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Preventive Effect of Low Molecular Weight Glycosaminoglycan from *Amussium pleuronectus* (Linne) on Oxidative Injury and Cellular Abnormalities in Isoproterenol-Induced Cardiotoxicity in Wistar Rats

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Abstract The present work explores suspicious consequence of low molecular weight glycosaminoglycan (LMW-GAG) on oxidative stress and cellular abnormalities in isoproterenol (ISO)-induced myocardial infarction in an experimental model. Group-III male Wistar rats (140±10 g) were administrated by ISO (85 mg ISO/ml subcutaneously (SC) injected at the last two days of a 2–week period). Group-IV rats were treated LMW-GAG plus ISO (300 μg/day per rat SC for 1 week followed by 85 mg/kg ISO on the end last two days of the 2 - weeks). Untreated control (Group-I) and LMW-GAG drug control (Group-II) were also included. Serum and tissue lactate dehydrogenase, aminotransferases, and creatine kinase activities were increased in ISO group, which were normalized by LMW-GAG pretreatment rats. Antioxidant enzymes – superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) activities and non-enzymatic enzyme reduced glutathione (GSH) were decreased in the ISO induced rats, and this was increased by LMW-GAG pretreatment. Increased level of thiobarbituric acid reactive substances (TBARS) in plasma and the heart of ISO treated rats; pre s.c. injected with LMW-GAG to ISO-induced rats decreased the levels of TBARS. Histological examination revealed that the ISO-induced deleterious changes in the heart tissues were offset by LMW-GAG treatment. LMW-GAG affords considerable protection to the tissues challenged by cardiotoxicity, evidenced by its correction and restoration of serum and tissue indices of injury, to normalcy.

Keywords LMW-GAG · Isoproterenol · Myocardial infarction · Lipid peroxidation · Antioxidants

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Introduction

Heparin and low molecular weight heparins (LMWH) are glycosaminoglycans (GAG) consisting of chains of alternating residues of D-glucosamine and uronic acid, either gluconic acid or iduronic acid [1]. A sulfated GAG, heparin is a well-known anticoagulant used to prevent or limit clotting and thrombus formation in cardiovascular diagnostic and surgical procedures, as with -dwelling venous catheters, cardiopulmonary catheters, surgery of the heart and vessels, metal and plastic prostheses, and extracorporeal circulation of artificial organs and transplants [2].

Myocardial infarction (MI) is the most lethal manifestation of cardiovascular disease and has been the object of intense investigation by clinicians and basic medical scientists [3]. ISO is a synthetic β-adrenergic agonist that causes severe stress in the myocardium and necrosis in the heart muscle. ISO-induced myocardial necrosis showed membrane permeability alterations, which bring about the loss of function and integrity of myocardial membranes [4]. Heparin is also used in the treatment of unstable angina and acute myocardial infarction and in some patients with disseminated intravascular coagulation [5].

Proteoglycans are macromolecules characterized by a core protein and one or more attached GAG. They have been demonstrated to function as cell surface receptors, as ligands for growth factors and cytokines, and as signals for cell growth, migration, and differentiation [6]. A better understanding of the processes involved in myocardial injury has stimulated the search for new drugs, which could limit the myocardial damage. We have already isolated the LMW-GAG from *Amussium pleuronectus* [7]. No prior study examined the cardioprotective effect of LMW-GAG, which is extracted from marine bivalve molluscs *A. pleuronectus* on ISO-induced myocardial injury in rats. This communication evaluates the possible usefulness of LMW-GAG in ameliorating ISO-induced cardiotoxicities.

Methods 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×34 cm×20 cm) lined with husk renewed every 24 h under a 12-h light–12-h dark cycle at around 22 °C 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 fiber, 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 March 2007).

Drugs and Chemicals

Isoproterenol hydrochloride, (Sigma Chemical Company, St. Louis, MO, USA) and the extracts of LMW-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 [8].

Experimental Design

The animals were grouped into six rats in each group: Group I, normal control rats; Group II, drug control group. LMW-GAG was administrated subcutaneously at a dosage of 300 μg/day per rat for 1-week treated rats; Group III, ISO-treated rats; Group IV, rats were administrated LMW-GAG subcutaneously at a dosage of 300 μg/day per rat for 1 week [1] and administrated a double dose (85 mg/kg) ISO injection at the last two days of experimental period. All the rats were anesthetized with sodium pentobarbital (35 mg/kg, i.p.) and killed by cervical decapitation. Serum samples were prepared for enzyme 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 histopathologic processing.

Enzymatic Indices of Cellular Damage

Creatine kinase (CK) was assayed in the serum and heart tissue by the method of Okinaka et al. [9], activity of lactate dehydrogenase (LDH) by the method King [10], aminotransferases-aspartate and alanine transaminases, AST and ALT, respectively, were determined and expressed in terms of micromoles of pyruvate liberated per minute per milligram of protein at 37 °C [10]. Protein estimations were carried out according to the method of Lowry et al. [11].

Assessment of Oxidative Stress in the Cardiac Tissues

Lipid peroxidation in the heart tissue was determined by the method of Hogberg et al. [12], where malondialdehyde (MDA) produced during peroxidation of lipids, served as an index of lipid peroxidation; MDA reacts with thiobarbituric acid to generate a colored product which absorbs at 532 nm. The peroxidation system contained 10 mM ferrous sulfate and 0.2 mM ascorbate as inducers [13]. The degree of inhibition of the autoxidation of pyrogallol at an alkaline pH by SOD was used as a measure of the total enzyme activity [14]. GPx activity was assessed in terms of utilization of glutathione [15]. 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, [16], 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. [17].

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 tissue was 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

A single high dose injection of ISO (85 mg/kg) induced biochemical changes as well as oxidative damage in the heart tissues. The assessment of compromised cellular integrity in the ISO group was made by determining the activities of CK, LDH, AST, and ALT in serum and tissues (Table 1). These parameters showed a significant increase in ISO group with respect to control (P<0.05). LMW-GAG treatment of ISO-induced rats resulted in near normal activities of these enzymes. The dosage schedule of LMW-GAG followed in the present study was not toxic to heart; the activities of enzymes in the LMW-GAG control (Group II) did not reveal any significant changes when compared with the normal control group. Groups I and II present normal cardiac histology [Fig. 1(a) and (b), respectively]. Figure 1(c) reveals cardiac muscle fiber destruction and hypertrophy. In the photomicrograph, the swollen and flabby muscle fibers of the ISO-induced group can be clearly observed. LMW-GAG plus ISO treatment [Fig. 1(d)], wherein the changes are less marked and an almost normal cardiac muscle fibre picture is presented.

The activities of SOD, catalase and GPx in heart of normal and experimental rats are depicted in Table 2. Rats induced with ISO, exhibited a significant (P<0.05) decrease in the activities of these antioxidant enzymes in the heart on comparison with normal control rats. Pretreatment with LMW-GAG (300 μg/day) to ISO-induced rats significantly (P<0.05) increased the activities of these 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 LMW-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 LMW-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 [Fig. 1(a)–(d)].

Table 3 shows the levels of TBARS in plasma and the heart of normal and experimental rats. Rats induced with ISO, showed a significant (P<0.05) increase in the levels of

Table 1 Changes in serum and tissue enzyme activities in the early phase ISO-induced cardiotoxicity and effect of LMW-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; LMW-GAG	Group III; ISO	Group IV; LMW-GAG+ISO
Serum				
CK	187.01±0.05	183.84±0.83	337.92±0.67 [a]	225.92±0.67 [b]
LDH	103.33±0.83	99.64±0.01	196.43±0.83 [a]	122.48±0.33 [b]
AST	28.43±0.01	27.02±0.83	57.86±0.50 [a]	35.35±0.33 [b]
ALT	16.23±0.17	14.92±0.50	39.44±0.83 [a]	21.83±0.33 [b]
Heart				
CK	15.62±0.67	15.93±0.50	8.35±0.33 [a]	10.51±0.67 [b]
LDH	85.79±0.50	87.94±0.17	50.82±0.01 [a]	78.41±0.83 [b]
AST	38.61±0.50	39.53±0.33	20.32±0.83 [a]	33.55±0.01 [b]
ALT	13.61±0.17	13.81±0.67	8.35±0.33 [a]	12.12±0.01 [b]

Enzyme units: CK: μmoles$\times 10^{-3}$ of phosphorus liberated/min; LDH: μmoles$\times 10^{-1}$ of pyruvate liberated/min; AST, ALT: μmoles$\times 10^{-2}$ of pyruvate liberated/min.

The symbols represent statistical singnicance: $P<0.05$, (DMRT).

[a,b] Comparisons were made between Groups III and IV

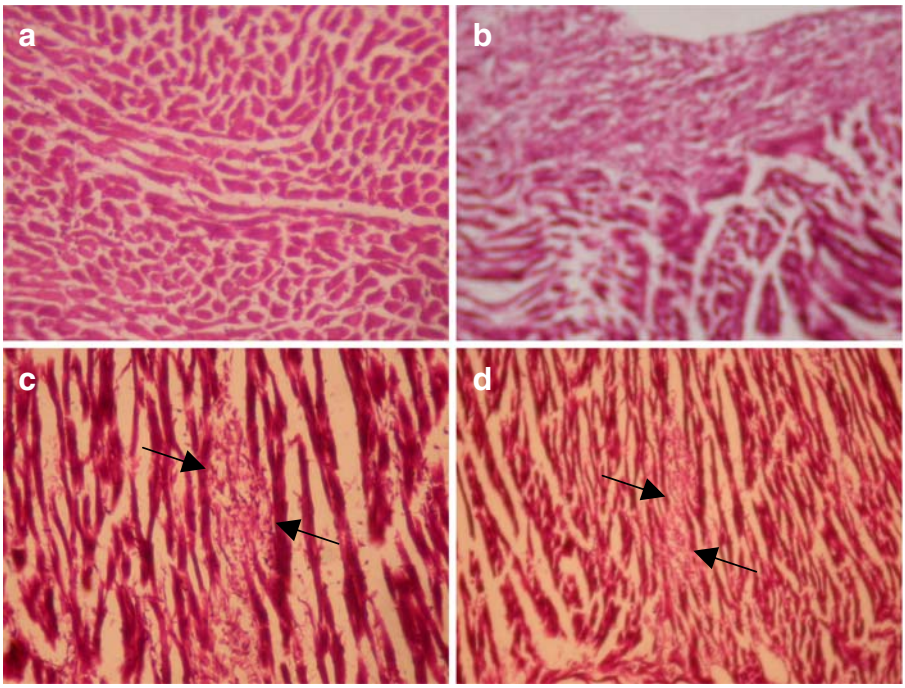

Fig. 1 Histological evaluation of the cardiac tissue corresponding to untreated ISO group and LMW-GAG treated groups (a) normal untreated group showing normal cardiac fibers without any infarction oedema and inflammatory cells; (b) LMW-GAG treated heart tissue showing normal cardiac fibers without any infarction, oedema and inflammatory cells; (c) ISO alone treated myocardium showing infracted zone with (➞) oedema and inflammatory cells in the infracted area and separation of muscle fibers; (d) minimal histophalogical changes observed (➞) in the LMW-GAG treated group

Table 2 Estimation of antioxidant status in the ISO-induced and LMW-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; LMW-GAG	Group III; ISO	Group IV; LMW-GAG + ISO
Enzymatic antioxidant				
SOD	8.37±0.01	8.57±0.33	3.11±0.83 [a]	6.71±0.01 [b]
Catalase	25.40±0.50	26.02±0.01	12.17±0.50 [a]	22.33±0.33 [b]
GPx	8.16±0.83	8.47±0.17	3.82±0.33 [a]	7.01±0.17 [b]
Non-enzymatic antioxidant (Plasma) GSH	8.57±0.17	9.36±0.33	10.16±0.33 [a]	16.52±0.67 [b]
Non-enzymatic antioxidant (Heart) GSH	12.60±0.33	12.90±0.83	7.24±0.67 [a]	10.81±0.83 [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,b] Comparisons were made between Groups III and IV

TBARS in plasma and the heart when compared to normal rats. Pre s.c. injected (group II) with LMW-GAG (300 μg/day) to ISO-induced rats for 1-week significantly ($P<0.05$) decreased the levels of TBARS in plasma and the heart when compared with ISO-alone induced rats.

Discussion

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 proposed as a cardiotoxic agent due to its ability to destruct myocardial cells. We have observed a significant elevation in the levels of diagnostic marker enzymes (CK,

Table 3 Effect of LMW-GAG on the levels of thiobarbituric acid reactive substances (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)
Normal control	4.84±0.01	1.26±0.01
LMW-GAG-treated (300 μg/day)	4.65±0.83	1.19±0.01
ISO-treated (85 μg/kg)	11.17±0.01[a]	2.72±0.67[a]
LMW-GAG+ISO-treated	6.21±0.01[b]	1.62±0.01[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,b] Comparisons were made between: Groups III and IV

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 accordance with previous reports [8]. 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 release of cellular enzymes reflects the alterations in plasma membrane integrity and/or permeability as a response to β-adrenergic stimulation. Pretreatment with LMW-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 LMW-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 levels 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 LMW-GAG to ISO-induced rats significantly decreased the levels of TBARS in serum and the heart. In this context Deepa and Varalakshmi [1] have reported that commercially available low molecular weight heparin reduces the levels of lipid peroxidation in adriamycin-induced cardiotoxicity in rats.

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, catalase, 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 catalyzes the destruction of hydrogen peroxide. During MI, 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 ISPH. 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. The observed increase in the hearty weight in ISO-induced rats might be due to the increased water content, oedematous intramuscular space and extensive necrosis of cardiac muscle fibers followed by the invasion of damaged tissues by the inflammatory cells.

LMW-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. LMW-GAG exerts anti-free radical effects and restores the antioxidant balance to normal. The rise in the activities of the primary enzymatic antioxidant defenses-SOD, catalase GPx and GSH in the LMW-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 LMW-GAG enhanced the antioxidant activity of SOD and contribute to the inhibition of free radical mediated tissue injury. Dinwoody and Anselt [18] have reported that heparin alleviates the effects of free radical production, and enhances the in vivo activity of SOD.

LMWH, as its name suggests, is a glycosaminoglycan that is approximately one third the molecular weight of UFH. It is derived from UFH through various depolymerization processes. LMWH binds to antithrombin, which in turn neutralizes factors Xa and IIa. Because LMWH lacks many of the longer chains required for binding to thrombin, it has less ability to neutralize thrombin relative to its ability to neutralize factor Xa. LMWH also has less nonspecific binding to other proteins. There is greater bioavailability with more predictable pharmacokinetics that eliminates the need for monitoring. The smaller size also allows for enhanced subcutaneous absorption when compared with UFH [19]. Low-molecular-weight heparin derivatives (LMWHs) are fragments of commercial grade heparin produced by either chemical or enzymatic depolymerization. LMWHs are potentially more advantageous than heparin due to their reduced risk of bleeding, greater bioavailability at low doses and longer half life [20].

Only bovine lung or porcine intestine tissues are currently used raw materials to prepare commercial, pharmaceutical heparins. But the appearance of bovine spongiform encephalopathy, 'mad cow disease', and its apparent link to the similar prion-based Creutzfeldt–Jakob disease in humans, has limited the use of bovine heparin. Moreover, it is not easy to distinguish bovine and porcine heparins, making it were difficult to ensure the species source of heparin [21]. GAG of marine molluscs have more advantage to use pharmacological activity.

To conclude, the present work projects the protection afforded by LMW-GAG against cardiac damage induced by ISO on the basis of biochemical assessment and oxidative stress management, confirmed by histopathological examination.

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