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Effect of ursolic acid on cardiac marker enzymes, lipid profile and macroscopic enzyme mapping assay in isoproterenol-induced myocardial ischemic rats

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ABSTRACT

This study investigates the antihyperlipidemic effect of ursolic acid (UA) on isoproterenol (ISO) induced male albino Wistar rats. Myocardial ischemia was induced by subcutaneous injection of ISO (85 mg/kg BW) twice at an interval of 24 h, for two consecutive days. A significant increase in the activities of the serum marker enzymes [creatine kinase, creatine kinase-MB and lactate dehydrogenease (LDH)], a prominent expression of LDH 1 and LDH 2 isoenzymes, increased levels of plasma total cholesterol (TC), low density lipoprotein-cholesterol, very low density lipoprotein-cholesterol, triglycerides (TG), free fatty acids (FFA), phospholipids (PL) and atherogenic index and decreased level of high density lipoprotein-cholesterol were observed in ISO-induced rats. The levels of TC, TG and FFA increased and the level of PL decreased in the heart tissue of ISO-induced rats. Further, there was an increased DNA damage (Comet assay) and myocardium infarct size as observed by staining with triphenyltetrazolium chloride (TTC). UA was administered subcutaneously for 7 days at a dose of 40 mg/kg BW. UA administration to ischemic rats brought all these parameters to near normality showing the protective effect of UA on ISO-induced rats.

1. Introduction

Myocardial infarction (MI) is one of the main causes of mortality and morbidity in the developed world and most of the developing countries. MI is an acute condition of necrosis of myocardium that occurs as a result of imbalance between coronary blood supply to any part of heart, resulting in death of cardiac tissue (Myocardial necrosis). Consequences of MI include hyperlipidemia, peroxidation of membrane lipids and loss of plasma membrane integrity (Gürgün et al., 2008).

Isoproterenol hydrochloride (ISO) is a β-adrenergic agonist that causes severe stress in myocardium resulting in the infarct like necrosis of heart muscle (Osadchii et al., 2007). ISO- induces damage in cardiac myocytes which include hypoxia due to myocardial hyper activity, coronary hypotension, calcium overload, depletion of energy reserves and excessive production of free radicals due to oxidative metabolism of catecholamines (Mohanty et al., 2004). Oxidation of catecholamine forms quinonoid compounds giving rise to the production of superoxide anions and subsequently hydrogen peroxide, which in the presence of iron forms highly reactive hydroxyl radicals and causes protein, lipid, DNA damage (Dhalla et al., 2000) and myocardial infarct size.

ISO causes an increase in the levels of circulatory and myocardial lipids, indicating its hyperlipidemic effect. High levels of circulating cholesterol and its accumulation in cardiac tissue are well associated with cardiovascular damage. Moreover, lipoprotein levels are also altered in ISO-induced rats. Thus, the lipids and lipoproteins play an important role in the pathogenesis of myocardial infarction (Chagoya de sanchez et al., 1997).

Plants constitute an important source of active natural products which differ widely in terms of structure and biological properties. In recent years, the prevention of cardiovascular disease (CVD) has been associated with the consumption of fresh fruits, vegetables or plants rich in natural antioxidants. ursolic acid (UA), a pentacyclic triterpenoid exists in natural plants in the form of free acid or aglycones (Fig. 1) (Ovesna et al., 2006). UA (3β-hydroxy-urs-12-en-28-oic acid) is present in apples, basil, berries, leaves, flowers and many kinds of medicinal herbs such as *Perilla frutescens* (Chen et al., 2003), *Rosmarinus officianalis* and *Eriobotrya japonica* (Liu, 1995). It exhibits a wide range of pharmacological effects such as antiinflammatory (Harmand et al., 2005), anticarcinogenic (Liu, 2005), antiulcer (Shih et al., 2004), cardiotonic (Somova et al., 2004), antihyperglycemic (Liu, 1995), hepatoprotective (Saravanan et al., 2006) and neuroprotective (Lu et al., 2007) activities.

The present study was aimed to investigate the effect of UA on the activities cardiac marker enzymes, lipid profile, atherogenic index, DNA damage and myocardial infarct size in ISO-induced myocardial ischemic rats.

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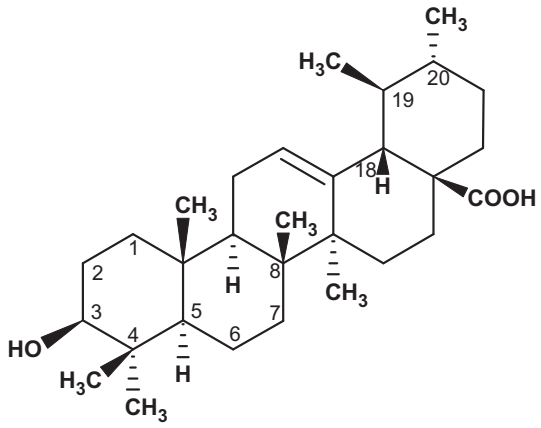

Fig. 1. Structure of ursolic acid.

2. Materials and methods

2.1. Chemicals

Isoproterenol hydrochloride (ISO) and ursolic acid (UA) were purchased from Sigma–Aldrich (St. Louis, Missouri, USA). The purity of UA is ⩾90% (HPLC). All other chemicals used in this study were of analytical grade obtained from E. Merck and HIMEDIA, India.

2.2. Experimental animals

All the experiments comply with the recommendations and guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India and the experimental protocol was approved by the Institutional Animal Ethics Committee of Rajah Muthiah Medical College and Hospital (RMMC&H), Annamalai University (Reg. No. 160/1999/CPCSEA: 560/2010). Healthy male albino Wistar rats (160–180 g), were obtained from the Central Animal House, Department of Experimental Medicine, RMMC&H, 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.

2.3. Experimental induction of myocardial ischemia

Myocardial ischemia was induced by subcutaneous (s.c.) injection (thigh muscle) of ISO (85 mg/kg BW dissolved in physiological saline) for the first two consecutive days (Seth et al., 1998).

2.4. Experimental design

The rats were randomly divided into four groups with six rats in each group. UA (40 mg/kg BW) was completely dissolved in 5% dimethyl sulfoxide (DMSO) and diluted with physiological saline.

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 (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.

2.5. Serum preparation

Blood samples were collected in dry test tubes and allowed to coagulate at an ambient temperature for 30 min. Serum was separated by centrifugation at 2000 rpm for 10 min.

2.6. Preparation of plasma

Blood samples collected in heparinised centrifuge tubes were centrifuged at 2000 rpm for 10 min and the plasma was separated by aspiration. The separated plasma was used for lipid estimations.

2.7. Preparation of heart tissue lipids

Lipids were extracted from tissues by the method of Folch et al. (1957) using chloroform methanol mixture (2:1 v/v). The tissues were rinsed in cold physiological saline thoroughly and dried by pressing between the folds of filter paper. The samples were homogenized in cold chloroform:methanol (2:1 v/v) and the contents were extracted after 24 h. The extraction was repeated four times. The combined filtrate was washed with 0.7% KCl and the aqueous layer discarded. The organic layer was made up to a known volume with chloroform and used for various estimations.

2.8. Myocyte preparation

Cardiomyocytes were obtained by using well established method of Van der Heide et al. (1990). Excised heart was quickly placed into chilled dissociation buffer (NaCl 137 mM, KCl 5.4 mM, $CaCl_2$ 1.8 mM, $MgCl_2$ 1.0 mM, KH_2PO_4 0.44 mM, Na_2HPO_4 0.34 mM, dextrose 5.6 mM, HEPES buffer (pH 7.5) 20 N, penicillin 50 U/mL, and streptomycin 50 μg/mL). The heart was cut into 1–2 mm cubes and dissociated by trypsinization (0.05% trypsin–EDTA at 37 °C for 10 min). Unfreed cells from the first treatment were discarded, and the sequence was repeated until all tissue was dissociated (approximately 5 times). Freed cells (1×10^6 cells) were collected in cold Dulbecco's modified Eagle's medium and used for the assessment of DNA damage.

2.9. Estimation of cardiac markers

The activities of creatine kinase (CK), creatine kinase-MB (CK-MB) and lactate dehydrogenase (LDH) were assayed by using commercial kits obtained from Agappe Diagnostics, Kerala, India.

2.10. Separation of serum LDH isoenzymes by agarose gel electrophoresis

LDH isoenzyme was separated by agarose gel electrophoresis method of Mckenzie and Henderson (1983). Agarose (1%) separating gel was prepared on the glass slide. Serum samples were loaded into wells and then electrophoresis at 37 °C for 1 h. After the run, gels were stained by solution which consists of 1.0 ml of 1.0 M lithium lactate, 1.0 ml of 0.1 M sodium chloride, 1.0 ml of 5.0 mM magnesium chloride, 2.5 ml of 0.1% (w/v) nitroblue tetrazolium, 0.25 ml of 0.1% phenazine methosulphate, 2.5 ml of 0.5 M phosphate buffer (pH 7.5) and 10 mg of nicotinamide adenine dinucleotide in a total volume of 10 ml. The gels were incubated with the staining solution at 37 °C in the dark for an hour. The separated LDH isoenzymes appeared as purple bands. The gels were washed with 7.5% acetic acid and preserved in 0.5% acetic acid.

2.11. Estimation of total cholesterol

The level of TC was estimated by the method of Allain et al. (1974). The reaction mixer consisted of 10 μL of plasma or 10 μL of lipid extract, 1.0 mL of enzyme reagent (4-aminoantipyrine, cholesterol esterase, phenol, cholesterol oxidase and horseradish peroxidase) which was mixed well and kept at 37 °C for 5 min. 10 μL of cholesterol standard and distilled water (blank) were also processed similarly. The absorbance was measured at 510 nm.

2.12. Estimation of triglyceride

The level of TG was estimated by the method of McGowan et al. (1983). To 10 μL of plasma or 10 μL of lipid extract, 1.0 mL of enzyme reagent (Lipase, glycerol kinase, glycerol 3-phosphate oxidase, peroxidase, 4-aminoantipyrine, ATP, 3, 5-dichloro-2-hydroxybenzene sulfonate) was added, mixed well and incubated at room temperature for 10 min. 10 μL of triglycerides standard and distilled water (blank) were also processed similarly. The absorbance was measured at 510 nm.

2.13. Estimation of free fatty acids

FFA levels in the plasma and heart tissue were estimated by the method of Falholt et al. (1973). 0.2 ml of plasma or 0.5 mL of lipid extract were evaporated to dryness and dissolved in 6.0 mL chloroform-heptane-methanol solvent and 2.5 mL of copper reagent were added. All the tubes were shaken vigorously for 90 s and were kept aside for 15 min. The tubes were centrifuged and 3.0 mL of the copper layer was transferred to another tube containing 0.5 mL of diphenyl carbazide and mixed carefully. The color developed was read at 540 nm against a reagent blank containing 3.0 mL solvent and 0.5 mL diphenyl carbazide.

2.14. Estimation of phospholipids

PL was estimated by the method of Zilversmit and Davis (1950). 0.5 mL of the lipid extract was evaporated to dryness in water bath. To the extract or 0.2 mL of plasma, 1.0 mL of 5.0 N sulphuric acid was added and digested till light brown. Then