Pharmacology

Cardioprotective Effects of *Lagenaria siceraria* Fruit Juice on Isoproterenol-induced Myocardial Infarction in Wistar Rats: A Biochemical and Histoarchitecture Study

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ABSTRACT

The present study was designed to evaluate the cardioprotective effects of *Lagenaria siceraria* fruit juice in isoproterenol-induced myocardial infarction. Rats injected with isoproterenol (200 mg/kg, s.c.) showed a significant increase in the levels of serum uric acid, tissue Na$^+$ and Ca$^{2+}$ ions and membrane-bound Ca$^{2+}$-ATPase activity. A significant decrease in the levels of serum protein, tissue K$^+$ ion, vitamin E level, and the activities of Na$^+$/K$^+$-ATPase and Mg$^{2+}$-ATPase was observed. Isoproterenol injected rats also showed a significant increase in the intensity of lactate dehydrogenase isoenzyme and histopathologic alterations in the heart. Treatment with *L. siceraria* fruit juice (400 mg/kg/day, p.o.) for 30 days and administration of isoproterenol on 29th and 30th days showed a protective effect on altered biochemical and histopathologic changes. These findings indicate the cardioprotective effect of *L. siceraria* fruit juice in isoproterenol-induced myocardial infarction in rats.

Key words: Antioxidant activity, isoproterenol, *Lagenaria siceraria*, myocardial infarction

INTRODUCTION

*Lagenaria siceraria* (LS), commonly known as bottle gourd, is reported in Ayurvedic Pharmacopoeia. Traditionally, the fruit of LS is used for its cardioprotective, cardiotonic, diuretic, and aphrodisiac activities and as an antidote to certain poisons.¹² Modern pharmacological studies have shown that fruit of LS possesses various positive effects in preventing various conditions including pain and inflammation, has diuretic activities, and is also effective in hyperlipidemic condition,¹³‐¹⁵ cardiotoxicity and stress.¹⁶⁻¹⁷

Isoproterenol (ISO), a β-adrenergic agonist, has been found to produce stress in the myocardium due to the generation of free radicals by its auto-oxidation. Some of the mechanisms proposed to explain ISO-induced damage to cardiac myocytes include hypoxia, coronary hypotension, calcium overload, energy depletion and excessive production of free radicals as a result of catecholamine autoxidation.¹⁸⁻¹⁹ Several natural products are reported to prevent ISO-induced myocardial infarction (MI) due to their antioxidant activity. In our previous study, we had reported the protective effects of *Lagenaria siceraria* fruit juice (LSFJ) in electrocardiographic, hemodynamic,
endogenous antioxidant status and lipid peroxidation in ISO-induced MI in rats.

Till date, no study has been carried out regarding the effect of LSFJ on membrane-bound phosphatases and histopathologic alteration induced by ISO. So, an attempt was made to evaluate the cardioprotective activity of LSFJ in ISO-induced MI by evaluating biochemical and histopathologic changes discussed below.

MATERIALS AND METHODS

Drugs and chemicals

(±)-Isoproterenol hydrochloride (ISO) was purchased from Sigma Aldrich Co. (St. Louis, MO, USA). Fresh fruits of LS were collected from a nearby farm at Baroda, Gujarat. All the reagents and chemicals used in the entire study were of analytical grade.

Collection and authentication of Lagenaria siceraria fruits

Fresh fruits of LS were collected from a nearby farm at Baroda. The fruits collected were semi-ripe, bottle shaped, weighed between 500 and 700 g and of the same plant. The fruit of LS was authenticated by the authority from Botany Department, The M. S. University of Baroda, Vadodara.

Preparation of Langenaria siceraria fruit juice

Fresh juice of LS was prepared with the help of a juicer without adding water. 250 g of fresh fruit was chopped into small pieces and 135 ml juice was collected. The juice was filtered with a sterile cloth and the resultant filtrate was used for oral dosing to animals. 10 ml of fresh juice was subjected to drying in a previously dried and weighed Petri dish, the juice was evaporated to complete dryness in a hot air oven (45ºC) and then the weight of Petri dish containing dry residue of juice was taken. The milligram equivalent dose of 10 ml juice was calculated by subtracting the initial weight of dried petridish. The same procedure was repeated for six times on different days. It was clear from the mean that 10 ml of juice gives 602.00 mg of total solid residue in dried juice, which is equivalent to 250 g of fresh fruit of LS. The dose of fresh juice of LS in (ml) equivalent of 100, 200 and 400 mg/kg/day was administered orally to rats for 30 days.

Fixation of optimum dosage of Langenaria siceraria fruit juice

A pilot study was carried out to establish the optimum dose of the drugs which exhibits maximum cardioprotective effect during 30 days. Rats were treated with (LSFJ) (100, 200 and 400 mg/kg/day, p.o.) for 30 days and ISO was administered on 29th and 30th days. At the end of treatment period, serum lactate dehydrogenase (LDH) and creatine phosphokinase-MB levels were evaluated. 400 mg/kg/day, p.o. was found to be the most effective dosage of LSFJ in functional recovery of the above biochemical alterations and this dose was selected for further evaluation in the present study.

Experimental animals

All experiments were carried out on male albino Wistar rats weighing 200–250 g, obtained from in-house animal breeding. They were housed in polypropylene cages (47 × 34 × 20 cm) lined with husk, renewed every 24 h under a 12:12 h light-dark cycle at around 22ºC and had free access to water and food. The animals were fed on a standard pellet diet (Pranav Agro Industries Ltd., Maharashtra, India). All the protocols of animal experiments were approved by the Institutional Animal Ethics Committee (IAEC) in accordance with the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India, New Delhi.

Experimental protocol

Group I: Control rats received distilled water for 30 days and normal saline (1 ml/kg, s.c.) on 29th and 30th days.
Group II: Rats received distilled water for 30 days and ISO (200 mg/kg, s.c.) in normal saline on 29th and 30th days at an interval of 24 h.
Group III: Rats received LSFJ (400 mg/kg/day, p.o.) for 30 days.
Group IV: Rats received LSFJ (400 mg/kg/day, p.o.) for 30 days and ISO on 29th and 30th days.

Twenty-four hours after the second injection (i.e. at 48th h after first injection of ISO) of ISO was given, blood was collected and serum was separated. The animals were sacrificed and the heart was dissected out and weighed. Serum and heart tissue homogenate were used for the estimation of following biochemical parameters.

Assessment of serum uric acid and total protein

On day 31, blood was collected from the retro-orbital plexus under mild ether anesthesia. Serum was separated and the levels of serum uric acid and total protein were determined by using standard kits (Reckon Diagnostic Ltd., Vadodara, Gujarat, India).
Estimation of vitamin E

The level of vitamin E was estimated as suggested by Desai.[11] To 1 ml of homogenate, 1 ml of ethanol was added and thoroughly mixed. 3 ml of petroleum ether was added, shaken rapidly and centrifuged. 2 ml of supernatant was taken and evaporated to dryness. To this, 0.2 ml of bathophenanthroline (0.2% 4,6-diphenyl-1,10-phenanthroline in ethanol) was added. The assay mixture was protected from light and 0.2 ml of ferric chloride (0.001 M) was added followed by 0.2 ml of e-phosphoric acid (0.001 M). The total volume was made upto 3 ml with ethanol. The color developed was read at 530 nm. The level of vitamin E is expressed as μg/mg protein.

Estimation of membrane-bound phosphatases (Na⁺/K⁺, Mg⁺ and Ca⁺⁺ ATPases)

The sediment after centrifugation of tissue homogenate was resuspended in ice-cold tris buffer (10 mM, pH 7.4) to get a final concentration of 10% w/v and was used for the estimation of Na⁺/K⁺-ATPase,[13] Ca²⁺-ATPase[13] and mg²⁺-ATPase.[14] Protein was estimated according to the method of Lowery et al.[15]

Estimation of Na⁺, K⁺ and Ca⁺⁺ levels

The concentrations of Na⁺ and K⁺ were estimated using commercial kits (Monozyme India Ltd., Secunderabad, India). Ca⁺⁺ in the heart homogenate was measured by the o-cresolphthalein complexone method using a reagent kit (Span Diagnostioc Ltd., Gujarat, India). In this method, o-cresolphthalein complexone binds to calcium tightly in alkaline solution to form a deeply colored complex, owing to lactone formation in the phthalein part of molecule.[16]

Separation of LDH isoenzymes

LDH isoenzymes were separated and quantified by agarose gel electrophoresis.[17] Agarose gel (1%) was prepared and applied immediately to the glass slide. After the agarose gel had set properly, serum samples were applied into wells and separated by electrophoresis. After the run, the gels were removed and stained by using staining solution. The gels were incubated with the staining solution at 37°C in the dark for a suitable period (approx. 30 min). The separated LDH isoenzymes appeared as purple bands. The gel was washed with 7.5% acetic acid and preserved in 5% acetic acid. The photographs were taken using AlphaEase Fc Imaging system, USA.

Hemotoxylin and eosin staining

After the treatment period, the animals were sacrificed and the organs were excised, blotted free of blood and tissue fluids and preserved in 10% v/v formal saline solution. The specimens were given for further processing to Baroda Pathological Laboratory, Vadodara, where routine procedure for sectioning, staining and mounting was observed by the laboratory personnel. Briefly, after a week, the tissues were washed thoroughly in repeated changes of 70% alcohol and then dehydrated in ascending grades of alcohol (70–100%). After dehydration, the tissues were cleaned in xylene and embedded in paraffin wax. Sections of 5 μm thickness were cut with a microtome and taken on glass slides coated with albumin. The sections were deparaffinated in xylene and downgraded through 100, 90, 70, 50, 30% alcohol and then finally in water. The hemotoxylin-stained sections were stained with eosin for 2 minutes and were then quickly passed through ascending grades of alcohol, cleaned in xylene and mounted on Canada balsam. The stained sections were examined under Olympus (Magnus MIX series) India Pvt. Ltd. photomicroscope and photographed (10×). The pathologist was blinded to the treatment. Analyses of the heart sections were carried out. Briefly, the findings were graded as no changes (A); (+) mild (focal myocyte damage or small multifocal degeneration with slight degree of inflammatory process); (++) moderate (extensive myofibrillar degeneration and/or diffuse inflammatory process); (+++) marked (necrosis with diffuse inflammatory process).

Statistical analysis

Results are presented as mean ± SEM. Analysis was done using one-way analysis of variance (ANOVA) followed by Bonferroni multiple comparisons using a computer-based fitting program (Prism). Differences were considered to be statistically significant when P<0.05.

RESULTS

Effect of LSFJ on serum uric acid, total protein and vitamin E levels

ISO injected rats showed a significant increase (P<0.001) in serum uric acid levels and significant decrease (P<0.001) in total protein and tissue vitamin E levels as compared to control rats. Administration of LSFJ to ISO injected rats (LSFJ + ISO) showed a significant (P<0.05) increase in the total protein level and tissue vitamin E contents as compared to ISO injected groups. LSFJ administered rats showed decrease in uric acid level compared to ISO injected
Effect of LSFJ on ATPases and electrolytes' levels

The effects of LSFJ on membrane-bound ATPase and electrolyte levels in normal and ISO injected rats are shown in Figure 1 and Table 2. Rats injected with ISO showed a significant ($P<0.001$) decrease in $\text{Na}^+$/K$^+$-ATPase and mg$^{2+}$-ATPase and a significant ($P<0.001$) increase in Ca$^{2+}$-ATPase activity as compared to control rats. Treatment with LSFJ in ISO injected rats (LSFJ + ISO) showed a significant ($P<0.05$) decrease in Ca$^{2+}$-ATPase as compared to ISO injected group [Figure 1]. ISO injected rats also showed a significant ($P<0.001$) increase in the levels of tissue sodium and calcium; however, the level of potassium was significantly ($P<0.001$) decreased compared to normal control rats. Administration of LSFJ for 30 days significantly ($P<0.05$) improved the level of tissue sodium concentration. However, it showed non-significant effects on calcium and potassium level.

Effect of LSFJ on separation of LDH isoenzymes levels

Agarose gel electrophoretic separation of serum LDH isoenzyme patterns of normal and ISO injected rats is shown in Figure 2. Rats injected with ISO showed an increase in the intensity of LDH-1 and LDH-2 isoenzyme bands compared to control rats. Treatment with LSFJ in ISO injected rats (LSFJ + ISO) resulted in a decrease in the intensity of LDH-1 isoenzymes compared to ISO injected rats.

DISCUSSION

Phytopharmaceuticals are gaining importance in allopathic as well as in traditional medicine owing to their non-addictive and less toxic nature. Novel antioxidants may offer an...
Many dietary antioxidants and some non-nutrient based antioxidants from plants such as garlic, green tea, anthocyanins in red berries, lycopene in tomatoes, red and white wines from grape seeds are increasingly being recognized as potential health promoters in reducing the risk of cardiovascular disease. In the present study, a significant increase in serum uric acid levels and a decrease in total protein levels were observed in ISO injected rats, which is in line with the previous reports. Large group studies have shown that uric acid is an important independent risk factor for cardiovascular mortality and in the development of MI. In hypoxic tissue, ATP depletion occurs, causing accumulation of hypoxanthine. When tissues are disturbed, the enzyme xanthine dehydrogenase is converted to xanthine oxidase by the oxidation of essential –SH groups. Xanthine oxidase catalyzes the conversion of hypoxanthine to xanthine, uric acid and superoxide. This could be one of the reasons for the elevated levels of serum uric acid in ISO injected rats. A decrease in the level of serum total proteins in ISO injected rats could be due to increased free radical production by ISO. Administration of LSFJ did not show significant improvement in serum uric acid level, whereas it showed improvement in serum protein levels as compared to ISO injected rats.

Vitamin E is a major lipid-soluble non-enzymatic antioxidant present in cell membranes and lipoproteins and protects against oxidative modification. The observed decrease in the vitamin E level in the present study might be due to increased utilization for the neutralization of ISO-mediated free radicals and lipid peroxidation. Administration of LSFJ improved the level of vitamin E which might be due to its antioxidant activity.

ATPases play a significant role in the contraction and relaxation of the cardiac muscle by maintaining the normal ion levels inside the myocyte. In the present study, ISO injected rats showed a significant decrease in Na⁺/K⁺-ATPase and Mg²⁺-ATPase activities and correspondingly Ca²⁺-ATPase activity was increased. The changes in the ATPase activity are in line with the previous reports. Na⁺/K⁺-ATPase is a lipid-dependent enzyme containing sulfhydryl (–SH) group. Oxidants are known to initiate lipid peroxidation and thus destroy phospholipids which are required for the normal activity of membrane-bound protein. Increased in lipid peroxidation and decrease in the phospholipid content in myocardium following ISO injection was reported in our previous study. This could be the reason for alteration in the ATPase activity.

Results of the present study also displayed enhanced activity of Ca²⁺-ATPase; this may be due to the activation of adenylate cyclase activity. ISO-induced MI has been reported to enhance adenylate cyclase activity, resulting in increased formation of cAMP. This may be one of the reasons for enhanced Ca²⁺-ATPase activity and thereby increased Ca²⁺ level. Calcium overload in the myocardial cells during ischemia activates the Ca²⁺-dependant ATPase of the membrane, depleting high high-energy phosphate stores, thereby indirectly inhibiting Na⁺ and K⁺ transport and inactivating Na⁺/K⁺-ATPase.

In the cell, ATPases are intimately associated with the plasma membrane and participate in the energy-dependent transport of sodium, potassium, magnesium and calcium translocation. An increase in sodium and calcium along with decrease in potassium were observed in ISO injected rats.
rats. As discussed above, an alteration in ATPase activity brings about a change in the concentration of electrolytes. Treatment with LSFJ in ISO injected rats (LSFJ + ISO) slightly altered the ATPase activity and electrolyte levels, but was found to be statistically non-significant.

LDH is a cytosolic enzyme existing in five different isoforms (LDH-1–LDH-5). LDH-1 predominates in cardiac tissues. Variation in the serum LDH isoenzyme pattern can be considered as definitive diagnostic criterion for assessing the myocardial damage, as the rate of appearance and disappearance of LDH in the blood indicates the size of infarction.[29] In the present study, an increase in LDH-1 and LDH-2 isoenzyme bands in ISO injected rats was observed which is supported by previous findings.[30] Administration of LSFJ significantly decreased the intensity of LDH-1 isoenzyme, suggesting its membrane stabilizing activity.

Biochemical findings were further confirmed by histopathologic studies. Histopathologic examination of myocardial tissue in control rats depicted clear integrity of the myocardial cell membrane. Histopathologic sections of the heart injected with ISO displayed necrosis of muscle fibers with inflammatory cell infiltration, edema and fragmentation of muscle fibers, which indicates involvement of oxidative stress and inflammatory processes in ISO induced myocardial injury. Administration of LSFJ in ISO injected rats (LSFJ + ISO) resulted in moderate changes in histopathologic alterations [Figure 3 and Table 3].

In conclusion, the present study showed the protective effect of LSFJ on ISO-induced MI in rats. These effects might be due to the presence of polyphenolic components in the fruit of LS. Further study is required with higher doses/duration of treatment with LSFJ to confirm its cardioprotective activity.

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