in the plasma and heart tissue. Activities of enzymatic antioxidants were increased significantly in the erythrocytes and heart tissue and also non-enzymatic antioxidants levels were increased significantly in the plasma and heart tissue in UA-administered rats. UA influenced decreased DNA fragmentation and an apoptosis by upregulation of anti-apoptotic proteins such as Bcl-2, Bcl-xL and down-regulation of Bax, caspase-3, -8 and -9, cytochrome c, TNF-α, Fas through mitochondrial pathway. Histopathological observations were also found in line with biochemical parameters. Thus, results of the present study demonstrated that the UA has anti-apoptotic properties in ISO-induced rats.

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1. Introduction

Myocardial infarction (MI) is an acute condition of myocardial necrosis that occurs as a result of imbalance between coronary blood supply and myocardial demand. Although clinical care, public awareness, health innovations have been improved against MI, it remains the leading cause of death worldwide [1]. It is well recognized that there is an excessive generation of reactive oxygen species such as superoxide anion and hydroxyl radicals and other reactive species in heart failure, which involved in the formation of lipid peroxides, damage of cell membrane, and antioxidative defense system [2]. Therapeutic intervention via suppression of free radical generation and/or enhancement of endogenous antioxidants may limit the infarct size and attenuate myocardial dysfunction [3].

Growing evidence implicates that apoptosis mainly contributes to acute myocardial infarction (AMI) [4]. Apoptosis or programmed cell death is a genetically regulated cell suicide that plays a pivotal role in a variety of cell homeostatic and pathological processes. An important pathway of apoptosis is mitochondrial or intrinsic pathway. A pathway via mitochondrial injury leads to over-expression of Bax which accelerate apoptotic cell death [5–8]. Bcl-2

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is a cytosolic protein with a lipid-anchoring domain that can target this protein to the mitochondria or the nucleus [9]. It plays a role in the inhibition of apoptosis [10–12]. Caspase-3 plays a central role in the execution-phase of cell apoptosis [13]. Activated caspase-3, in turn, cleaves other protein substrates within the cell, and results in the apoptotic process. In contrast, in death receptors pathway (or extrinsic pathway), the cell surface transmits apoptotic signals initiated by specific ligands such as Fas ligand, TNF-α and TNF-related apoptosis-inducing ligand (TRAIL). These play an important role in apoptosis and can activate a caspase cascade, then leading cell to apoptosis [14].

In recent years, the prevention of cardiovascular diseases has been associated with ingestion of fresh fruits, vegetables or plants rich in natural antioxidants. New therapies are needed to treat myocardial ischemia because current treatments have limited impact on survival and annual costs. Triterpenoids exist widely in nature and are the major components of many traditional medicinal herbs. Ursolic acid (UA), a pentacyclic triterpenoid exists in natural plants in the form of free acid or aglycones [15]. UA (3β-hydroxyl-urs-12-en-28oic acid) is present in berries, leaves, flowers and many kinds of medicinal herbs such as *Perilla frutescens* [16], *Rosmarinus officianalis* and *Eriobotrya japonica* [17]. It has wide range of pharmacological effects such as antiinflammatory [18], anticarcinogenic [19], antiulcer [20], antihyperlipidemic [21], cardiotonic [22], antihyperglycemic [17], hepatoprotective [23] and neuroprotective [24] activities.

In the present study, attempt has been made to demonstrate the molecular mechanisms underlying the therapeutic effect of UA by analysing the activities/expressions of cardiac and apoptotic markers, evaluating DNA damage and studying the histopathological changes.

2. Materials and methods

2.1. Experimental animals

Healthy male albino Wistar rats (160–180 g) were obtained from the Central Animal House, Department of Experimental Medicine, Rajah Muthiah Medical College and Hospital, Annamalai University, and maintained in an air-conditioned room (25 ± 3 °C) with a 12 h light/12 h dark cycle. Feed and water were provided *ad libitum* to all the animals. The study protocols were approved by the Institutional Animal Ethics Committee of Rajah Muthiah Medical College and Hospital (Reg No. 160/1999/CPCSEA, Proposal number: 560), Annamalainagar.

2.2. Chemicals

Isoproterenol hydrochloride (ISO), Ursolic acid (UA) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Polyvinylidenedifluoride membrane was purchased from Millipore, Bax, Bcl-2, Bcl-xL, caspase-3, -8 and -9, cytochrome c, TNF-α and Fas antibodies were purchased from Santa-cruz Biotechnology, Inc, USA and Goat anti-rabbit, anti-mouse and Rabbit anti-goat secondary antibodies were purchased from Genei, Bangalore, India. Enhanced chemiluminescence (ECL)-kit was purchased from GenScript ECL kit, USA. All other chemicals used in this study were of analytical grade obtained from E. Merck and HIMEDIA, India.

2.3. Experimental induction of myocardial ischemia

Myocardial ischemia was induced by subcutaneous (s.c.,) injection of ISO (85 mg/kg BW dissolved in physiological saline) for the first two consecutive days [25].

2.4. Experimental design

The rats were randomly divided into four groups with six rats each. The test compound (UA) was completely dissolved in DMSO (5%) and diluted with saline to the volume of 5:100 ratio.

Group I: Control (5% DMSO s.c., from 3rd day to 9th day)
Group II: Control + UA (40 mg/kg BW, s.c., from 3rd day to 9th day)
Group III: ISO control (85 mg/kg BW, s.c., for 1st and 2nd day)
Group IV: ISO (85 mg/kg BW, s.c., for 1st and 2nd day) + UA (40 mg/kg BW, s.c., from 3rd day to 9th day)

The total duration of the study was 9 days. On 10th day, the rats were sacrificed by cervical dislocation. Blood samples were collected in a dry test tube and allowed to coagulate at ambient temperature for 40 min. Serum was separated by centrifugation at 2000 rpm for 10 min. The heart tissue was excised immediately, washed with chilled isotonic saline and used for the various parameters analysis.

2.5. Estimation of cardiac markers

The activity of CK-MB was assayed by a commercial kit obtained from Agappe Diagnostics, Kerala, India. Serum cTn T and I were quantitatively measured with highly specific enzyme immunoassay kits [26,27].

2.6. Estimation of lipid peroxidative markers and antioxidants assay

The levels of TBARS, HP and CD were estimated by the methods of Niehaus and Samuelson [28], Jiang et al. [29], Rao and Recknagel [30], respectively. The levels of vitamin C, vitamin E and GSH were estimated by the methods of Roe and Kuether [31], Baker et al. [32], Ellman et al. [33] respectively. The protein content was determined by the method of Lowry et al. [34]. SOD, CAT, GPx, GST and GR were assayed with the methods of Kakkar et al. [35], Sinha [36], Rotruck et al. [37], Habig et al. [38], Pinto and Bartley [39], respectively.

2.7. DNA fragmentation assay

Agarose gel electrophoresis was performed in order to verify DNA fragmentation (Hebert et al., 1996) [40]. The heart tissue was homogenized using 5 ml of lysis buffer (50 mM Tris–HCl pH 8.0, 10 mM NaCl, 10 mM EDTA, 100 μg/ml proteinase K and 0.5% SDS) and incubated for 1 h at 50 °C. 10 μl of 100 μg/ml ribonuclease A (RNase A) was added to the mixture and incubated for an additional 1 h at 50 °C. Tissue samples were treated with 1 ml phenol followed by extraction with chloroform/isoamyl alcohol. The aqueous phase was treated with 25–50 μl of 3 M sodium acetate (pH 5.2) and one volume of ethanol, shaken gently, and left at −20 °C overnight. The precipitate was collected by centrifugation at 12,000× *g* for 20 min. The pellet was rinsed with 1 ml of 70% ethanol and spin for 10 min. The supernatant was discarded and the pellet was air dried at room temperature and later dissolved in 0.5–1.0 ml of double distilled water. DNA was precipitated in cold ethanol at −20 °C and finally dissolved in 0.5 ml of buffer. DNA sample was loaded in 1.0% agarose gel containing 0.5 μg/ml ethidiumbromide, electrophoresed at 80 V and visualized under UV transilluminator.

2.8. SDS-PAGE and Western blot analysis

Western blotting was performed to analyze the expression pattern of Bax, Bcl-2, Bcl-xL, caspase-3, -8 and -9, cytochrome c, TNF-α and Fas (Laemmli, 1970) [41]. The heart tissue samples were homogenised in an ice-cold RIPA buffer (1% Triton, 0.1% SDS, 0.5%

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