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The cytoprotective role of a low-molecular-weight heparin fragment studied in an experimental model of glomerulotoxicity

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Abstract

Abnormal glomerular glycosaminoglycan metabolism is involved in the onset of the morphological and functional aberrations of glomerulopathies. In the present study, a heparin derivative, low-molecular-weight heparin, was tested for its ability to afford renoprotection in an established model of experimental glomerulopathy. Two groups of male albino rats of the Wistar strain (140 ± 10 g) received a single intravenous injection of adriamycin (7.5 mg/kg) to induce glomerulopathy, and one of them received low-molecular-weight heparin (Certoparin Sodium, Troparin®; 300 μg/day/rat s.c.) treatment, commencing on day 8, for a week. Urinary protein/creatinine ratio, serum albumin, urea, uric acid and creatinine clearance were evaluated. Renal cell injury was assessed in terms of renal tissue lactate dehydrogenase, aminotransferases (aspartate and alanine transaminases) and alkaline phosphatase activities, as well as renal antioxidant status (superoxide dismutase, catalase and glutathione peroxidase, reduced glutathione, vitamins E and C). The kidney tissue was subjected to histopathologic examination. Low-molecular-weight heparin significantly reduced proteinuria and improved creatinine clearance and serum albumin levels in the rats with glomerulopathy. The significant rise in serum uric acid in the rats with glomerulopathy was reversed by low-molecular-weight heparin. Altered tissue enzyme activities in response to injury, oxidative stress challenged renal antioxidant system and abnormal renal histology were observed in the untreated nephrotic rats, while low-molecular-weight heparin treatment protected the nephrotic rats against these changes. Thus, in this study, low-molecular-weight heparin was evaluated for its role in combating glomerular injury, on the basis of some salient biochemical parameters, oxidative injury indices and histologic picture. The ability of low-molecular-weight heparin to restore glomerular anatamo-functional features in this nephrotoxic condition illuminates its multi-faceted renoprotective role.

Keywords: Low-molecular-weight heparin; Adriamycin; Glomerulopathy; Glycosaminoglycan; Oxidative stress

1. Introduction

Heparin and low-molecular-weight heparins are glycosaminoglycans consisting of chains of alternating residues of D-glucosamine and a uronic acid, either gluconic acid or iduronic acid. Heparin is a heterogeneous polydispersed mixture of sulfated polysaccharides ranging in molecular weight from 5000 to 30,000 (Johnson and Mulloy, 1979). Low-molecular-weight heparins are fragments of commercial-grade heparin produced by either chemical or enzymatic depolymerization. They are potentially more advantageous than heparin due to their reduced hemorrhagic to antithrombotic ratio, reduced risk of bleeding, greater bioavailability at low doses, longer half-life and more predictable anticoagulant response at fixed doses (Green et al., 1994). Long-term administration of exogenous glycosaminoglycans has been demonstrated to have a favorable effect on renal morphologic and functional abnormalities and glomerular hemodynamics in diabetic nephropathy (Gambaro et al., 1992). It has been reported that the full expression of adriamycin-induced nephrotic syndrome, involving significant changes in the glomerular epithelial cells with accompanying heavy proteinuria, develops 13 to 15 days after a single intravenous injection of adriamycin (Bertani et al., 1982). Renal morphologic changes and proteinuria are the consequences of a common primary event, the loss of glomerular fixed negative charges, which is comparable

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with aminonucleoside nephrosis (Michael et al., 1970). Exogenous glycosaminoglycan administration increases the negative electrical potential of the vessel wall (Hiebert and Jaques, 1986). Whether glycosaminoglycan therapy in the form of low-molecular-weight heparin exerts a favorable influence on the kidney, both at the cellular as well as the functional level, was investigated in the present study.

2. Materials and methods

2.1. Experimental protocol

Male albino Wistar rats (140 ± 10 g) were divided into four groups, each consisting of six animals. Group I served as the control. In Group II, rats were administered adriamycin (7.5 mg/kg) as a single injection intravenously through the tail vein. Group III rats received low-molecular-weight heparin alone for 7 days, while Group IV comprised of rats given the single adriamycin injection followed by low-molecular-weight heparin treatment commencing 1 week later. Low-molecular-weight heparin (Certoparin Sodium, Troparin®; average molecular weight of 4200–6200) was administered subcutaneously at a dosage of 300 μg/day/rat for 7 days.

The rats were fed on a normal diet and water was available ad libitum. The animals were placed in metabolic cages to obtain 24-h urine samples prior to death by decapitation on day 15 of the experimental period. Blood samples were collected for urea, uric acid, creatinine and albumin estimations. The kidneys were excised and a 10% homogenate was prepared for biochemical assays; also a section was set aside for histologic processing.

Experimental animals were handled according to the University and institutional legislation, regulated by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India.

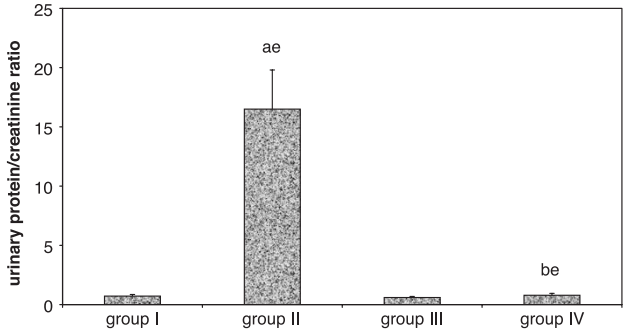

Fig. 1. Urine protein/creatinine ratio in adriamycin-injected and adriamycin/low-molecular-weight heparin-treated rats, along with normal and low-molecular-weight heparin-control groups (Comparisons are made between: [a]Group I and Groups II, III, IV; [b]Group II and Group IV; [e]$P<0.001$).

Table 1
Biochemical evaluation of adriamycin-induced nephrotoxicity and the renoprotective role of low-molecular-weight heparin (compared with control groups)

Blood parameters	Group I	Group II	Group III	Group IV
Urea (mg/dl)	22.15 ± 2.09	43.33 ± 3.95[a,e]	20.79 ± 2.82	26.94 ± 2.28[b,e]
Uric acid (mg/dl)	1.95 ± 0.13	5.80 ± 0.35[a,e]	1.88 ± 0.10	2.20 ± 0.10[b,e]
Creatinine clearance (ml/min)	0.91 ± 0.05	0.26 ± 0.01[a,e]	0.91 ± 0.07	0.87 ± 0.05[b,e]
Albumin (g/dl)	2.94 ± 0.05	2.13 ± 0.12[a,d]	3.20 ± 0.10	2.90 ± 0.19[b,d]

Values are expressed as means ± S.D. for six animals in each group.
Comparisons are made between: [a]Group I and Groups II, III, IV; [b]Group II and Group IV.
The symbols represent statistical significance: [d]$P<0.01$; [e]$P<0.001$.

2.2. Biochemical estimations in blood and urine

Plasma and urinary creatinine levels were estimated (Owen et al., 1954); creatinine clearance was calculated by using a standard formula. Urinary protein excretion is expressed relative to urinary creatinine excretion(urinary protein/creatinine ratio). Serum albumin was assayed by the method of Reinhold (1953). Blood urea (Natelson, 1971) and uric acid (Caraway, 1965) were estimated by standard procedures.

2.3. Renal enzymic indices of cellular integrity

Renal lactate dehydrogenase and aminotransferases (aspartate and alanine transaminases) were assayed and activities expressed in terms of μmol of pyruvate liberated/min/mg of protein at 37 °C; alkaline phosphatase activity in

Table 2
Alterations in renal tissue enzyme activities in the early phase of adriamycin-induced glomerulopathy and the effect of low-molecular-weight heparin treatment, compared with the control groups

Enzyme assays (U/mg protein)	Group I	Group II	Group III	Group IV
Lactate dehydrogenase	9.38 ± 1.03	16.25 ± 1.79[a,e]	9.65 ± 0.97	10.18 ± 1.02[b,e]
Aspartate transaminase	0.12 ± 0.02	0.31 ± 0.05[a,e]	0.12 ± 0.01	0.13 ± 0.01[b,e]
Alanine transaminase	0.26 ± 0.03	0.47 ± 0.05[a,e]	0.27 ± 0.02	0.29 ± 0.04[b,e]
Alkaline phosphatase	1.31 ± 0.15	1.96 ± .20[a,e]	1.30 ± 0.12	1.50 ± 0.08[b,d]

Values are expressed as means ± S.D. for six animals in each group.
Enzyme units: lactate dehydrogenase—μmol × 10^{-1} of pyruvate liberated min^{-1}; aspartate transaminase, alanine transaminase—μmol × 10^{-2} of pyruvate min^{-1}; alkaline phosphatase—μmol × 10^{-2} of phenol min^{-1}.
Comparisons between groups are as in Table 1.
The symbols represent statistical significance: [d]$P<0.01$; [e]$P<0.001$.

Table 3
Lipid peroxidation levels, an index of oxidative stress, in nephrotoxic and low-molecular-weight heparin-treated rats compared with the control groups

Lipid peroxidation	Group I	Group II	Group III	Group IV
Basal	1.60 ± 0.16	3.69 ± 0.37[a,c]	1.60 ± 0.10	1.72 ± 0.17[b,c]
$FeSO_4$-induced	8.94 ± 0.71	12.91 ± 1.23[a,c]	9.15 ± 0.70	9.27 ± 0.88[b,d]
Ascorbate-induced	4.80 ± 0.48	7.62 ± 1.07[a,d]	4.50 ± 0.43	4.78 ± 0.67[b,d]

Values are expressed as means ± S.D. for six animals in each group.
Units: lipid peroxidation—nmol of malondialdehyde formed/mg protein.
Comparisons between groups are as in Table 1.
The symbols represent statistical significance: [d]$P<0.01$; [e]$P<0.001$.

renal tissue was assayed using disodium phenyl phosphate as substrate and expressed as μmol of phenol liberated/min/mg of protein (King, 1965a,b,c).

2.4. Assessment of oxidative stress in the renal tissue

Renal lipid peroxide level was determined by the method of Hogberg et al. (1974), where the release of malondialdehyde served as an index of lipid peroxidation. The peroxidation system contained 10 mM ferrous sulfate and 0.2 mM ascorbate as inducers (Devasagayam, 1986). The degree of inhibition of the autoxidation of pyrogallol at alkaline pH by superoxide dismutase was used as a measure of enzyme activity (Marklund and Marklund, 1974). Glutathione peroxidase activity was assessed in terms of the utilization of glutathione (Rotruck et al., 1973). Catalase activity was assayed by the method of Sinha (1972). Total reduced glutathione was estimated in the renal tissue by the method of Moron et al. (1979). Vitamin E (Baker and Frank, 1951) and ascorbic acid (Omaye et al., 1979) were quantified in the renal tissue using standard protocols.

2.5. Histopathologic studies

A sample of renal tissue collected immediately after death was fixed in 10% formalin. The washed tissue was dehydrated in descending grades of isopropanol and cleared in xylene. The tissue was then embedded in molten paraffin wax. Sections were cut at 5-μm thickness and stained with hematoxylin and eosin. The sections were then viewed under light microscope for histopathological changes in the kidney.

2.6. Statistics

The results are expressed as mean values ± S.D. Differences between groups were assessed by one-way analysis of variance (ANOVA), using the SPSS system for Windows.

3. Results

A single high dose of the potent cytotoxic drug, adriamycin, resulted in a severe nephrotic syndrome within 2 weeks. We found a marked increase in urinary protein excretion with a concomitant fall in urinary creatinine level. This pattern is expressed as a ratio, thereby correcting for abnormalities in the glomerular filtration rate. The adriamycin group had a urinary protein/creatinine ratio of 16.50 ± 3.30 compared with the control values of 0.72 ± 0.11 (Fig. 1). The low-molecular-weight heparin-treated adriamycin-injected group had values within the normal range (0.79 ± 0.16). A two-fold and three-fold increase in serum levels of urea and uric acid, respectively, and a reduction in creatinine clearance by 71.43% mark the severity of the glomerular disease in the adriamycin group (Table 1). These values were restored to normalcy by low-molecular-weight heparin treatment ($P<0.001$). Hypoalbuminemia (adriamycin vs. control, a 27.55% de-

Table 4
Assessment of antioxidant status in the adriamycin-injected and adriamycin/low-molecular-weight heparin-treated groups compared with the controls

Renal antioxidants	Group I	Group II	Group III	Group IV
Enzymic antioxidants				
Superoxide dismutase	4.90 ± 0.49	3.71 ± 0.41[a,d]	4.95 ± 0.40	4.82 ± 0.36[b,c]
Catalase	153.85 ± 15.4	93.29 ± 7.46[a,c]	156.22 ± 17.18	137.00 ± 15.22[b,d]
Glutathione peroxidase	15.71 ± 1.75	21.84 ± 2.62[a,d]	15.87 ± 1.75	16.21 ± 0.90[b,d]
Non-enzymic antioxidants				
GSH	13.09 ± 1.18	10.49 ± 1.30[a,c]	13.10 ± 1.18	13.23 ± 1.30[b,c]
Ascorbate	1.50 ± 0.12	1.06 ± 0.08[a,d]	1.51 ± 0.12	1.27 ± 0.11[b,c]
α-Tocopherol	0.57 ± 0.06	0.39 ± 0.03[a,d]	0.60 ± 0.05	0.66 ± 0.07[b,e]

Values are expressed as means ± S.D. for six animals in each group.
Enzyme activities are expressed as follows: superoxide dismutase, units (mg protein)$^{-1}$ (1 unit = amount of enzyme that inhibits the autoxidation reaction by 50%); catalase, μmol of H_2O_2 consumed min^{-1} (mg protein)$^{-1}$; glutathione peroxidase, μg of reduced glutathione utilised min^{-1} (mg protein)$^{-1}$.
Non-enzymic antioxidants are expressed as: GSH and ascorbate, μg/mg protein; α-tocopherol, mg/g tissue.
Comparisons between groups are as in Table 1.
The symbols represent statistical significance: [c]$P<0.05$; [d]$P<0.01$; [e]$P<0.001$.

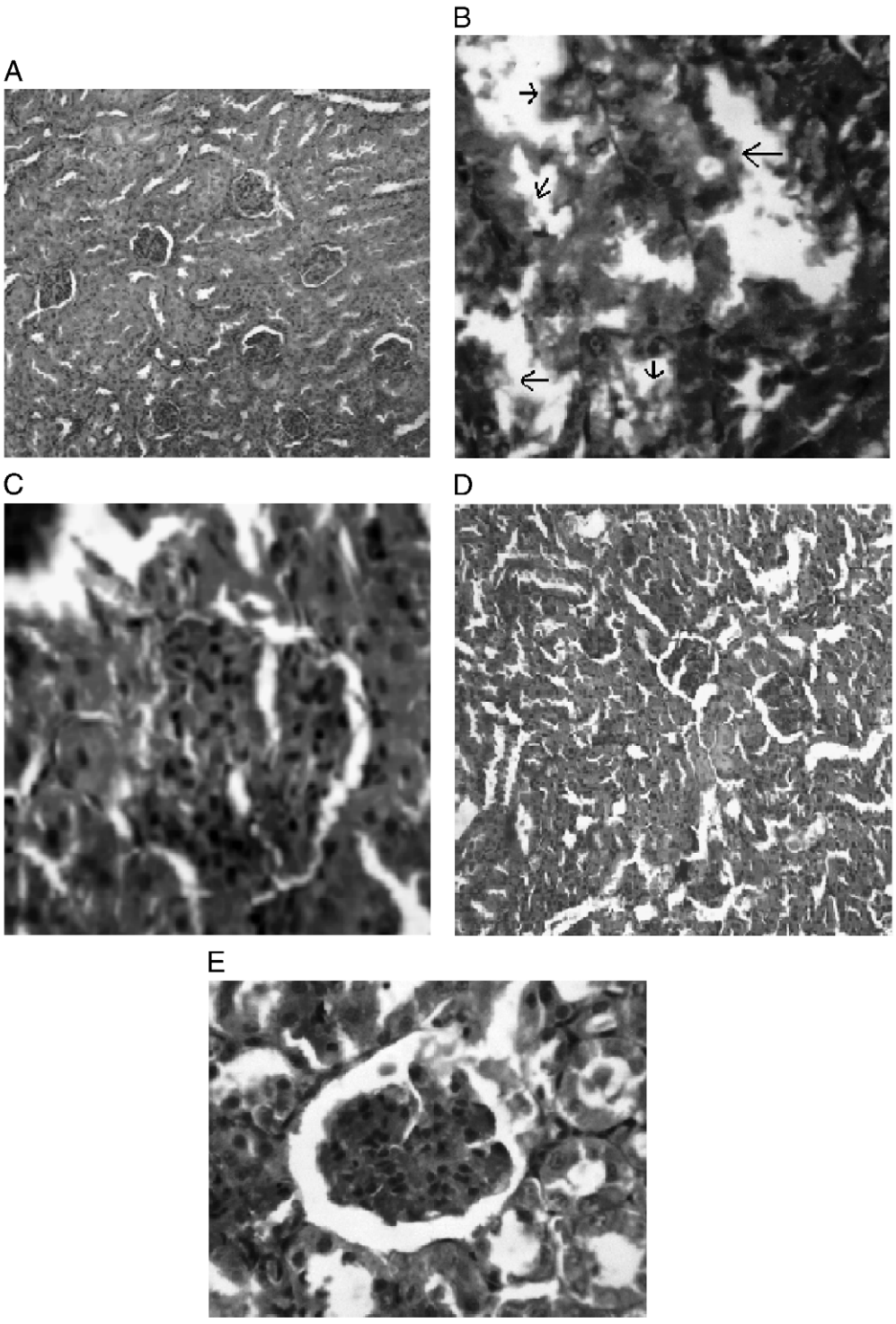

Fig. 2. Histological findings in renal tissue from the four experimental groups. (A) Group I—normal renal architecture(H&E, × 100); (B) Group II—adriamycin-induced tubular damage manifested as epithelial cell detachment from the basement membrane (indicated by arrows, H&E, × 400); (C) Group II—adriamycin-induced glomerular lesion (H&E, × 400)-glomeruli with mesangial cellular proliferation with subsequent reduction of the glomerular Bowman's space; (D) Group III—low-molecular-weight heparin control shows normal renal picture as in group I (H&E, × 100); (E) Group IV—low-molecular-weight heparin treatment of the adriamycin-injected rats improved the renal histology (H&E, × 400). Normal glomeruli with no obliteration of glomerular Bowman's space and absence of glomerular cellular proliferation. Notable absence of tubular damage.

crease in serum albumin level) in the proteinuric rats was not observed in the low-molecular-weight heparin/adriamycin group.

There was a significant rise in tissue enzyme activities in the adriamycin group ($P < 0.001$) as compared with the control; low-molecular-weight heparin treatment reversed this increase to nearly normal values (Table 2).

Renal lactate dehydrogenase, aspartate transaminase, alanine transaminase and alkaline phosphatase activities were increased by 1.73-, 2.6-, 1.81- and 1.5-fold, respectively, in the nephrotoxic condition ($P < 0.001$).

Renal lipid peroxidation was increased 2.31-fold in the adriamycin group compared with the control. The low-molecular-weight heparin treatment brought the level of peroxidation down by 53.4%, which was close to control values (Table 3). Table 4 shows the antioxidant status of the adriamycin group and the low-molecular-weight heparin-treated group. They were compared with the control values and statistically expressed at $P < 0.05$, $P < 0.01$ and $P < 0.001$. There was a significant decrease in the antioxidants superoxide dismutase, catalase, GSH, vitamin C (ascorbic acid) and vitamin E (α-tocopherol), and an increase in glutathione peroxidase activity in the adriamycin group. All these abnormalities were normalized to near control values in the low-molecular-weight heparin/adriamycin group.

Histopathologic changes have been presented in Fig. 2B and C. In Fig. 2B, the tubulorrhexis lesion is highlighted, showing the damaged tubular epithelial cells becoming detached from the basement membrane. Fig. 2C shows glomeruli with proliferation of mesangial cells, resulting in a reduction of the Bowman's space. The photomicrograph of renal tissue from the low-molecular-weight heparin/adriamycin rats shows near normal morphology (Fig. 2E). The absence of tubulorrhexis indicates the protection rendered by the heparin derivative against tubular damage induced by adriamycin. There were normal glomeruli with no obliteration of glomerular Bowman's space, indicating no glomerular cellular proliferation in the low-molecular-weight heparin/adriamycin group. A higher magnification was chosen to appreciate the histologic changes in the adriamycin group and their minimization in the low-molecular-weight heparin-treated group. The normal histologic findings in the control as well as low-molecular-weight heparin alone groups are also presented (Fig. 2A and D).

4. Discussion

A single intravenous injection of 7.5 mg/kg adriamycin leads to the total absence of polyanions after 13 days (Bertani et al., 1982). The same drug dosage in a separate study caused a few foci of mononuclear inflammatory infiltrates in the renal cortex after 2 weeks that eventually intensified along with interstitial fibrosis and tubular atrophy, culminating in pronounced tubulointerstitial nephropathy, a significant lesion that mediates renal damage in adriamycin glomerulopathy (Guezmes et al., 1992). The consequences of these renal perturbations are manifested as extensive biochemical derangements at the cellular level, as reflected in the results of our study.

Elevated proteinuria, observed by us and others, can be viewed as a preliminary yet significant indicator of glomerular lesion initiation. It has been suggested that the polyanionic changes associated with the loss of sialic acid, by reducing the electrostatic barrier of glomeruli, are responsible for the enhanced filtration of circulating polyanions, such as albumin, which then penetrate the urinary space (Blau and Michael, 1972), leading to ultrastructural changes in the glomeruli and to the onset of proteinuria in the adriamycin-injected rats. Treatment of adriamycin-injected rats with the heparin derivative tested in this study decreased proteinuria. This effect may be attributed to the normalization of the electronegative glomerular charges (Chen et al., 1992).

The albumin component of plasma proteins was markedly decreased in the rats with nephropathy, in keeping with earlier reports (Bertani et al., 1982). The decrease in serum albumin might be attributed to its increased urinary excretion due to the glomerular barrier abnormality and to the increased permeability of vascular tissue to albumin as a result of increased levels of cAMP induced by inflammatory mediators (Kahn et al., 1976). The increase in albumin levels induced by low-molecular-weight heparin may therefore be due to restoration of normal glomerular function and to a decrease in albumin extravasation in response to inflammation.

The significant increase in serum uric acid level seen in the adriamycin group serves as a prognostic marker for renal deterioration. Serum uric acid is reported to increase in proportion to the decrease in creatinine clearance and correlates with urinary protein excretion and the degree of renal tubulo-interstitial damage (Ohno et al., 2001).

Creatinine clearance is an index of the glomerular filtration rate, which in turn reflects the physiologic functioning of the glomeruli. As the toxic effect of adriamycin is directly exerted on the glomerular barrier, an agent like low-molecular-weight heparin, which restores the electronegative glomerular charge, thereby normalizing one of the fundamental cytotoxic changes, can reverse the abnormalities caused by the initial glomerular damage. Glycosaminoglycan administration has been shown to slow down progression to uremia in rats with subtotal renal ablation (Purkerson et al., 1988). We found that low-molecular-weight heparin reversed the increase in serum urea levels induced by adriamycin.

Levels of enzyme markers of cytotoxic damage with concomitant inflammation, such as renal lactate dehydrogenase, aspartate transaminase, alanine transaminase and alkaline phosphatase, increased sharply in the adriamycin group. Increases in tissue enzyme activities have been reported in experimental inflammatory conditions (Naik and Sheth, 1978). Phosphatase activity on the endothelial cell surface is responsible, in part, for the conversion of adenosine nucleotides to adenosine, a potent vasodilator

and anti-inflammatory mediator that can protect tissues from the ischemic damage that results from injury. Therefore, following injury, accumulation of interleukin-6 can lead to the production by alkaline phosphatase of adenosine and to subsequent protection from ischemic injury (Gallo et al., 1997). This may account for the induction of alkaline phosphatase activity in our adriamycin-induced inflammation model.

In the present study, low-molecular-weight heparin treatment of adriamycin-injected rats afforded considerable protection of cellular integrity, as is evident from the near-normal activity of lactate dehydrogenase, aminotransferases and alkaline phosphatase. The findings of this study further substantiate the positive influence exerted by low-molecular-weight heparin in curtailing the inflammatory sequelae (Benchetrit et al., 2001; Nelson et al., 1993) that disturb the normal cellular enzyme machinery.

Adriamycin glomerulopathy is characterized by pronounced oxidative stress with increased production of reactive oxygen species and lipid peroxidation. Oxidative stress may upregulate or downregulate the antioxidant defense system. On the one hand, an increase in serum uric acid has negative implications of renal function as discussed above, while on the other hand, being an antioxidant molecule, an increase in its level may boost the antioxidant defense in reactive oxygen species-challenged nephrotoxic rats. However, the levels of other free radical scavenger molecules, ascorbic acid, α-tocopherol and reduced glutathione, were decreased in the rats with glomerulopathy. The adriamycin group also had lower levels of the antioxidant enzymes superoxide dismutase and catalase and enhanced glutathione peroxidase activity. These observations can be attributed to the stage of oxidant injury in which early cellular adaptive tactics to control the oxidative stress, by upregulating the antioxidant defense system, are gradually replaced by the toxicity-induced inhibition of the innate cellular defense mechanism, leading to its eventual collapse. Catalase is more vulnerable than superoxide dismutase and glutathione peroxidase in renal disease, hence, the tissues may be expected to rely longer on superoxide dismutase and glutathione peroxidase for their defense against oxidant injury in its early stage, since their activity is better preserved (Van den Branden et al., 2000). The diminished activities of superoxide dismutase and catalase in the adriamycin group indicate the progressive generation of reactive oxygen species that serve as substrate for the enzyme glutathione peroxidase, thereby enhancing its activity. Excess glutathione peroxidase mediated utilization of glutathione (reduced form) resulted in tissue GSH depletion in the adriamycin group. Moreover, GSH may be partly involved in the reduction of the oxidized forms of ascorbic acid (Bigley et al., 1981) in the tocopherol regenerating system that consists of ascorbic acid, which is converted to the semidehydroascorbic acid radical and then to dehydroascorbic acid.

Low-molecular-weight heparin administration to the adriamycin-injected rats prevented the build up of oxidative stress, as can be seen by the low levels of tissue lipid peroxides. This in turn prevents the depletion of antioxidant molecules, namely vitamins C and E and GSH. The decrease in oxidative stress in the low-molecular-weight heparin/adriamycin rats is reflected by the return to normal levels of glutathione peroxidase activity, which was enhanced in the reactive oxygen species-challenged adriamycin group. The inhibition of free radical effects and favorable modulation of antioxidant enzymes by heparin have been summarized in an enlightening review by Engelberg (1996). In the present investigation, the levels of superoxide dismutase and catalase, which were decreased by adriamycin-induced oxidative injury, increased to normal levels after low-molecular-weight heparin treatment.

The onset of proteinuria coincided with epithelial cell detachment from the glomerular basement membrane. Sialic acid, the main polyanion responsible for establishing the charge barrier properties (Venkatachalam and Rennke, 1978), has been shown to be important for the maintenance of normal foot process and slit architecture (Andrews, 1979). In the present study, photomicrographs of the renal tissue revealed glomerular and tubular damage induced by adriamycin. Low-molecular-weight heparin treatment preserved the renal structure, as evidenced by the normal glomerular picture and notable absence of tubulorrhexis. This in turn has a significant bearing on the permselective filtration barrier function of the glomeruli, enabling normal renal functioning.

In conclusion, low-molecular-weight heparin treatment ameliorated the renal cellular and functional abnormalities occurring in the early phase of adriamycin-induced glomerulopathy. The cytoprotective role of low-molecular-weight heparin is supported by its protection against biochemical perturbations, oxidative stress and histological abnormalities.

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