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Original Article

Protective Effect of Glycosaminoglycans from *Meretrix* (Gmelin) Against Isoproterenol - Induced Myocardial Infarction in Male Wistar Rats

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ABSTRACT

Rats (140 ± 20 g) were divided into four groups (n=6); control (Group I), ISO Group II (85 mg/kg of ISO subcutaneously injected at 13th and 14th days), GAG treated (300 µg/day per rat s. c. for 2 weeks - Group III), and GAG plus ISO Group IV (300 µg/day per rat s. c. for 12 days and then by ISO on 13th and 14th days). At the end of experimental period, all rats were anesthetized with sodium pentabarbitol and sacrificed by survical decapitation. Plasma, serum and heart tissues were separated for enzymes assays. Lactate dehydrogenase (LDH), aminotransferases (AST and ALT) and creatine kinase (CK) activities were increased in serum and decreased in heart tissue of ISO group, which were normalized by GAG pretreatment rats. Superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) activities and reduced glutathione (GSH) were abnormal in the ISO induced rats, and this was minimized by GAG pretreatment. Increased level of thiobarbituric acid reactive substances (TBARS) in plasma and the heart of ISO treated rats; pre s.c. injected with GAG to ISO-induced rats decreased the levels of TBARS. Restoration of cellular normalcy accredits GAG has protective role against ISO-induced cardiotoxicity.

Keywords: *M. meretrix*, GAG, Isoproterenol, Myocardial infarction, Lipid peroxidation.

INTRODUCTION

Although clinical care is improved, public awareness is raised and health innovations are widely used, myocardial infarction remains the leading cause of death

worldwide[1]. It is the acute condition of myocardial necrosis that occurs as a result of imbalance between coronary blood supply and myocardial demand. The patient may experience significant disability or die[2]. Experimental and clinical studies have shown that there is increased generation of reactive oxygen species such as superoxide anion ($.O_2^-$) and hydroxyl radicals (.OH) in heart failure, which involved in the formation of lipid peroxides, damage of cell membrane, and destruction of antioxidative defense system[3]. Therapeutic intervention via suppression of free radical generation and/or enhancement of endogenous antioxidant enzymes may limit the infarct size and attenuate myocardial dysfunction[4]. ISO, a β-adrenergic agonist, is a well-known inducer of myocardial hypertrophy[5] and its supramaximal dosages produce acute myocardial necrosis and interstitial fibrosis[6].

For >30 years, the role of heparin in the treatment of myocardial infarction has been controversial with regard to safety and efficacy[7]. Heparin is a highly sulphated and polydispersed linear GAG of alternating 1→4 linked hexuronic acid and D-glycosamine residues, with a molecular weight (Mw) of 3,000–37,000 Da and an average Mw of 15,000 Da[8]. The marine environment is an exceptional reservoir of bioactive natural products, many of which exhibit structural features not found in terrestrial natural products. Research into the pharmacological properties of marine natural products has led to the discovery of many potentially active agents considered worthy of clinical application[9]. The GAGs / proteoglycans from various animals perform powerful blood anticoagulant activity. The most widely known and therapeutically used GAGs are "heparin"[10].

Marine clam *M. meretrix* is an Indian bivalve mollus of great local economic importance. We have already deliberated the possible convenience of GAG from marine *M. meretrix*[11]. Considering the above said properties of GAG and its derivatives, the present study has been designed to evaluate the cardioprotective activity of GAG from marine clamp on isoproterenol (ISO)-induced myocardial damage in rats.

MATERIALS AND METHODS

Experimental animals

All the experiments were carried out with male albino Wistar rats weighing 140-160 g, obtained from the Central Animal House, Rajah Muthiah Institute of Health Sciences, Annamalai University, Tamil Nadu, India. They were housed in polypropylene cages (47 cm x 34 cm x 20 cm) lined with husk, renewed every 24-h under a 12-h light: 12-h dark cycle at around 22ºC and had free access to tap water and food. The rats were fed on a standard pellet diet (Pranav Agro Industries Ltd., Maharastra, India). The pellet diet consisted of 22.02% crude protein, 4.52% crude oil, 3.25% crude fibre, 7.5% ash, 1.38% sand silica, 0.8% calcium, 0.6% phosphorus, 2.46% glucose, 1.8% vitamins and 56.17% nitrogen free extract (carbohydrates). The diet provided metabolisable energy of 3000 kcal. The experiment was carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India and approved by the Animal Ethical Committee of Annamalai University (Approval No. 428; dated 21.03.07).

Drugs and chemicals

Isoproterenol hydrochloride, (Sigma Chemical Company, St. Louis, MO, USA) and the extracts of GAG were used. Thiobarbituric acid and reduced glutathione were purchased from Sisco Research Laboratories, Mumbai, India. Glucose, uric acid, total protein and A/G ratio kits were purchased from Qualigens Diagnostics,

Mumbai, India. All other chemicals used in this study were analytical grade.

Induction of experimental myocardial infarction

ISO (85 mg/kg) was dissolved in normal saline and injected subcutaneously to rats at an interval of 24 h for 2 days to induce experimental MI[12].

Experimental design

The animals were grouped as six rats in each group-Group I: control rats; Group II: ISO treated; Group III: GAG was administrated s.c. at a dosage of 300 µg/day per rat for 2-weeks; Group IV: rats were injected s.c. 300 µg/day/rat of GAG for 12 days and then 85 mg/kg of ISO at the last two days of experimental period. All the rats were anesthetized with sodium pentobarbital (35 mg/kg, i.p.) and sacrificed by cervical decapitation. Serum and plasma samples were prepared for enzymes assays. The excised heart tissues were rinsed in ice-cold physiological saline and homogenized in 100 mM Tris-HCl buffer (pH 7.4) to give a 10% homogenate. Aliquots of the tissue homogenate were suitably processed for biochemical assays, lipid peroxidation and antioxidant studies. Aliquots of heart tissues were set aside for histopathological processing.

Enzymatic indices of cellular damage

CK was assayed in the serum and heart tissue by the method of Okinaka *et al*[13]; activities of LDH by the method of King,[14] AST and ALT were determined and expressed in terms of µmoles of pyruvate liberated/min/mg of protein at 37ºC[14]. Protein estimations were carried out according to the method of Lowry *et al* [15].

Assessment of oxidative stress in the cardiac tissues

Lipid peroxidation in the heart tissue was determined by the method of Hogberg et al [16], where malondialdehyde (MDA) produced during peroxidation of lipids, served as an index of lipid peroxidation, MDA reacts with thiobarbituric acid to generate a coloured product which absorbs at 532 nm. The peroxidation system contained 10 mM ferrous sulfate and 0.2 mM ascorbate as inducers[17]. The degree of inhibition of the autoxidation of pyrogallol at an alkaline pH by superoxide dismutase (SOD) was used as a measure of the total enzyme activity[18]. Glutathione peroxidase (GPx) activity was assessed in terms of utilization of glutathione[19]. It is based on the reaction between glutathione (present in the reaction mixture), remaining after the action of GPx (present in the tissue homogenate aliquot), and 5,5' –dithio-bis(2-nitrobenzoic acid) resulting in a complex that absorbs maximally at 412 nm. Catalase activity was assayed by the method of Sinha[20] where in the enzyme present in the tissue homogenate aliquot was allowed to split hydrogen peroxide (H_2O_2) for different time periods. The reaction was stopped at specific time intervals by the addition of dichromate/acetic acid mixture and the remaining H_2O_2 was determined by colorimetric measurement of the resulting chromic acetate after heating the reaction mixture. Total reduced glutathione was estimated in the cardiac tissues by the method of Moron *et al*[21].

Histopathological studies

Portions of heart tissues were fixed in 10% formalin. The washed tissues were dehydrated in the descending grades of isopropanol and finally cleared in xylene. The tissues were then embedded in molten paraffin wax. Sections were cut at 5 µm thickness, stained with haematoxylin and eosin. The sections were then viewed under

light microscope (320x) for histopathological changes.

Statistics

Statistical analysis was performed using one-way analysis of variance (ANOVA) using SPSS Software 9.05 followed by Duncun's multiple range test (DMRT). Results were expressed as mean ± S.D. from six rats in each group and triplicate estimates in each rat. *P* values <0.05 were considered as significant.

RESULTS & DISCUSSION

The appraisal of compromised cellular integrity in the ISO group was made by determining the activities of CK, LDH, AST, ALT and in serum and tissues (Table 1). These cardiac marker enzymes showed a significant increase in ISO group with respect to control (*P* <0.05). GAG pretreatment of ISO-induced rats resulted in near normal activities of these cardiac marker enzymes. The dosage agenda of GAG followed in the present study was not toxic to heart; the activities of enzymes in the GAG control (Group III) did not reveal any significant changes when compared with the normal control group. Groups I and III represents normal cardiac histology [Fig. 1 (A) and (C), respectively]. Fig. 1 (B) reveals cardiac muscle fibre destruction and hypertrophy. In the photomicrograph, the swollen and flabby muscle fibres of the ISO-induced group (Group II) can be clearly observed. GAG plus ISO treatment (Group IV) [Fig. 1. (D)], wherein the changes are less marked and an almost normal cardiac muscle fibre picture is presented.

Rats induced with ISO, exhibited a significant (*P* <0.05) decrease in the activities of these antioxidant enzymes in the heart tissue when compared with control rats. The activities of SOD, catalase and GPx in heart of normal and experimental rats are depicted in Table 2. Pretreatment with GAG (300 µg/day) to ISO-induced rats significantly (*P* <0.05) increased the activities of these antioxidant enzymes when compared with ISO-alone induced rats. Table 2 shows the assessment of non-enzymatic antioxidant status of GSH in the ISO-induced and GAG in plasma and heart rats. Rats induced with ISO, showed a significant (*P* <0.05) decrease in the activity of this antioxidant enzyme and the levels of GSH on comparison with normal control rats. S.C. injection of GAG (300 µg/day) to ISO-induced rats significantly (*P* <0.05) increased the activities of these antioxidant enzymes and the levels of GSH when compared with ISO-alone induced rats.

Rats induced with ISO, showed a significant (*P* <0.05) increase in the levels of TBARS in plasma and the heart when compared to normal rats. Table 3 shows the levels of TBARS in plasma and the heart of normal and experimental rats. Pre s.c. injected (group IV) with GAG (300 µg/day) to ISO-induced rats for 2-weeks significantly (*P* <0.05) decreased the levels of TBARS in plasma and the heart tissue when compared with ISO-alone induced rats. ISO, a synthetic catecholamine and β-adrenergic agonist, causes severe stress in the myocardium. Amongst various mechanisms proposed to explain ISO-induced cardiac damage generation of highly toxic free radicals through auto-oxidation of catecholamine has been implicated as one of the important causative factors. This free radical-mediated peroxidation of membrane phospholipids and consequent changes in membrane permeability is the primary target responsible for cardiotoxicity induced by ISO.

ISO is anticipated as a cardiotoxic mediator due to its ability to destruct myocardial cells. We have observed a significant elevation in the levels of diagnostic marker enzymes (CK, LDH, AST and ALT) in serum with subsequent decrease in the heart of ISO-induced rats. This could be due to nectrotic damage of the myocardial

membrane caused by ISO. Our results are in friendship with previous reports of Saravanan and Shanmugam[12]. When myocardial cells are damaged or destroyed due to the deficiency of oxygen supply the cell membrane becomes permeable or may rupture and results in the leakage of enzymes. The discharge of cellular enzymes reflects the alterations in plasma membrane integrity and/or permeability as a response to β-adrenergic stimulation. Pretreatment with GAG to ISO-induced rats significantly decreased the levels of these enzymes in serum with significant increase in the heart. This could be due to reduction in the damage of the myocardium by GAG there by it reduces the release of the enzymes from the myocardium.

Lipid peroxidation, a type of oxidative degeneration of polyunsaturated fatty acids has been linked with altered membrane structure and enzyme inactivation, is an indication of the severity of ISO-induced damage of the heart. Activated lipid peroxidation is an important pathogenic event in myocardial infarction, with increased levels of TBARS reflecting the major stages of the disease and its complications. The observed increase in the levels of serum and heart TBARS in ISO-induced rats shows the excessive formation of free radicals and activation of lipid peroxidation. Pretreatment with GAG to ISO-induced rats significantly decreased the levels of TBARS in serum and the heart. Low molecular weight heparin reduces the levels of lipid peroxidation in adriamycin-induced cardiotoxicity in rats[22].

Reactive oxygen species are generated from the leakage of electrons into oxygen from various systems in our body and the endogenous antioxidant defense is a very important source to neutralize the oxygen free radical mediated tissue injury. A significant decrease in the activities of SOD, CAT, GPx and the levels of GSH observed in the heart ISO-induced rats. SOD plays an important role in protecting the cells from oxidative damage by converting superoxide radicals into hydrogen peroxide, which is further metabolized by catalase, which catalyses the destruction of hydrogen peroxide. During myocardial infarction, SOD and catalase are structurally and functionally impaired by free radicals resulting in myocardial damage. The decrease in SOD and catalase may be due to the involvement of superoxide and hydrogen peroxide free radicals in myocardial cell damage mediated by GAG. Reduced availability of GSH in ISO induced rats also reduces the activity of GPx upon ISO administration. Inactivation of GPx in the heart leads to accumulation of oxidized glutathione which in turn inactivates many enzymes containing the SH group and inhibits protein synthesis.

GAG pretreatment to the ISO-induced rats prevented the build up of oxidative stress. This in turn prevents the depletion of antioxidant molecules, namely SOD, catalase, GPx and GSH. GAG exerts anti-free radical effects and restores the antioxidant balance to normalcy. The rise in the activities of the primary enzymatic antioxidant defenses-SOD, catalase GPx and GSH in the GAG treated ISO-induced group highlight the protection rendered by the heparin derivative in combating the oxidative insult. It has been suggested that small amounts of GAG enhanced the antioxidant activity of SOD and contribute to the inhibition of free radical mediated tissue injury. Heparin alleviates the effects of free radical production, and enhances the *in vivo* activity of SOD[23].

To conclude, the present work projects the protective effect afforded by GAG against cardiac damage induced by ISO on the basis of biochemical assessment and oxidative stress management, confirmed by histopathological examination. The efficacy of marine clam GAG usefulness in pharmacology can be tested by *in vitro* also in future.

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Table 1. Changes in serum and tissue enzyme activities in the early phase ISO-induced cardiotoxicity and effect of GAG treatment (values are expressed as mean ± S.D. for 6 animals in each group)

Enzyme assays (U/mg protein)	Group I; Control	Group II; ISO	Group III; GAG	Group IV; GAG + ISO
Serum				
CK	200.82 ± 0.67	335.95 ± 0.38[a, ***]	191.70 ± 0.50	203.44 ± 0.50 [b, ***]
LDH	106.20 ± 0.33	203.51 ± 0.01 [a, ***]	100.96 ± 0.01	120.40 ± 0.01 [b, ***]
AST	27.56 ± 0.33	55.32 ± 0.83 [a, ***]	25.57 ± 0.67	33.58 ± 0.01 [b, ***]
ALT	14.43 ± 0.83	35.07 ± 0.01 [a, ***]	13.12 ± 0.01	19.88 ± 0.01 [b, ***]
Heart				
CK	16.94 ± 0.83	10.04 ± 0.33 [a, ***]	18.15 ± 0.33	14.46 ± 0.71 [b, ***]
LDH	85.83 ± 0.67	50.87 ± 0.01 [a, ***]	94.35 ± 0.50	88.28 ± 0.33[b, ***]
AST	34.82 ± 0.33	25.06 ± 0.67 [a, ***]	38.20 ± 0.33	29.42 ± 0.50 [b, ***]
ALT	41.52 ± 0.33	34.19 ± 0.50 [a, ***]	43.54 ± 0.67	28.68 ± 0.33 [b, ***]

Enzyme units: CK: µmoles x 10^{-3} of phosphorus liberated/min; LDH: µmoles x 10^{-1} of pyruvate liberated/min; AST, ALT: µmoles x 10^{-2} of pyruvate liberated/min.
The symbols represent statistical singnicance: *** P <0.05, (DMRT).
[a]Comparisons were made between: group I and groups II, III, IV
[b]Groups II and IV

Table 2. Estimation of antioxidant status in the ISO-induced and GAG treated groups compared with the controls (values are expressed as mean ± S.D. for six animals in each group)

Antioxidant activity	Group I; Control	Group II; ISO	Group III; GAG	Group IV; GAG + ISO
Enzymatic antioxidant SOD Catalase GPx	 9.05 ± 0.33 26.54 ± 0.50 5.62 ± 0.67	 6.83 ± 0.33 [a, ***] 16.05 ± 0.17 [a, ***] 4.00 ± 0.50 [a, ***]	 11.52 ± 0.67 24.72 ± 0.83 7.27 ± 0.17	 7.30 ± 0.01 [b, ***] 23.12 ± 0.50 [b, ***] 6.29 ± 0.01 [b, ***]
Non-enzymatic antioxidant (Plasma) GSH	7.29 ± 0.83	11.59 ± 0.50 [a, ***]	7.48 ± 0.63	16.21 ± 0.67 [b, ***]
Non-enzymatic antioxidant (Heart) GSH	11.18 ± 0.01	9.47 ± 0.83 [a, ***]	14.52± 0.17	12.76 ± 0.01 [b, ***]

Enzyme activities are expressed as follows SOD: U/mg protein (1 U = amount of enzyme that inhibits the autoxidation reaction by 50 %); Catalase: µmol of H_2O_2 consumed/min/mg protein. Non-enzymatic antioxidant is expressed as: GSH: µg/mg protein. GPx: µg of reduced glutathione utilized/min/mg protein. Comparisons between groups are as in Table 1.
The symbols represent statistical singnicance: *** P <0.05, (DMRT).
[a]Comparisons were made between: group I and groups II, III, IV
[b]Groups II and IV

Table 3. Effect of GAG on the levels of TBARS in plasma and the heart in normal isoproternol (ISO)-induced myocardial infarction (MI) in rats

Groups	Plasma TBARS (nM/ml)	Heart TBARS (mM/100 g wet tissue)
Control	6.80 ± 0.50	2.33 ± 0.01
Group II	15.43 ± 0.67[a]	3.93 ± 0.50[a]
Group III	6.03 ± 0.17	1.79 ± 0.50
Group IV	8.22 ± 0.01[b]	2.51 ± 0.33[b]

Each value is mean ± S.D. for six rats in each group. Values not sharing a common letter differ significantly at $P < 0.05$ (DMRT)
[a]Comparisons were made between: group I and groups II, III, IV
[b]Groups II and IV

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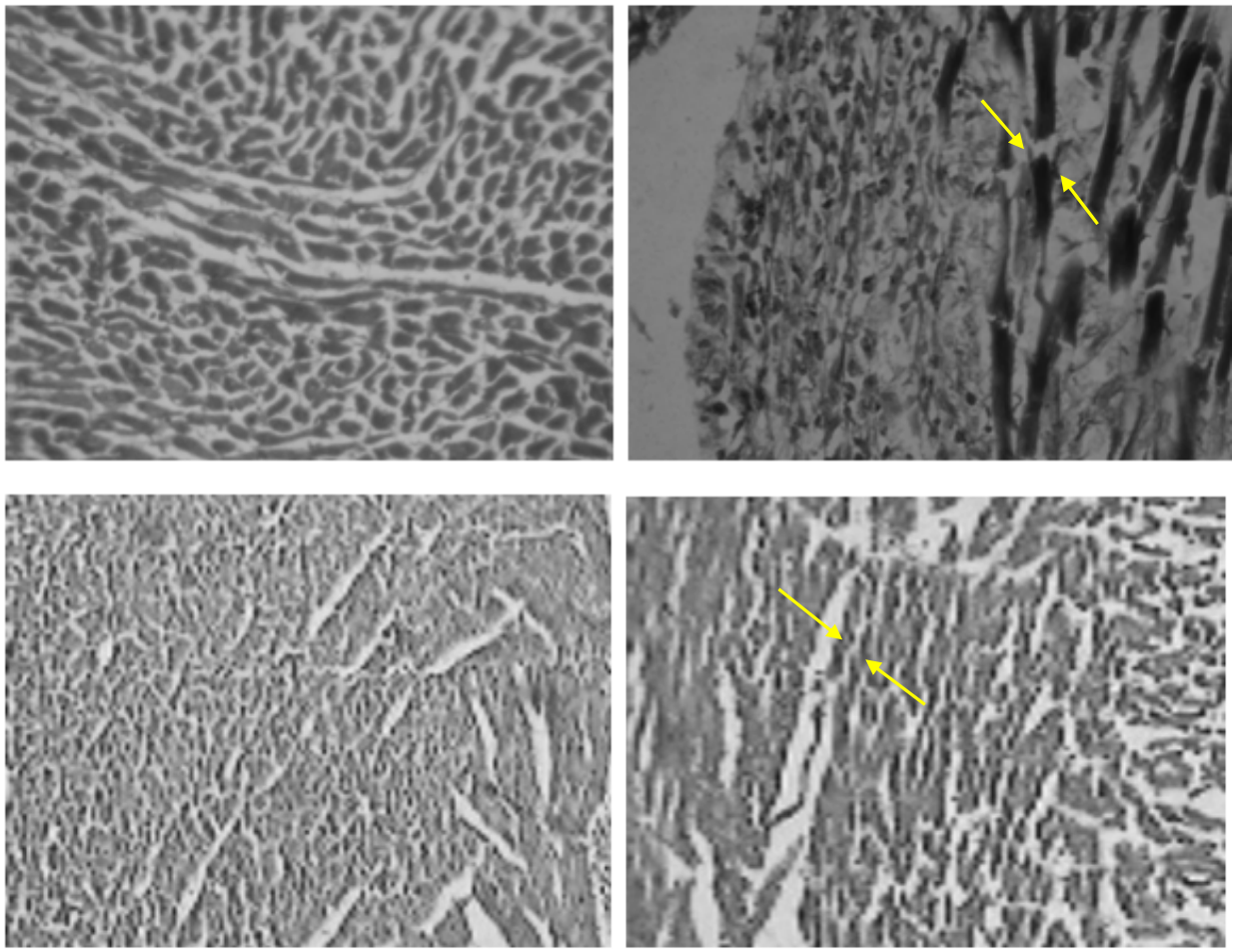

Figure.1. Histological evaluation of the cardiac tissue corresponding to untreated ISO group and molluscan GAG treated groups (A) normal untreated group showing normal cardiac fibres without any infarction oedema and inflammatory cells, (B) ISO alone treated cardiac muscle fibre swelling, hypertrophy and fibre destruction seen (→) in group II, (C) Molluscan GAG treated heart tissue showing normal cardiac fibres without any infarction and inflammatory cells, (D) minimal histophalogical changes observed (←) in the molluscan GAG treated group.