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Beneficial role of sulfated polysaccharides from edible seaweed *Fucus vesiculosus* in experimental hyperoxaluria

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Abstract

Sulfated polysaccharides from marine algae are known to possess numerous properties of pharmacological importance. The present study is an attempt to evaluate the efficacy of the sulfated polysaccharides from edible seaweed, *Fucus vesiculosus* in ameliorating the abnormal biochemical changes in experimental hyperoxaluria. Two groups of male albino rats of Wistar strain (140 ± 20 g) received 0.75% ethylene glycol for 28 days to induce hyperoxaluria, and one of them received sulfated polysaccharides (fucoidan from *F. vesiculosus*, 5 mg/kg b.w. s.c.) treatment, commencing from the 8th day of the experimental period. One group was maintained as a control group and another group served as a drug control, which received only sulfated polysaccharides. Incongruity in the renal tissue enzymes (ALP, β-Glu and γ-GT) were observed during hyperoxaluria along with an increased activity of oxalate metabolizing enzymes like LDH, GAO and XO. These changes were reverted to near normalcy with sulfated polysaccharide administration. Alterations were observed in the activities/levels of tissue enzyme (superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione S-transferase and glucose-6-phosphate dehydrogenase) and non-enzymes (reduced glutathione, ascorbate and γ-tocopherol) antioxidants, along with high malondialdehyde levels in the hyperoxaluric group. However, normalized lipid peroxidation status and antioxidant defences were noticed with sulfated polysaccharide administration. Biochemical discrepancies observed in hyperoxaluria disrupt membrane integrity, favouring a milieu for crystal retention. Administration of sulfated polysaccharides enhanced the antioxidant status, thereby preventing membrane injury and alleviating the microenvironment favourable for stone formation.

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Keywords: Hyperoxaluria, Sulfated polysaccharides, *Fucus vesiculosus*, Lipid peroxidation, Antioxidants.

1. Introduction

Seaweeds have been used as a foodstuff in the Asian diet for centuries and are considered an under-exploited resource (Nisizawa, Noda, Kikuchi, & Watamaba, 1987). They have also proven to be rich sources of structurally diverse bioactive compounds with valuable pharmaceutical and biomedical potential. Seaweeds have been used as thickening and gelling agents in foods. Seaweeds are low in fats but contain vitamins and bioactive compounds, like terpenoids and sulfated polysaccharides, the latter being a potential natural antioxidant not found in land plants (Lahaye & Kaffer, 1997). Sulfated polysaccharides, the naturally occurring glycosaminoglycans, are a class of compounds containing hemi-ester sulphate groups in their sugar residues (Shanmugam & Mody, 2000). Sulfated polysaccharides from brown algae are generally known as fucoids, as they are rich in the sugar, fucose. They are reported to have blood anticoagulant, anti-tumour, anti-mutagenic, anti-complementary, immunomodulating, hypoglycaemic, antiviral, hypolipidemic and anti-inflammatory activities (Shanmugam & Mody, 2000). Exploring the biomedical potential of sea-
weeds has opened a new era of research, resulting in the application of seaweeds, to remodel the treatment regime in various pathologies. Recently, the potential of seaweeds to ameliorate chronic renal failure in rats has also been reported (Zhang, Li, Xu, Niu, & Zhang, 2003).

Hyperoxaluria is considered as one of the major risk factors for idiopathic calcium oxalate stones. Supersaturation, the initial event in the sequence of stone formation is influenced by oxalate rather than calcium (Kok & Khan, 1994). Oxalate, an inert end product of carbon assimilation, is mainly excreted by the kidney. Abnormalities in oxalate metabolism have been suggested as a cause for the pathogenesis of stone disease, as an excessive excretion of oxalate leads to calcium oxalate crystalluria. Calcium oxalate stones have been known to haunt mankind for centuries, despite the development of novel means of treatment, as the basic mechanism of stone formation and the identity of predictors of recurrence are still largely shrouded in uncertainty. Application of exogenous glycosaminoglycans (GAGs) to prevent stone formation and recurrence is considered as a promising prophylactic approach (Cao et al., 1997). Reports have stated a significant difference in GAG excretion between the stone formers and non-stone formers (Cao et al., 1997). Exogenous supplementation of the GAGs, like sodium pentosan polysulfate (SPP), has been effective in the treatment of urolithiasis (Subba, Basak, & Varalakshmi, 1992). In experimental diabetic nephropathy, Gambro, Venturini, and Noonan (1994) demonstrated that the administration of glycosaminoglycans prevents morphological and functional alterations of the kidney and appeared to reverse established diabetic lesions. Synthetic polysaccharides have been studied in our and other laboratories as potential inhibitors of crystal agglomeration and nucleation (Senthil, Subba, Saravanan, & Varalakshmi, 1996). Verkoeien, Romijn, Boeve, De Bruyn, & Schroder (1995) Synthetic polysaccharides, like low molecular weight heparin (LMWH), have been reported to have renoprotective effects (Deepa & Varalakshmi, 2003). The sulfated polysaccharides isolated from marine organisms bear similarity with heparin, possessing less anticoagulant activity and greater pharmacological approach (Shanmugam & Mody, 2000). Hence, the nephroprotective action of heparin derivatives like LMWH and SPP can also be extended to sulfated polysaccharides. Moreover, the potential of the sulfated polysaccharides to ameliorate nephrotic lesions and their accumulation in the kidneys has led us to investigate the beneficial role of sulfated polysaccharides, fucoidan, from the edible seaweed 

2. Materials and methods

2.1 Animal model

Male Wistar albino rats of body weight 140 ± 20 g were purchased from Tamil Nadu Veterinary and Animal Sciences University, Chennai, India. The animals were maintained under standard conditions of humidity, temperature (25 ± 2°C) and light (12 h light/12 h dark). They were fed standard rat pelleted diet (M/s Pranav Agro Industries Ltd., India), under the trade name Amrut rat/mice feed, and had free access to water. The experiments were conducted in accordance with the guidelines of the Institutional Animal Ethics Committee (IAEC).

2.2 Experimental protocol

The rats were randomly divided into four groups consisting of six animals each. Group I rats served as vehicle treated control. Group II received ethylene glycol (EG, 0.75% in drinking water) for 28 days, to induce a chronic low grade hyperoxaluria and generate calcium oxalate deposition in kidneys. Group III rats served as drug controls and were given sulfated polysaccharides, fucoidan from Fucus vesiculosus (Sigma Chemicals, St Louis, MO, USA), 5 mg/kg body weight dissolved in saline and passed through a 0.2 um filter before subcutaneous administration. Group IV rats received ethylene glycol for 28 days and sulfated polysaccharide commencing on day 8 of the experimental period.

At the end of the 28 days, the animals were sacrificed and the liver and kidney were excised, rinsed in ice-cold physiological saline and homogenized in Tris-HCl buffer (0.1 M, pH 7.4) to give a 10% homogenate. Tissue homogenates were suitably processed for the assessment of enzymes, lipid peroxidation and antioxidant status.

2.3 Enzyme indices of cellular damage

Alkaline phosphatase (ALP) was assayed using disodium phenyl phosphate as substrate (King, 1965) γ-Glutamyl transferase (γ-GT) and β-glucuronidase (β-Glu) were measured by the methods of Orlowski and Meister (1965) and Kawai and Anno (1971) with L-γ-glutamyl p-nitroanilide and p-nitrophenyl-β-D-glucuronide as substrates, respectively.

2.4 Assessment of oxalate metabolizing enzymes

Lactate dehydrogenase (LDH) was assayed by the method of Liao and Richardson (1973), using glyoxylate as substrate. Glycolic acid oxidase (GAO) was assayed by the method of Liu and Roels (1970). Xanthine oxidase (XO) was assayed by the method of Fried and Fried (1966).

2.5 Assessment of lipid peroxidation

Lipid peroxidation (LPO) was determined by the procedure of Hogberg, Larson, Kristoferson, and Orrenius (1974). The formation of malondialdehyde (MDA), a thioarbituric acid reactive end product served as the index of LPO. The coloured product formed gave an absorption maximum at 532 nm. The ferrous sulphate and ascorbate-induced LPO system contained 10 mM
ferrous sulphate and 0.2 mM ascorbate as inducers (Devasa-
gagayam, 1986)

2.6 Determination of the activities of enzymic antioxidants

Superoxide dismutase (SOD) was assayed by the method
of Marklund and Marklund (1974). The unit of the
zyme activity is defined as the enzyme required for 50% inhibition of pyrogallol auto-oxidation. The activity of cata-
lyase (CAT) was assayed by the method of Sinha (1972). It is based on the reduction of dichromate to chromic acetate when heated in the presence of hydrogen peroxide, with the formation of perchloric acid as an unstable intermediate. The chromic acetate thus formed is measured colorimetrically at 510 nm Glutathione peroxidase (GPx) was assayed by the method of Rotruck et al (1973), which is based on the reaction between the glutathione remaining after the action of GPx and 5,5'-dithiobis(2-nitrobenzoi acid), to give a compound that absorbs light at 412 nm Glutathione S-transferase (GST) was assayed by the method of Habig, Pabst, and Jakoby (1974) Glutathione reductase (GR), which utilizes NADPH to convert oxidized glutathione (GSSG) to the reduced form (GSH), was assayed by the method of Staal, Visser, and Veeger (1969). The estimation of glucose-6-phosphate dehydroge-
nase (G6PD) was carried out according to the method of Beulter (1983). It is based on the increase in absorbance on addition of the substrate glucose-6-phosphate. Protein estimations were carried out according to the method of Lowry, Rosebrough, Farr, and Randall (1951)

2.7 Estimation of non-enzymic antioxidants

Total reduced glutathione (GSH) was estimated by the method of Moron, Depierre, and Mannervik (1979), where the colour developed was read at 412 nm Ascorbic acid (Vitamin C) was assayed by the method of Omaye, Turn-
bull, and Sauberlich (1979). Ascorbic acid was oxidized by copper to form dehydroascorbic acid and diketoglutaric acid, and was treated with 2,4-dimethylphenylhydrazine to form the derivative of 2-t-butylphenylhydrazine which undergoes rearrangement, to form a product which was measured at 520 nm α-Tocopherol (Vitamin E) was estimated by the method of Desai (1984)

2.8 Data analysis

The results are expressed as mean ± standard deviation (SD). Differences between groups were assessed by one-way ANOVA using the SPSS software package for Windows. Post hoc testing was performed for inter-group comparisons, using the least significance difference (LSD) test. Significance at P-values <0.001, 0.01 and <0.05 have been given respective symbols in the tables.

3. Results

Table 1 delineates the activities of renal enzymes in experimental animals. The activity of ALP, β-Glu and γ-GT were markedly decreased in Group II animals when compared to that of the control animals (P < 0.001). This abnormal enzymic profile was reverted to near normalcy on administration of sulfated polysaccharides (P < 0.001) suggesting the membrane protective effects of the sulfated polysaccharides.

Table 2 shows the abnormal rise in the oxalate metaboliz-
ing enzymes of liver and kidney. The marked increase in the LDH and XO activity in the kidney (P < 0.001) and liver (P < 0.01, P < 0.001) was normalized with sulfated polysaccharide administration. The liver GAO activity which showed an upsurge in hyperoxaluria, was also normalized with sulfated polysaccharide administration.

Table 3 shows the lipid peroxidative damage in kidney, induced by increased concentration of oxalate/calcium oxalate and the protection rendered by the sulfated polysaccharides. LPO was increased by 1.37-, 1.34- and 1.45-fold in basal, ferrous/sulfate and ascorbate-induced conditions, respectively, in hyperoxaluric rat kidneys, when compared with the control animals. Increase in LPO was culminated by the administration of sulfated polysaccharides.

Table 4 presents the altered activities of enzymic antioxidants in the kidney of control and experimental animals. Compared with the control group, SOD, CAT and GPX activities decreased by 23.35%, 31.80% and 34.02%, respectively, in group II rat kidneys. The activities of GST, GR and G6PD were also significantly (P < 0.001) decreased in hyperoxaluric rats. The abnormal alterations associated with hyperoxaluria were effectively prevented (P < 0.001) with sulfated polysaccharides treatment.

Table 1

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Group I control</th>
<th>Group II EG</th>
<th>Group III sulfated polysaccharide</th>
<th>Group IV, EG + sulfated polysaccharide</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALP</td>
<td>1.55 ± 0.17</td>
<td>1.20 ± 0.11*</td>
<td>1.58 ± 0.13</td>
<td>1.55 ± 0.11**</td>
</tr>
<tr>
<td>γ-GT</td>
<td>8.68 ± 0.77</td>
<td>6.18 ± 0.74*</td>
<td>8.33 ± 0.83</td>
<td>8.35 ± 0.78**</td>
</tr>
<tr>
<td>β-Glu</td>
<td>0.62 ± 0.08</td>
<td>0.36 ± 0.01*</td>
<td>0.59 ± 0.05</td>
<td>0.60 ± 0.06**</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD for six animals in each group. Unit ALP nmol of phenol liberated/mg protein, γ-GT nmol x 10 p-nitroaniline liberated/mg protein. β-Glu, nmol of p-nitrophenol liberated/mg protein. Comparisons are made between " group I and group II, III IV," group II and group IV. The symbols represent statistical significance *P < 0.05, **P < 0.01, ***P < 0.001.
Table 2
Effect of sulfated polysaccharides on oxidase metabolizing enzymes of kidney and liver

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Group I control</th>
<th>Group II EG</th>
<th>Group III sulfated polysaccharide</th>
<th>Group IV EG + sulfated polysaccharide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDH</td>
<td>2.13 ± 0.19</td>
<td>3.99 ± 0.35***</td>
<td>1.99 ± 0.27</td>
<td>2.51 ± 0.24***</td>
</tr>
<tr>
<td>XO</td>
<td>0.91 ± 0.084</td>
<td>1.43 ± 0.15***</td>
<td>0.91 ± 0.089</td>
<td>1.36 ± 0.16***</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDH</td>
<td>2.36 ± 0.16</td>
<td>2.72 ± 0.19**</td>
<td>2.41 ± 0.20</td>
<td>2.44 ± 0.20**</td>
</tr>
<tr>
<td>XO</td>
<td>1.34 ± 0.12</td>
<td>2.49 ± 0.20**</td>
<td>1.35 ± 0.15</td>
<td>1.38 ± 0.22**</td>
</tr>
<tr>
<td>GAO</td>
<td>2.80 ± 0.21</td>
<td>5.41 ± 0.46***</td>
<td>2.65 ± 0.23</td>
<td>2.49 ± 0.34***</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD for six animals in each group. LDH, XO units/mg protein (1U = the amount of enzyme that brings about a change in O.D of 0.01 in 1 min) GAO nmol of glyoxylate formed/mg protein. Comparisons are made between *group I and group II III IV group II and group IV. The symbols represent statistical significance *P < 0.05 **P < 0.01 ***P < 0.001

Table 3
Lipid peroxidation levels in the kidney of treated and untreated animals in experimental hyperoxaluria

<table>
<thead>
<tr>
<th>Lipid peroxidation</th>
<th>Group I control</th>
<th>Group II EG</th>
<th>Group III sulfated polysaccharide</th>
<th>Group IV EG + sulfated polysaccharide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>2.07 ± 0.24</td>
<td>2.84 ± 0.32***</td>
<td>1.91 ± 0.24</td>
<td>2.12 ± 0.32***</td>
</tr>
<tr>
<td>Ascorbate-induced</td>
<td>4.23 ± 0.43</td>
<td>6.15 ± 0.62***</td>
<td>4.25 ± 0.49</td>
<td>4.75 ± 0.38***</td>
</tr>
<tr>
<td>Ferric sulfate induced</td>
<td>12.62 ± 1.26</td>
<td>16.85 ± 1.96***</td>
<td>12.96 ± 1.57</td>
<td>13.06 ± 1.62***</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD for six animals in each group. Unit LPO nmol of MDA formed/mg protein. Comparisons are made between *group I and group II III IV b group II and group IV. The symbols represent statistical significance *P < 0.05 **P < 0.01 ***P < 0.001

Table 4
Activities of antioxidant enzymes in hyperoxaluric and sulfated polysaccharides treated rats

<table>
<thead>
<tr>
<th>Antioxidants</th>
<th>Group I control</th>
<th>Group II EG</th>
<th>Group III sulfated polysaccharide</th>
<th>Group IV EG + sulfated polysaccharide</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD</td>
<td>5.44 ± 0.48</td>
<td>4.17 ± 0.42***</td>
<td>5.49 ± 0.51</td>
<td>5.43 ± 0.45***</td>
</tr>
<tr>
<td>CAT</td>
<td>165.20 ± 16.48</td>
<td>112.67 ± 10.29**</td>
<td>167.57 ± 15.27</td>
<td>150.83 ± 16.72**</td>
</tr>
<tr>
<td>GPX</td>
<td>8.2 ± 0.80</td>
<td>5.41 ± 0.58***</td>
<td>8.05 ± 0.82</td>
<td>7.63 ± 0.68***</td>
</tr>
<tr>
<td>GR</td>
<td>1.51 ± 0.19</td>
<td>0.95 ± 0.92***</td>
<td>1.47 ± 0.16</td>
<td>1.36 ± 0.14***</td>
</tr>
<tr>
<td>GST</td>
<td>1.44 ± 0.17</td>
<td>0.87 ± 0.99***</td>
<td>1.51 ± 0.21</td>
<td>1.30 ± 0.24***</td>
</tr>
<tr>
<td>G6PD</td>
<td>1.88 ± 0.13</td>
<td>1.06 ± 0.92***</td>
<td>1.90 ± 0.15</td>
<td>1.64 ± 0.22***</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD for six animals in each group. Units SOD units/mg protein (1U = amount of enzyme required to bring about 50% inhibition of auto oxidation of pyrogallol) CAT μmol of H2O utilized/mg protein GPX μg of GSH utilized/mm/mg protein GR μmol of NADPH oxidized/mm/mg protein GST nmol of 1-chloro-2,4-dinitro benzene GSH conjugate formed/mm/mg protein G6PD nmol of NADPH reduced/mm/mg protein. Comparisons are made between *group I and group II III IV b group II and group IV. The symbols represent statistical significance *P < 0.05 **P < 0.01 ***P < 0.001

Table 5
Effect of sulfated polysaccharides on the non-enzymic antioxidants in the kidney of experimental animals

<table>
<thead>
<tr>
<th>Non-enzymic antioxidants</th>
<th>Group I control</th>
<th>Group II EG</th>
<th>Group III sulfated polysaccharide</th>
<th>Group IV EG + sulfated polysaccharide</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH</td>
<td>4.60 ± 0.56</td>
<td>2.88 ± 0.34****</td>
<td>4.67 ± 0.49</td>
<td>4.62 ± 0.39****</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>2.04 ± 0.24</td>
<td>1.04 ± 0.19***</td>
<td>2.01 ± 0.2</td>
<td>1.97 ± 0.24***</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>1.00 ± 0.11</td>
<td>0.50 ± 0.02****</td>
<td>1.01 ± 0.091</td>
<td>1.06 ± 0.15***</td>
</tr>
</tbody>
</table>

Values are mean ± SD for six rats. Units GSH Vit C and Vit E μg/mg protein. Comparisons are made between *group I and group II III IV b group II and group IV. The symbols represent statistical significance *P < 0.05 **P < 0.01 ***P < 0.001

The values reported in Table 5 shows the effect of the sulfated polysaccharides on the levels of non-enzymatic antioxidants in rat kidney. Significant reduction in the level of non-enzymatic antioxidants (P < 0.001) GSH, vitamin C and vitamin E in hyperoxaluric animals were normalized with sulfated polysaccharide administration.

4. Discussion

In recent years, a broad series of polysaccharides from edible seaweeds have emerged as an important class of bioactive natural products, possessing many important properties of pharmacological relevance (Shanmugam &...
Mody, 2000) Sulfated polysaccharides are widespread in nature, occurring in a great variety of marine organisms. It is well established that the damaged epithelium of the kidney, due to an increased oxidative stress and with reduced anti-adherent glycosaminoglycan layer, might act as a nidus for stone formation (Selvam, 2002). Culminating the production of free radicals or regeneration of the kidney epithelial membrane might be an effective treatment in stone pathogenesis. It is known that sulfated polysaccharides from marine organisms are poorly taken up from the intestine because of their size and electrical charge but it is found that the sulfated polysaccharides, upon systemic administration, accumulated predominantly in the kidney (Guimaraes & Mourao, 1977), suggestive of the possible role of these naturally-occurring glycosaminoglycans in renoprotection.

Abnormal changes in renal enzyme levels can indicate tubular dysfunction and the specific tubular segments that are involved. Calcium oxalate crystals are known to damage proximal tubular epithelium and are generally associated with shedding of the brush border membrane and leakage of enzymes in urine (Thamilselvan & Menon, 2005). ALP present in the brush border of the kidney is implicated in the calcification process and the observed decrease in ALP activity in the present study might be due to the decreased translocation of the enzyme across the epithelial membrane or the leak of the enzyme in general circulation from the collateral circulation, due to the damage of the membranes by calcium oxalate crystals γ-GT, an amphiphatic dimeric protein with the ability to accumulate glutamyl peptides in kidney, was also found to be decreased in hyperoxaluric condition. Altered enzyme activity might implicate a defective transport mechanism as the hydrophobic part of the enzyme spans the membrane and may be directly involved in the transport of cations. These enzymes are localized in the proximal tubule, the part of the nephron where the renal handling of oxalate is maximum (Hautman & Oswald, 1985). Decreased excretion of these enzymes has already been reported in hyperoxaluric state and can be directly correlated with the extent of membrane damage. In the present study, the abnormal decrease in renal enzymes was in line with the increased oxalate metabolizing enzymes. Decrease in β-Gla, another membrane-bound enzyme, is due to the damage to the membrane inflicted by the calcium oxalate crystals. This abnormal enzyme profile was corrected to near normalcy with sulfated polysaccharides administration. The sulfated polysaccharides maintained the integrity of the cell membrane and, consequently, prevented the adhesion of calcium oxalate crystals, as calcium oxalate crystals are known to adhere to damaged epithelium. These observations are in line with our previous findings, where administration of SPP, a heparin analogue, was found to have membrane protective effect in experimental hyperoxaluria (Subha et al., 1992).

Increase in oxalate synthesizing enzymes observed in the present study, might be due to the increased availability of their substrate GAO, a flavoprotein, catalyses the two step oxidation of glycolate to oxalate, with glyoxylate as an intermediate. This enzyme is localized in the liver and its activity is found to be increased during hyperoxaluria (Pragasam, Kaluselvi, Sumitra, Srinivasan, & Varalakshmi, 2005). LDH and XO of kidney and liver were also increased during hyperoxaluria. LDH, a cytosolic enzyme, catalyses the coupling of oxidation and reduction of glyoxylate, resulting in the formation of glycolate and oxalate (Luo & Richardson, 1973). Due to the breaks encountered in the electron transport pathway and the inhibition of oxidative phosphorylation, a high concentration of FADH₂/NADH ratio is easily provided in the hyperoxaluric rats, which favour the increase in GAO and LDH activity (Ichiyama et al., 2000). Apart from increasing the concentration of the oxalate, GAO and XO release hydrogen peroxide and/or superoxide anions as end products. In normal tissues XO exists as a dehydrogenase, utilizing NAD⁺ as coenzyme, instead of oxygen as electron-acceptor. Xanthine dehydrogenase can be converted to XO by oxidation of its critical sulfhydryl groups (Nishino, 1994). Depletion of antioxidants in hyperoxaluric condition favours the formation of XO from xanthine dehydrogenase, further increasing the oxalate load. Increased XO can react with free iron in uricolithic conditions resulting in the generation of hydroxyl radicals (Biemond, Swaak, Beundorff, & Koster, 1986). Oxalate, and the free radicals generated during the metabolism of oxalate, impose an oxidative stress on hyperoxaluric animals, as evident from the increased LPO in the present study. The increase in oxalate metabolizing enzymes was circumvented with administration of sulfated polysaccharide, a potential antioxidant which corroborates well with the previous observation, where administration of antioxidants, vitamin E and selenium was effective in hyperoxaluric condition (Kumar & Selvam, 2003).

LPO, a degenerative pathway of the membrane components mediated through the free radicals produced in the cell, is a hallmark feature of oxidative stress. In the present study, there is an upsurge in LPO in rat kidney during hyperoxaluric condition and this was further increased in the presence of inducers like ascorbate and ferrous sulphate. LPO was found to increase when LLC-PK1 cells were incubated with oxalate and this was further elevated on incubation with calcium oxalate crystals (Thamilselvan, Khan, & Menon, 2003). The kidney, being highly vascular in nature, is more susceptible to the toxic effects of lipid peroxides secondary to erythrocyte membrane LPO (Sumathi, Jayanthi, & Varalakshmi, 1993). The evidence of the involvement of oxalate in free radical-mediated LPO reaction is further strengthened by previous observations (Huang, Ma, Chen, & Chen, 2003; Selvam, 2002). Ascorbic acid, a precursor of oxalate biosynthesis, has been shown to promote LPO in vitro in tissue non-enzymatically. Decrease in antioxidant enzymes in the present study might also be partly attributed to the elevation in the LPO Abnormal rise in LPO was reverted back to near normalcy.
with sulfated polysaccharide administration, due to their antioxidant activity, emphasized through numerous in vitro experiments (Rupererez, 2001; Xue, Yu, Hirata, Terao, & Lin, 1991). This is in line with the previous observation where LMWH supplementation could circumvent the elevated LPO, associated with nephrotoxic condition (Deepa & Varalakshmi, 2003).

Complexes of free radical scavenging enzymes, including SOD, CAT and GPX have evolved to prevent excessive oxidant stress. The development of tissue injury probably depends on the balance of the generation of reactive oxygen species and the enzymes antioxidant defense mechanism. SOD is the primary enzyme involved in the dismutation of the superoxide radical to hydrogen peroxide. CAT and GPX are involved in the splitting of hydrogen peroxide to water and hydrogen. Decrease in the activities of antioxidant enzymes observed in the present study might be due to the increase in free radicals during hyperoxaluria. The free radicals when present in high concentrations are capable of interacting with the enzymes and inactivating them (Pigeon et al., 1990). Direct evidence of decrease in antioxidant enzymes in hyperoxaluria and restoration to normal values on antioxidant therapy has been reported (Thamshelvan & Menon, 2005). Decrease in the activity of CAT might be attributed to direct inhibition of CAT by oxalate and decreased regeneration of CAT from its inactive form, due to lesser availability of NADPH (Kirkman & Gaetani, 1984). Reduction in the activity of GPX might be due to the decreased availability of its substrate GSH and partly due to its inhibition by the superoxide radicals accumulated, due to the decreased activity of SOD. Supplementation with exogenous sulfated polysaccharides was found to increase the activity of the antioxidant enzymes and correlates positively with the observation made with LMWH supplementation on nephrotoxicity (Deepa & Varalakshmi, 2003). Zhang et al. (2003) have reported an increase in antioxidant status in aging mice on supplementation with polysaccharide fraction from Porphyra haitanensis.

Reduced glutathione, an important oxidant defense, functions in the reduction of oxidized tissue components. The observed decrease of GSH in hyperoxaluric animals might be due to its increased conversion to GSSG. Increased oxidative stress increases the formation and efflux of GSSG (Rashed, Menon, & Thamshelvan, 2004). Anundi, Hogberg, and Stead (1979) reported that GSH depletion induces LPO and ultimately cell lysis. Replenishing the GSH levels is, therefore, necessary for the maintenance of the overall thiol status in the cell. GSH reduction can additionally explain a decreased concentration of the non-enzymatic antioxidant vitamin C, which enters the cell mainly in the oxidized form, where it is reduced by GSH (Packer, 1992). The diminution of this vitamin is very detrimental, because additionally to its antioxidant function, vitamin C plays a role in sparing other antioxidants like vitamin E (Packer, 1992). The decrease in the levels of these vitamins increases the risk of LPO. This correlates with previous observations where induction of hyperoxaluria causes a significant decrease in non-enzymic antioxidants (Farooq, Asokan, Sakthivel, Kalaiselvi, & Varalakshmi, 2004).

Decrease in the levels of GSH may be correlated with decreased activities of the glutathione synthesizing enzymes GR and G6PD, both of which are influenced by oxidative stress. G6PD, the first enzyme in the hexose monophosphate shunt, is responsible for the production of the reducing equivalent NADPH, which is used by the cell for GSH synthesis (GSSG). The modification of thiol groups in G6PD during oxidative stress leads to loss of activity, consequently leading to decreased NADPH levels (Yoshida & Huang, 1986). The observed decrease in the activity of the GR might be due to decreased availability of the cofactor NADPH. Reports demonstrate that decrease in glutathione metabolizing enzymes during hyperoxaluria can be normalized with supplementation of an antioxidant (Rashed et al., 2004). GST forms the group of multifunctional proteins catalyzing the detoxification of electrophilic compounds, to protect cells against peroxidaive damage (Liebau, Wildenburg, Walter, & Henkle-Duhrsen, 1994). Diminution in the level of GST may be due to lack of the substrate GSH. Administration of sulfated polysaccharides to hyperoxaluric rats is found to increase the reduced milieu of the cell, thereby preventing oxidative stress mediated renal injury.

To summarize, the exogenous supplementation of sulfated polysaccharides to hyperoxaluric rats is effective in decreasing the oxidative stress, by increasing the activities of antioxidant enzymes like SOD, CAT, GPX, and limiting lipid peroxidation. Furthermore, sulfated polysaccharides were able to prevent crystal retention, by averting the membrane damage induced by the calcium oxalate crystals. It also had an appreciable effect on the levels of non-enzymic antioxidants. Similarity of the sulfated polysaccharides with heparin derivatives can be attributed as the possible reason for the protective effect of the sulfated polysaccharide observed in the present study, as the heparin derivatives like SPP and LMWH have renoprotective effects. Further studies are warranted to explore the mechanisms underlying the protective action of this drug as its pharmacological properties could be of therapeutic use to mankind.

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Physico-chemical alterations of urine in experimental hyperoxaluria: a biochemical approach with fucoidan

Coothan Kandaswamy Veena, Anthony Josephine, Sreenivasan P Preetha and Palaninathan Varalakshmi

Abstract

Urinary supersaturation-induced crystal formation has been attributed as one of the key factor for the pathogenesis/progression of lithogenesis. This study was aimed at investigating whether fucoidan, a naturally occurring sulfated glycosaminoglycan, could ameliorate the biochemical changes in urine induced by stone formation. Two groups of male albino Wistar rats (120 ± 20 g) received 0.75% ethylene glycol (EG) for 28 days to induce hyperoxaluria, and one of them received sulfated polysaccharides (fucoidan from Fucus vesiculosus, 5 mg kg⁻¹ s.c.), commencing from the 8th day of the experimental period. One group was maintained as normal control group and another group served as drug control, which received sulfated polysaccharides. The urine collected from all the groups was analysed for changes in pH, volume, oxalate, calcium, phosphorus, uric acid, magnesium, citric acid and glycosaminoglycans. Urinary crystals were analysed with a light microscope. Renal tissues were studied under polarized light for deposition of crystals and also analysed for their oxalate and calcium content. The changes in extracellular matrix on crystal deposition were also evaluated. The urinary pH and volume were altered in rats treated with EG along with an increase in weight of the kidney. Further, administration of EG to rats increased the supersaturation of urine by escalating the levels of the stone-forming constituents, such as oxalate, calcium, phosphorus and uric acid, which was completely restored by fucoidan treatment. The decrease in the inhibitors, like citrate, magnesium and glycosaminoglycans, in urine was prevented by the co-treatment with fucoidan in hyperoxaluric rats, there was an increased excretion of calcium oxalate monohydrate crystals in urine along with crystal deposition in renal tissues, this was prevented by fucoidan treatment. Fucoidan administration reversed even the tissue levels of calcium and oxalate. The increased accumulation of collagen and expression of transforming growth factor β, in hyperoxaluria was normalized on fucoidan administration. These results suggest that the physico-chemical alterations in urine produced during hyperoxaluria can be reversed by fucoidan administration.

Introduction

Nephrolithiasis is a real problem for public health and its increased prevalence and peak of frequency among men between the third and sixth decade explains its high socio-economic cost (Sowery et al. 1998). The advent of minimally invasive surgical options, such as extracorporeal shockwave lithotripsy and laser lithotripsy, has significantly changed the treatment strategies for urolithiasis. However, a preventive prophylactic programme is essential because besides the high recurrence rate of kidney stones, exposure to shock waves even in therapeutic doses may cause acute renal injury and decrease in renal function (Willis et al. 2006). In general, kidney stone formation is a multifactorial process involving a cascade of events, including supersaturation, nucleation, aggregation, growth and retention of crystals in the renal tubules (Jonassen et al. 2005). Disturbance in the balance between supersaturating and inhibiting factors in urine is considered to be a primary aspect in altering the supersaturation of urine and initiating stone formation. The production of concentrated urine frequently initiates crystal formation in the kidney and persistent inadequate elimination of crystal material with the urine can eventually lead to formation of a stone in the urinary tract (Selvam 2002). Retention of microliths in the urinary tract facilitates further development.
of crystals into stones and reports suggest that alteration in extracellular matrix (ECM) plays a crucial role in stone retention (Khan 2004). Hence, drugs that can alter the excretion of stone-forming constituents/modulators in urine and prevent the disturbance in ECM would be beneficial in the treatment of urolithiasis.

Glycosaminoglycans (GAGs) are recognized as potent inhibitors of urolithiasis through numerous in-vitro and in-vivo experiments (Cao et al. 1997a). GAGs are polysaccharide chains formed from alternating hexosamine and uranyl residues. Except in the urinary tract these polymers are not found as free chains but are constituents of proteoglycans that usually contain one or more GAG chains. As a consequence of proteoglycans degradation, GAGs appear in the urine after being filtered through the glomerulus. Although numerous in-vitro studies have described the inhibitory effect of GAGs on stone formation, controversy exists in published reports that have compared GAG levels in patients and controls (Michelacci et al. 1989, Nikkila 1989). Recent research on these molecules has shown their biphasic nature. GAGs from marine algae, especially fucoxanthin, a sulfated polysaccharide extracted from brown algae are endowed with important properties, including anti-oxidant anti-coagulant antithrombotic, anti-angiogenic and anti-inflammatory activity, which are of potential value (Berteau & Mulloy 2003). Zhang et al. (2003) showed that fucoxanthin could also alleviate chronic renal failure in rats suggesting a possible nephroprotective role for fucoxanthin. Sodium pentosan polysulfate a heparin analogue was able to alter the stone-forming constituents in urolithic rats (Subba & Varalakshmi 1993). Our previous studies have also shown that fucoxanthin was able to modulate the oxidative stress associated with hyperoxaluria (Veena et al. 2007). Therapeutic agents that can modulate the damage in renal tissue as well as alter the urinary biochemical composition would be beneficial in the prognosis of urolithiasis. Hence, this study was initiated to explore the effect of fucoxanthin on urinary stone-forming constituents/modulators.

Materials and Methods

Chemicals

Fucoxanthin from _Fucus vesiculosus_ and BSA were procured from Sigma Chemical Co (St Louis, MO). All other chemicals and reagents used were of analytical grade.

Animal model

Male Wistar albino rats, 120±20 g, were purchased from Tamil Nadu Veterinary and Animal Sciences University, Chennai, India. The rats were maintained under standard conditions of humidity, temperature (25 ± 2°C) and light (12-h light–dark cycle) and were fed with standard rat pellets diet (Amrut rat/mice feed, M/S Pranav Agro Industries, Ltd, India) and allowed free access to water. Experimental rats were handled with humane care according to the guidelines of the institutional animal ethics committee (IAEC).

Experimental setup

The rats were randomly divided into four groups consisting of six rats each as shown in Table 1. In the ethylene glycol (EG) model of hyperoxaluria induction, different concentrations are established (0.5% EG + 25-hydroxy cholesterol, 1% EG, 2% EG, EG + ammonium chloride, etc.). The dose used in this study was 0.75% w/v EG in drinking water for 28 days. The provision of this dose of EG has generated hyperoxaluria in as little as 3 days (Huang et al. 2002) and as long as 60 days (Thamilselvan et al. 1997), with no discernible effect on renal function as judged by creatinine clearance (Huang et al. 2003). At the end of 28 days, the rats were housed in metabolic cages for 24-h urine collection. Urine collected was used for analysis of urinary components and to measure urinary volume, pH and crystalluria. The rats were sacrificed and the kidneys were rinsed in ice-cold physiological saline. A portion of the tissue was fixed in 10% formalin for polarized microscopy of crystals and the remainder was homogenized in Tris-HCl buffer (pH 7.4) to give a 10% homogenate and was suitably processed.

Biochemical analysis of urine and renal tissue

Urine oxalate and tissue oxalate were estimated after acid extraction as described by Hodgkinson & Williams (1972). Urinary uric acid, phosphorus and citrate were estimated by the methods of Caraway (1963), Fiske & Subbarow (1925) and Rajagopal (1984), respectively. Calcium and magnesium measurements were performed by atomic absorption spectrophotometry. For the measurement of GAG it was precipitated with cetylpyridinium chloride and then reacted with dimethylmethane blue to produce a complex with the polyanionic molecule of sulfated GAGs (Panin et al. 1986). Protein was estimated by the method of Lowry et al. (1951).

Urinary crystal study

Twenty-four-hour urine was collected and a drop of it was allowed to spread over a clean glass slide and visualized under a light microscope (ECLIPSE E400, Nikon, Japan).

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
</tr>
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<tbody>
<tr>
<td>I</td>
<td>Vehicle treated control rats</td>
</tr>
<tr>
<td>II</td>
<td>Rats were administered ethylene glycol (0.75% w/v in drinking water) for 28 days</td>
</tr>
<tr>
<td>III</td>
<td>Rats were administered sulfated polysaccharide (Fucus vesiculosus Sigma Chemicals St Louis, MO) 5 mg kg⁻¹ dissolved in saline and passed through a 0.2 μm filter before subcutaneous administration</td>
</tr>
<tr>
<td>IV</td>
<td>Rats were administered ethylene glycol for 28 days and sulfated polysaccharide commencing on day 8 of the experimental period</td>
</tr>
</tbody>
</table>
Polarized microscopic studies

Kidneys harvested from rats were examined for crystals after they were fixed in formalin and embedded in paraffin. Sections (5 μm thick) were stained by eosin solution and examined by polarized light microscopy (Euromex Stereo microscope, Holland).

Evaluation of extracellular matrix (ECM) alterations

Collagen content of renal tissues was estimated by the method of Esteban et al. (2005) using Sirius red. Transforming growth factor-β1 (TGF-β1) expression in renal tissues was assessed using reverse transcriptase-polymerase chain reaction (RT-PCR). To determine the expression of TGF-β1 mRNA in each group, total RNA was isolated from kidney using a total RNA extraction kit (Trizol; Medox Biotech Pvt Ltd, India). The following primer pairs were used: sense: 5'-ACT GAT ACG CCT GAG TGG CTG T-3'; anti-sense: 5’-CTC TGG GGA GCT GAA GTA GAT G-3' (Shi et al. 2004). The expected product size of TGF-β1 mRNA was 303 bp. PCR amplification was carried out with a thermal cycler using a one-step RT-PCR kit (Quagen one step RT-PCR kit, Germany) according to a protocol: initial denaturing at 94°C for 15 min; then 39 cycles at 94°C for 15 s (denaturation), at 60°C for 1 min (annealing) and 72°C for 30 s (extension); and a further extension at 72°C for 5 min. The PCR products were resolved on a 2% agarose gel in Tris-borate-EDTA buffer. Ribosomal protein L19 (RPL-19) was used as an internal standard.

Data analysis

The results are expressed as mean ± standard deviation (s.d.) for six rats in each group. Differences between groups were assessed by one-way analysis of variance using the SPSS software package for Windows. Post-hoc testing was performed for inter-group comparisons using the least significance difference (LSD) test; significance at P values <0.001, <0.01 and <0.05 have been given respective symbols in the tables and figures.

Results

Table 2 delineates the changes in the body weight, kidney weight and urinary pH. There was no significant change in the body weight between the experimental groups. However, the weight of the kidney in group II rats was found to be increased from that of controls (P < 0.001). The pH of the ethylene glycol (EG)-treated rats was decreased (P < 0.001) when compared with the control, indicative of an acidic scenario favouring crystal retention. Treatment with fucoidan accentuated the abnormal decrease in the pH of the urine (P < 0.001) and also reversed the increase in kidney weight to near normalcy (P < 0.01).

Figure 1 shows the protein content and urinary volume of the different experimental groups. An increase in urinary volume was observed in EG-treated rats (P < 0.01). Treatment with fucoidan profoundly increased the urinary volume of group IV rats when compared with group II, illustrating the diuretic activity of fucoidan (P < 0.001). The excretion of protein in urine was increased in hyperoxaluric rats, indicating damage to the kidney (P < 0.001). Fucoidan administration decreased the protein excretion in urine (P < 0.001).

Table 3 shows the urinary content of the major stone-forming constituents, such as calcium, oxalate, uric acid and phosphorus. Oxalate, calcium and phosphorus were significantly increased (P < 0.001), more than uric acid (P < 0.05), in group II rats. In EG + fucoidan-treated rats, the stone-forming constituents were decreased when compared with the group II rats (P < 0.001). In EG-treated rats, the drastic decrease in the inhibitor constituents, such as citrate (P < 0.05), magnesium (P < 0.001) and GAGs (P < 0.001), favours a milieu for crystal nucleation and retention. Administration of fucoidan was able to alleviate these abnormal changes (P < 0.001).

Light microscopic observation of urinary crystals revealed the presence of aggregated calcium oxalate monohydrate crystals in EG-treated rats (Figure 2B) whereas calcium oxalate dihydrate crystals were present in EG + fucoidan-treated rats (Figure 2D). Control and rats treated with fucoidan showed the presence of calcium phosphate crystals (Figure 2A, C).

Table 4 shows the oxalate and calcium content of the kidney. There was a significant increase in the level of the stone-forming constituents, such as calcium and oxalate, in the kidney (P < 0.001). The increased accumulation of the stone-forming constituents in renal tissue was successfully reversed with fucoidan treatment (P < 0.001).

The examination of the renal sections under polarized microscope revealed that the EG-treated nephrolithic rats had large aggregated crystals in all the major areas of the kidney, especially the tubules (Figure 3B). In contrast, the kidneys treated with EG + fucoidan showed limited calcium oxalate deposition (Figure 3D). Sections from control and drug control rats showed no crystal deposition (Figure 3A, C).

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Effect of fucoidan on rat body weight, kidney weight and urinary pH in experimental hyperoxaluria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group I, control</td>
</tr>
<tr>
<td>Body weight (initial) (g)</td>
<td>125.83 ± 13.23</td>
</tr>
<tr>
<td>Body weight (final) (g)</td>
<td>142.67 ± 13.23</td>
</tr>
<tr>
<td>Kidney weight (g)</td>
<td>1.03 ± 0.12</td>
</tr>
<tr>
<td>Urinary pH</td>
<td>7.53 ± 0.96</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± s.d. for 6 rats in each group. Comparisons are made between: *Group I and Group II, III, IV; **Group II and Group IV (*P < 0.05, **P < 0.01, ***P < 0.001).
TGF-β1 mRNA and collagen levels in renal tissue showed an increase in ECM synthesis during hyperoxaluria. Rats treated with EG showed a 1.68 fold increase in collagen when compared with control (Figure 4) Elevated renal collagen indicates an increase in fibrosis during hyperoxaluria. Fucoidan administration significantly decreased the collagen levels in kidney tissue \( (P < 0.001) \). Figure 5A shows the RTPCR analysis of TGF-β1 mRNA. A single transcript was observed at 303 bp in all the groups. Densitometric analysis showed a 1.32 fold increase in TGF β1 mRNA expression under hyperoxaluric condition (Figure 5B). On fucoidan administration, the TGF-β1 mRNA was significantly modulated in hyperoxaluric rats \( (P < 0.001) \).

**Discussion**

Two forces are known to control crystallization in human urine: urinary supersaturation with respect to stone salts and the presence of crystallization modulators. Urinary chemistry is a determining factor of supersaturation and subsequent formation of crystals. Evidence has shown that inhibition of crystallization, by decreasing the supersaturation, might be an important defense against stones (Kato et al. 2004, Tungsanga et al. 2005). Our preliminary studies indicated that fucoidan was able to improve the antioxidant status of hyperoxaluric rats (Veena et al. 2007) and this interested us to further explore the changes in urinary chemistry after fucoidan administration to oxalate- loaded rats.

Hyperoxaluric rats showed a mild reduction in body weight with a marked increase in kidney weight which might be due to the increased synthesis of ECM. High oxalate concentrations in tubule fluid might have injured the renal epithelial cells resulting in disturbance of pH regulation (Coe et al. 2005), hence a low urinary pH was observed in this study. The low urinary pH was also indicative of an environment conducive to stone formation. The urinary volume was increased in hyperoxaluric rats, interestingly fucoidan + EG treated rats showed a profound increase in urine volume, attributing a diuretic property to fucoidan. Fucoidan-treated rats exhibited a 20% increase in urine volume when compared with control rats, indicative of a diuretic action. Besides these abnormal changes in urine, the protein excretion in urine was also increased, suggesting renal damage. Increased urinary protein could act as a nucleating platform, an architectural framework/cement, which was insufficient to resist the thermodynamic pressure of supersaturated urine and eventually results in stone formation (Ryall 1999). Further, oxidative damage to the kidney and increased protein excretion along with the supersaturating environment might initiate crystal nucleation (Jonasson et al. 2005). The elevation of protein excretion positively correlated with increased excretion of urinary enzymes in our previous studies (Veena et al. 2006).

The increased urinary excretion of oxalate, calcium and uric acid observed in this study corroborated positively with the previous report, which showed an upsurge in the excretion of these urinary stone-forming constituents in patients susceptible to stone formation (Coe et al. 2005). Increased urinary excretion of oxalate might be due to the increase in oxalate as a consequence of ingestion of EG or secretion of oxalate by the tubules (Coe et al. 2005). Data linking calcium excretion to stone risk is supportive of the idea that it is a graded risk factor (Curchan et al. 2001). Oxalate alters intracellular calcium level through its potential to mobilize calcium from intracellular stores (Iida et al. 2003). In addition, reactive oxygen species produced by oxalate can also damage the renal tubules leading to...

**Table 3** Alterations in rat urinary constituents on administration of fucoidan in experimental hyperoxaluria

<table>
<thead>
<tr>
<th>Urinary constituent (mg/24 h)</th>
<th>Group I, control</th>
<th>Group II, EG</th>
<th>Group III, fucoidan</th>
<th>Group IV, EG + fucoidan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxalate</td>
<td>0.29 ± 0.02</td>
<td>1.86 ± 0.19  ***</td>
<td>0.22 ± 0.03</td>
<td>0.50 ± 0.05  ***</td>
</tr>
<tr>
<td>Calcium</td>
<td>1.13 ± 0.19</td>
<td>2.16 ± 0.18  ***</td>
<td>1.12 ± 0.15</td>
<td>1.18 ± 0.16  ***</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>3.11 ± 0.32</td>
<td>4.30 ± 0.52  ***</td>
<td>3.15 ± 0.33</td>
<td>3.25 ± 0.28  ***</td>
</tr>
<tr>
<td>Uric acid</td>
<td>2.48 ± 0.23</td>
<td>2.77 ± 0.24  ***</td>
<td>2.43 ± 0.19</td>
<td>2.49 ± 0.23  ***</td>
</tr>
<tr>
<td>Citrate</td>
<td>1.19 ± 0.19</td>
<td>1.10 ± 0.19  ***</td>
<td>1.43 ± 0.20</td>
<td>1.38 ± 0.20  ***</td>
</tr>
<tr>
<td>Magnesium</td>
<td>2.25 ± 0.29</td>
<td>1.17 ± 0.17  ***</td>
<td>2.31 ± 0.16</td>
<td>2.23 ± 0.20  ***</td>
</tr>
<tr>
<td>GAGs</td>
<td>1.87 ± 0.20</td>
<td>0.47 ± 0.05  ***</td>
<td>2.21 ± 0.16  **</td>
<td>1.96 ± 0.17  ***</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± s d for 6 rats in each group. Comparisons are made between \*Group I and Group II \ III IV \*Group II and Group IV \( *P < 0.05 \) **P < 0.01 ***P < 0.001 \)
to decreased calcium reabsorption with a consequent increase in calcium excretion in urine (Selvam 2002). Further reactive oxygen species can peroxidize the membrane and perturb calcium homoeostasis by decreasing the activity of calcium ATPase thereby increasing calcium concentration (Selvam 2002). Our previous report has already shown a decrease in calcium-ATPase activity in experimental hyperoxaluria and its normalization with fucoidan (Veena et al 2006). Any of these mechanisms can increase urinary calcium level and subsequent supersaturation. The acidic pH of urine along with increased excretion of uric acid and phosphate, might favour the formation of uric acid and calcium phosphate crystals (Coe et al 2005). It has been suggested that calcium phosphate and uric acid crystals increase the propensity of calcium oxalate crystals by heterogeneous nucleation (Khan 2004). Furthermore increase in uric acid decreases the solubility of calcium oxalate in addition to aborting the inhibitory activity of GAGs (Grasev et al 1991). Increased degradation of protein in tubules might have contributed to the increase in uric acid. Studies have shown that hyperuricosuria independent of the formation of uric acid crystals, could also potentiate calcium oxalate monohydrate crystal retention in the kidney (Farell et al 2004). Further oxalate is known to induce apoptosis, which might also contribute to increase the uric acid.

Table 4  Oxalate and calcium content of rat kidney in experimental hyperoxaluria

<table>
<thead>
<tr>
<th></th>
<th>Group I, control</th>
<th>Group II, EG</th>
<th>Group III, fucoidan</th>
<th>Group IV, EG + fucoidan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxalate (mg g⁻¹)</td>
<td>1.97 ± 0.14</td>
<td>3.22 ± 0.36</td>
<td>1.94 ± 0.15</td>
<td>2.14 ± 0.26</td>
</tr>
<tr>
<td>Calcium (mg g⁻¹)</td>
<td>5.34 ± 0.52</td>
<td>10.27 ± 0.97</td>
<td>5.22 ± 0.56</td>
<td>6.05 ± 0.66</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± s.d for 6 rats in each group. Comparisons are made between a Group I and Group II b Group II and Group III c Group II and Group IV (****P < 0.001)

Figure 2  Light microscopic analysis of urinary crystals (400x) A. Control urine showing the presence of calcium phosphate crystals. B. Urine from hyperoxaluric rats showing the presence of calcium oxalate monohydrate crystals C. Urine from fucoidan treated rats showing the presence of calcium phosphate crystals. D. Urine from EG + fucoidan treated rats showing the presence of small calcium oxalate dehydrate crystals.
and phosphate (Sarica et al. 2001). Increase in the stone-forming constituents, as observed in this study, raises the supersaturation, thus increasing the chances of crystal nucleation and deposition in kidneys.

Increase in the concentration of stone-forming constituents was accompanied by a decrease in the concentration of inhibitors, like magnesium, citrate and GAGs. Magnesium exhibits its inhibitory activity by binding to oxalate and increasing its excretion by the formation of soluble magnesium oxalate (Kato et al. 2004). Previous studies have already suggested the role of citrate in binding calcium and inhibiting calcium crystallization (Lieske et al. 1996). Urine citrate concentration is determined mainly by tubule reabsorption, and diminution in the level of magnesium and citrate as observed in this study predicts the increased propensity to stone formation in hyperoxaluric rats. Decrease in the GAGs content during hyperoxaluria decreases the inhibitory potential of the urine and favours an environment for the precipitation of crystals. Reports have shown that increase in oxalate content, apart from increasing the supersaturation, also decreases the inhibitory potential of GAGs (Cao et al. 1997b). Decrease in these potential inhibitors of stone formation in this study indicates that they might have been utilized in the process of

Figure 3  Polarized microscopic analysis of rat renal tissues (40x) A Sections from control rats showing no crystals deposition B Sections from EG treated rats showing the presence of aggregated crystals C Sections from fucoidan-treated rats showing no crystal deposition D Sections from EG + fucoidan treated rats showing the presence of tiny crystals.

Figure 4  Effect of fucoidan on rat renal collagen level in experimental hyperoxaluria. Values are expressed as mean ± s.d for 6 rats in each group. Comparisons are made between *Group I and Group II, III, IV, +Group II and Group IV (**P < 0.01, ***P < 0.001)
Fucoidan reverses physico-chemical urine alterations in hyperoxaluria

Figure 5: Effect of fucoidan on TGF-β1 mRNA expression in kidney tissue of experimental rats in hyperoxaluria. A. The 303 and 194 bp fragments represent transcript TGF-β1 and RPL-19 as internal standard, respectively. Lane 1, 100 bp DNA ladder; lane 2, control; lane 3, EG: lane 4, fucoidan; lane 5, EG + fucoidan. B. Ratio of TGF-β1 mRNA expression relative to RPL-19 mRNA. Values are expressed as mean ± s.d. for 6 rats in each group. Comparisons are made between: aGroup I and Group II, III, IV; a, bGroup II and Group IV (*P < 0.05, ***P < 0.001).

stone formation, which results in decrease in their concentration in this study. Analysis of urinary stones has shown that these constituents are present in the stone matrix; further, it has also been proved that these components competitively inhibit the crystallization process (Lieske et al. 1995).

Fucoidan administration was able to decrease the supersaturation and crystal retention in EG-treated rats possibly due to its effect on glycolic acid oxidase and its anti-adherent property, as it is a naturally occurring GAG. The modulatory effect of fucoidan on glycolic acid oxidase and its diuretic property might be responsible for the decreased excretion of stone-forming constituents (Veena et al. 2007). Our previous report showed that fucoidan decreases the activity of glycolic acid oxidase, a flavoprotein that catalyses the two-step oxidation of glycolate to oxalate with glyoxylicate as an intermediate (Veena et al. 2007). This enzyme is localized in the liver and its activity increases during hyperoxaluria. Fucoidan was also able to modulate the activity of xanthine oxidase, which also contributes to increase oxalate to a minor extent. Additionally, it is also found to increase the inhibitor concentration and this might be responsible for its protective activity against lithogenesis. Similar effects have been reported for sodium pentosan polysulfate, a heparin analogue, under oxalate stress (Subba & Varalakshmi 1993).

The urinary stone analysis showed that administration of fucoidan resulted in calcium oxalate dihydrate crystals whereas free and aggregated calcium oxalate monohydrate crystals were found in hyperoxaluric rats. Although both type of crystals are found in hyperoxaluria, calcium oxalate monohydrate crystals predominate under oxalate stress (Khan 1997; Lieske et al. 1999). Moreover, it has been reported that the crystal morphology is mainly determined by the supersaturation of the urine. Carvalho & Vieira (2004) have shown that calcium oxalate dihydrate crystals are formed in urine at low supersaturation and, as the supersaturation increases, the number of crystals is increased as well as the morphology changes. Hence, the formation of calcium oxalate monohydrate crystals in EG-treated rats might be due to the increased supersaturation of urine. Increased excretion of calcium oxalate monohydrate is usually associated with stone formation as calcium oxalate monohydrate crystals have greater affinity for renal tubules than calcium oxalate dihydrate or calcium phosphate (Wesson et al. 1998). The formation of calcium oxalate dihydrate in fucoidan-administered rats is another standing evidence of the protective action of fucoidan against urolithiasis.

The selective reabsorption capacity of segments of nephron poses different risks for crystallization at the various sites in the nephron. Renal tissues in this study showed an increase in accumulation of calcium and oxalate. The concentration of these substances in the renal tissue plays a key role in the pathogenesis of papillary calcification and eventual stone formation. Decreased glutathione and calcium-ATPase activity might account for the increased calcium content. Increase in oxidized glutathione leads to the formation of protein-S-glutathione disulfide, which favours an increase in cytosolic calcium during peroxidation (Bellomo et al. 1983). Moreover, the loss of the critical –SH group of calcium-ATPase, which maintains the calcium pump, leads to the perturbation of cellular calcium homeostasis (Mark et al. 1995). Decreased calcium-ATPase activity and reduced glutathione have already been reported by us, nevertheless fucoidan was able to prevent the oxidation of –SH group of calcium-ATPase and increase the glutathione content of the renal tissues (Veena et al. 2006). These properties of fucoidan might be responsible for the decreased retention of oxalate and calcium in the renal tissues in this study.

Fucoidan’s effect in decreasing the retention of the crystals in the renal tissue was further demonstrated by the polarized microscopic studies, which showed decreased crystals in the renal tissue on treatment with fucoidan. Apart from decreasing the concentration of stone-forming constituents, like oxalate and calcium, which decrease the supersaturation of urine, fucoidan, being a GAG, can also bind to the potential growth sites of crystals and block crystal growth (Cao et al. 1997a). Besides, it can also cover the crystals and prevent their retention in the renal tissues (Cao et al. 1997b). These mechanisms might lie behind the decreased retention of crystals in the renal tissue of fucoidan + EG-treated rats.
Numerous reports support the fact that fucoidan is functionally similar to heparin and its mode of action can be related with heparin (Pereira et al 1999, Shannugam & Mody 2000) It has been suggested that exogenous GAG administration in the form of low-molecular-weight heparin improves the anionic charges on the tubular epithelium, which is depleted during calcineurigenesis and thereby decreases tubular uptake of crystals (Rajewski & Varalakshmi 2006) This coating phenomenon principally defies calcium oxide crystals and oxalate from gaining easy access to renal tubular epithelium by way of charge repulsion action Hence, it can be hypothesized that fucoidan exerts its protective effect similarly to heparin by decreasing the tubular uptake of crystals In this study, polarized images showed numerous crystals accumulated in the tubular lumen on EG administration and only a few interstitial crystals on EG + fucoidan treatment This further supports the hypothesis that fucoidan prevents tubular uptake of crystals Oxidative stress and associated injury have been recognized as the key factors in crystal retention and subsequent formation of stones Fucoidan was proved to decrease oxidative stress and renal injury through our previous studies (Veena et al 2007), hence, decrease in renal injury on fucoidan administration can also be attributed to the decreased crystal retention of the renal tissues

Recent studies have shown that crystal deposition is associated with increased ECM synthesis resulting in renal fibrosis (Khan 2004) Light and electron microscopic studies of a renal papilla and associated calcium oxalate kidney stones showed distinct signs of injury and inflammation and the presence of a large amount of collagen, indicative of renal fibrosis Increase in collagen content of the renal tissue in this study shows the increase in fibrotic nature as well as increase in the ECM favouring stone retention Toblli et al (1999) have shown an increase in collagen expression in experimental hyperoxaluria and have suggested that decreasing collagen accumulation is favourable for circumventing hyperoxaluria TGF-β1 has a central role in regulating renal fibrosis and increasing ECM synthesis during lithogenesis Numerous studies have reported that TGF-β1 inhibits matrix degradation, regulates type I, type III and type VI collagen synthesis and also participates in apoptosis Similar increase in collagen content and TGF-β1 expression has already been reported in hyperoxaluria (Toblli et al 1999) An increased ECM synthesis results in tissue remodelling and exposure of crucial crystal-binding molecules, which favours crystal retention (Toblli et al 1999, Umekawa 2004) Various actions of TGF-β1 are mediated by oxidative stress, fucoidan, due to its anti-oxidant potential, might have decreased the expression of TGF-β1 Apart from this indirect effect, fucoidan may act on the intracellular signalling machinery, such as the process of transcription factor activation Fucoidan is known to bear functional similarity with heparin and heparin is known to migrate into the nucleus and suppress AP-1 mediated transcription in smooth muscle cells and hepatoma cells (Dudas et al 2000) A similar effect of fucoidan is also possible in hyperoxaluria Decrease in TGF-β1 expression may account for the decrease in collagen as TGF-β1 can modulate the expression of collagen

**Conclusion**

In conclusion, we have demonstrated the impact of fucoidan on urinary supersaturation as well as crystal retention in the renal tissues Our findings imply that by decreasing supersaturation, fucoidan could modulate the urinary crystallization process Further, fucoidan was able to abort the stone retention Alteration in ECM, which is also an important determinant favouring stone retention, was normalized with fucoidan administration These findings highlight the protective role of fucoidan in experimental hyperoxaluria

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Fucoidan reverses physico-chemical urine alterations in hyperoxaluria


Renal peroxidative changes mediated by oxalate: The protective role of fucoidan

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Abstract

Oxalate, one of the major constituents of renal stones, is known to induce free radicals which damage the renal membrane. Damaged epithelia might act as nuclei for stone formation aggregating calcium oxalate precipitation during hyperoxalatemia. In the present study, the beneficial effects of fucoidan on oxalate-induced free radical injury were investigated. Male Wistar rats were divided into four groups. Hyperoxalatemia was induced in two groups by administration of 0.75% ethylene glycol in drinking water for 28 days and one of them was treated with fucoidan from Fucus vesiculosus at a dose of 5 mg kg−1 bw subcutaneously commencing from the 8th day of induction. A control and drug control (fucoidan alone) was also included in the study. The extent of renal injury in hyperoxalatemia was evident from the increased activities of alkaline phosphatase, γ-glutamyl transferase, β-glucuronidase, N-acetyl β-D-glucosaminidase in urine. There was a positive correlation between plasma malondialdehyde levels and renal membrane damage indicating a striking relation between free radical formation and cellular injury. Increased protein carbonyl and decreased thiol further exemplified the oxidative milieu prevailing during hyperoxalatemia. Decreased renal membrane ATPase accentuated the renal membrane damage induced by oxalate. Renal microscopic analyses showed abnormal findings in histology as an evidence of oxalate damage. The above biochemical and histopathological discrepancies were abrogated with fucoidan administration indicating its protective role in oxalate mediated peroxidative injury.

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Keywords: Oxalate, Nephrotoxicity, Protein Carbonyl, Malondialdehyde, Kidney, Fucoidan

Introduction

Nephrotoxicity of oxalic acid has been recognized since the nineteenth century. Oxalate plays no vital function and most of the urinary oxalate appears to be a product of endogenous biosynthesis from oxalate precursors (Harris and Richardson, 1980). Its injurious effects are considered to be a result of the physiological properties of its calcium salts, especially the calcium oxalate, which is insoluble at physiological pH. However, it is now well established that high oxalate levels have direct toxic effects on renal tubular cells and numerous experiments point out that oxalate itself may be injurious to renal epithelial cells and this toxicity may be exaggerated in the presence of calcium oxalate crystals (Khan et al., 1992; Scheid et al., 1996; Thamilselvan et al., 2001). Exposure to a high concentration of oxalate results in the production of free radicals, which induces oxidative stress as shown by increased lipid peroxidation (LPO), free radical generation, and arachidonic acid release with decreased glutathione (Kohiyama et al., 1999; Thamilselvan and Menon, 2005). Free radicals interact with renal epithelia damaging the renal membranes, there is strong evidence that tubular dysfunction or damage is the initial step involved in binding of crystals and subsequent pathology (Graves et al., 1998). Biological compounds with antioxidant properties and renal membrane-regenerating potential may be a boon in alleviating oxalate-induced toxicity.
Brown seaweeds have been the mainstay of the Japanese diet and are also documented in traditional Chinese medicine for over a 1000 years (McLellan and Jurd, 1992). Sulphated polysaccharides extracted from marine algae represent a source of marine compounds with potential applications in medicine. Fucoids, the sulphated polysaccharides extracted from brown algae, first isolated by Kylin almost a century ago, contain substantial percentages of 1-fucose and sulphate ester groups (Patanak et al., 1993). Earlier, fucoidan was considered as an alternative to heparin and focus was mainly on its anticoagulant activity. Recently, fucoidan is being studied extensively due to its numerous biological activities including antitumor, antiviral, anticomplement and anti-inflammatory activities (Chizhov et al., 1999, Berteu and Mullot, 2003). Recently, the supplement of fucoidan to rats with chronic renal failure demonstrated the renoprotective effects of fucoidan (Zhang et al., 2003a). Further, it was also found to be effective in decreasing the proteinuria associated with Heyman nephritis (Zhang et al., 2005). Previous studies from our laboratory have shown that fucoidan was able to increase the antioxidant enzymes in ethylene glycol (EG) treated rats (Veena et al., in press). Hence, the present study was conducted with an aim to examine whether fucoidan, could ameliorate the oxalate-induced peroxidative injury by assessing the thiol and carbonyl status along with histopathological studies.

**Materials and methods**

**Drugs and chemicals**

Fucoidan from *Fucus vesiculosus*, bovine serum albumin and 1,1,3,3-tetraethoxypropane were obtained from Sigma Chemicals, St Louis, MO, USA. All other chemicals and solvents used were of analytical grade.

**Animal model**

Male Wistar albino rats weighing 140±20 g were purchased from Tamil Nadu Veterinary and Animal Sciences University, Chennai, India. The animals were maintained under standard conditions of humidity, temperature (25±2 °C) and light (12 h light/12 h dark). They were fed with standard rat pelleted diet (M/s Pranav Agro Industries Ltd, India) under the trade name Amrut rat/mice feed and were allowed free access to water. Experimental animals were handled with human care 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.

**Experimental protocol**

Twenty four rats were randomly divided into four groups consisting of six each. Group I rats served as vehicle-treated control. Group II received EG (0.75% in drinking water) for 28 days to induce a chronic low-grade hyperoxaluria and generate calcium oxalate deposition in kidneys. Group III served as drug controls and received a subcutaneous injection of fucoidan from *F. vesiculosus* (5 mg/kg body weight) dissolved in saline and filtered through a 0.2 μm filter before administration. Group IV received EG for 28 days and fucoidan from day 8 of the experimental period.

At the end of 28 days, the animals were housed in metabolic cages for 24 h urine collection in ice jacketed flasks at 4 °C. Urine samples were centrifuged at 2000 ×g for 10 min to remove debris and the supernatant was used to assay urinary enzymes. Blood was collected with an anticoagulant and the plasma was separated to assess the malondialdehyde (MDA) content. The animals were sacrificed and the kidneys were rinsed in ice-cold physiological saline. A portion of the tissue was fixed in 10% formalin for histological processing and the remaining homogenized in Tris HCl buffer (0.1 M, pH 7.4) to give a 10% homogenate and was suitably processed for the assessment of biochemical indices.

**Urinary enzymes**

Alkaline phosphatase (ALP) was assayed using disodium phenyl phosphate as substrate (King, 1965). γ-Glutamyl transferase (γ-GT) and β-glucuronidase (β-Glu) were measured by the methods of Orloski and Meister (1965) and Kawakami and Anno (1971) with l-γ-glutamyl p-nitroanilide and p-nitrophe- nyl-p-β-glucuronide as substrates respectively. The activity of α-acetylglycosaminidase (NAG) was arrived by the method of Maruhn (1976). Urinary creatinine was estimated (Owen et al., 1954) and enzymuria was calculated in relation to urinary creatinine.

**Estimation of plasma MDA content by HPLC**

HPLC method previously described by Cords et al. (1998) and Pitz et al. (2000) was used to estimate the plasma MDA content, briefly 250 μl of plasma was treated with 50 μl of 6 M NaOH for alkaline hydrolysis of protein bound MDA and was incubated at 60 °C for 30 min. The hydrolyzed sample was acidified with 125 μl of 35% perchloric acid and centrifuged at 14000 ×g for 250 μl of supernatant was mixed with 25 μl DNPH and incubated for 10 min. After derivatization, the sample was filtered through a 0.2 μm filter. Aliquots of 20 μl of the filtrate were injected into the Nucleosil C18 column (5 μm particle size) in a Shimadzu liquid chromatograph using acetonitrile distilled water (38:62, v/v) containing 0.2% (v/v) acetic acid as the mobile phase. The DNPH derivatives were detected at 310 nm at a flow rate of 1 ml/min Concentrations of MDA were calculated from a standard curve prepared from 1,1,3,3 tetraethoxypropane and expressed as nmol/ml.

**Assessment of protein thools and protein carbonyls**

Total and non-protein thools were estimated by the method of Sedlak and Lindsay (1968). Protein carbonyl content was determined by a reliable method based on the reaction of carbonyl groups with DNPH to form 2,4-dinitrophenylhydrazone (Levine et al., 1990). Briefly, 100 μl of homogenate was incubated with 0.5 ml DNPH for 60 min and the protein was
precipitated from the solution with the use of 20% trichloroacetic acid. The pellet was washed after centrifugation (3400 × g) with ethyl acetate:ethanol (1:1 v/v) mixture thrice to remove excess of DNP and the final protein pellet was dissolved in 1.5 ml of 6 M guanidine hydrochloride. The carbonyl content was evaluated in a spectrophotometer at 370 nm. Protein content was estimated by the method of Lowry et al. (1951).

Assay of activities of ATPases

Na⁺K⁺-ATPases, Ca²⁺-ATPases and Mg²⁺-ATPases were determined by the method of Bonning (1970), Ohnishi et al. (1982), Hjerten and Pan (1983) respectively. Enzyme activity in each of these assays was expressed as a function of inorganic phosphorus liberated, which is due to the breakdown of ATP.

Histopathological studies

A portion of kidney tissue was fixed in 10% formalin. Tissues were washed and dehydrated in 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 analyzed under light microscope for histopathological changes.

Data analysis

The results are expressed as mean ± standard deviation (S.D.) for six animals in each group. Differences between groups were assessed by one way analysis of variance (ANOVA) using the SPSS software package for Windows. Post hoc testing was performed for inter-group comparisons using the least significance difference (LSD) test; significance at P-values < 0.001, < 0.01, < 0.05 have been given respective symbols in the tables and figures.

Results

Table 1 delineates the excretion of urinary enzymes in experimental animals. Urinary excretions of ALP and γ-GT were markedly increased in Group II animals when compared to that of the control animals (P < 0.001). Hyperoxaluria-induced cellular damage was demonstrated by the increased urinary excretion of the lysosomal enzymes like γ-Glu and NAG in

Table 1
Effect of furacold on the excretion of urinary enzymes in experimental hyperoxaluria

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Group I control</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
<th>Group IV EG-furacold</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALP</td>
<td>0.89±0.10</td>
<td>2.59±0.35***</td>
<td>0.94±0.12</td>
<td>0.95±0.12***</td>
<td></td>
</tr>
<tr>
<td>γ-GT</td>
<td>1.29±0.15</td>
<td>2.10±0.23**</td>
<td>1.23±0.13</td>
<td>1.42±0.11***</td>
<td></td>
</tr>
<tr>
<td>β-Glu</td>
<td>0.18±0.023</td>
<td>0.33±0.032**</td>
<td>0.19±0.018</td>
<td>0.21±0.024**</td>
<td></td>
</tr>
<tr>
<td>NAG</td>
<td>0.34±0.038</td>
<td>0.79±0.070***</td>
<td>0.64±0.050</td>
<td>0.40±0.029***</td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as mean ± S.D. for 6 animals in each group. ALP, μmol of phenol liberated/ h/mg creatinine; γ-GT, nmol of p-nitroaniline released/h/mg creatinine; β-Glu, nmol of p-nitrophenol released/h/mg creatinine. Comparisons are made between: *— Group I and Group II, III, IV; **— Group II and Group IV. The symbols represent statistical significance: *P < 0.05; **P < 0.01.

Table 2
Effect of furacold on renal membrane ATPases in experimental hyperoxaluria

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Group I control</th>
<th>Group II EG</th>
<th>Group III</th>
<th>Group IV</th>
<th>Group IV EG-furacold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺K⁺-ATPase</td>
<td>0.75±0.09</td>
<td>0.56±0.06***</td>
<td>0.74±0.09</td>
<td>0.73±0.08***</td>
<td></td>
</tr>
<tr>
<td>Ca²⁺-ATPase</td>
<td>0.32±0.04</td>
<td>0.20±0.03***</td>
<td>0.32±0.03</td>
<td>0.30±0.04***</td>
<td></td>
</tr>
<tr>
<td>Mg²⁺-ATPase</td>
<td>0.55±0.06</td>
<td>0.48±0.05**</td>
<td>0.55±0.05</td>
<td>0.54±0.05**</td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as mean ± S.D. for 6 animals in each group. Units: Na⁺K⁺-ATPase, Ca²⁺-ATPase and Mg²⁺-ATPase: μmol of inorganic phosphorus liberated/mg protein. Comparisons are made between: *— Group I and Group II, III, IV; **— Group II and Group IV. The symbols represent statistical significance: *P < 0.05; **P < 0.01.

Fig. 1. Effect of furacold on plasma MDA levels and renal protein carbonyls in hyperoxaluric rats. Units: plasma MDA — nmol of MDA/mL; protein carbonyls— μg/mg protein. Values are expressed as mean ± S.D. for 6 animals in each group. Comparisons are made between: a — Group I and Group II, III, IV; b — Group II and Group IV. The symbols represent statistical significance: ***P < 0.001; ****P < 0.001.

Fig. 2. Effect of furacold on protein thols in hyperoxaluric rat kidneys. Values are expressed as mean ± S.D. for 6 animals in each group. Comparisons are made between: a — Group I and Group II, III, IV; b — Group II and Group IV. The symbols represent statistical significance: ***P < 0.001.
hyperoxaluric animals. Abnormal elevations in the urinary enzymes were reverted to near normalcy on administration of fucoidan (P < 0.001) suggesting the renoprotective effect of the sulphated polysaccharides.

Plasma MDA levels were increased 2.5-fold in group II hyperoxaluric animals when compared with group I control animals (Fig 1). The increase in plasma MDA levels highlights the oxidative stress associated with hyperoxaluria. Significant (P < 0.001) reduction in the MDA levels was observed on supplementing fucoidan, as shown in group IV animals demonstrating the antioxidant activity of the sulphated polysaccharides.

Protein carbonyls were significantly increased (P < 0.001) in hyperoxaluric (Fig 1). Fucoidan administration was able to decrease the protein carbonyls formation.

Fig 2 depicts the alterations in the total protein and non-protein thiol levels. Marked decrease in the thiol levels (P < 0.001) indicative of the oxidative milieu prevailing in the hyperoxaluria was profoundly changed on fucoidan administration.

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Fig 3: Histopathological studies on renal tissues of hyperoxaluric rats (H & E 100×). A) Sections from control rats show normal glomeruli with tubules. B) sections from I-G treated rats show crystal deposition (arrow) with mild diffuse mononuclear infiltration. C) sections from EG treated rats show dilated tubules with epithelial desquamation and two shrunken glomeruli (arrows) with mild and diffuse mononuclear infiltration. D) sections from Fucoidan alone treated rats show normal glomeruli and tubules. E) sections from EG + Fucoidan treated rats show near normal glomeruli and tubules.
Table 2 shows the decrease in the renal membrane bound ATPases activity in hyperoxaluria. Na-K-ATPase and Ca2+-ATPase showed a marked decrease (P < 0.001) in group II when compared with group I animals whereas Mg2+-ATPase showed a moderate decrease in group II animals. The decrease in the renal membrane ATPases showed the profound effect of hyperoxaluria in damaging the renal membrane resulting in tonic imbalance. Fucoidan administration was able to circumvent these changes near normality.

Histopathological sections from control rats show normal glomeruli with tubules (Fig 3A). Hyperoxaluria rats show dilated tubules with epithelial denudation and two shrunken glomeruli with mild and diffuse mononuclear infiltration (Fig 3C). Dilated collecting system with crystal deposition was also observed (Fig 3B). Fucoidan administration protected the kidney from toxic effects of oxalate as evident by the section in Fig 3F, showing near normal glomeruli and tubules. The rats treated with fucoidan alone (Fig 3D) showed normal glomeruli depicting that fucoidan has no toxic effect on renal tissues.

Discussion

A change in the cellular redox environment to a more oxidizing state is characteristic of a variety of diseases that include cancer, diabetes, neurodegeneration, and vascular pathologies. Researchers have identified the role of free radicals in oxalate-induced toxicity (Thamilselvan and Menon 2005). Oxidative stress associated with oxalate toxicity might be the result of an increased concentration of free oxalate ions insoluble calcium oxalate interacting directly with renal epithelial cells or may originate from other inflammatory events. Oxalate toxicity results in an altered biochemical reaction including the alteration of the antioxidant defense system and increased LPO (Thamilselvan et al 1999). Fucoidan is a sulfated polysaccharide with antioxidant activity was administered to establish the protective role of these polysaccharides in oxalate toxicity.

Iovsen et al (1999) established that exposure of oxalate at a concentration of greater than or equal to 175 μM (free) produced membrane damage within 4 h in renal proximal tubular epithelial cell line derived from the human kidney and this effect was inhibited by Mn(III) tetrakis (1-methyl-4 pyridyl) porphyrin (MnTMPyP), a superoxide dismutase mimetic suggesting that oxalate exposure produces changes in renal membranes via a process dependent on reactive oxygen intermediates. Damage to the renal membrane results in the release of enzymes and hence in the present study there is a drastic increase in the excretion of brush border enzymes like ALP and γ-GT in hyperoxaluric animals. This corresponds with our observation of decreased levels of these enzymes in renal tissues (Veena et al in press). Increased excretion of these enzymes has already been reported in hyperoxaluria and can be directly correlated with the extent of renal membrane damage by oxalate-calcium oxalate (Thamilselvan and Menon 2005).

Abysmal increase in the urinary enzymes was found to correlate positively with the increased plasma MDA levels, observed in the present study. The increase in the excretion of lyosomal enzymes like β-Glu and NAG also emphasizes the tubular damage induced by hyperoxaluria. This abnormal enzymatic profile was corrected to near normalcy with fucoidan administration demonstrating the protective effect of the sulfated polysaccharides in maintaining the integrity of the renal membrane and is supported by the fact that fucoidan administration prevented enzyme leakage from renal tissues in oxalate toxicity (Veena et al in press). Sulfated polysaccharides are known to be beneficial in the treatment of wound healing suggesting their membrane-regenerative potential (O I, et al 2004). These observations are in line with our previous findings where administration of sodium pentosan polysulfate and heparin analogue was found to modulate the enzyme activity associated with experimental hyperoxaluria (Sabbà and Varalakshmi 1993).

Maintenance of redox homeostasis is critical to all mammalian cells especially in the kidney because of its high basal rate of aerobic metabolism. LPO is one of the manifestations of oxidative damage initiated by reactive oxygen species and it has been linked with impairment of membrane functioning, decreased fluidity inactivation of membrane bound receptors and enzymes and increased non specific permeability to ions (Sikk 2004). Increase in plasma MDA levels in EG treated rats observed in the present study might be attributed to the increased LPO of renal tissues due to an increase in oxalate concentration Schid et al (1996) reported increased free radical production in renal culture cells exposed to oxalate, ranging from 0.1 mM to 4.0 mM. Increased urinary MDA levels have also been observed in stone-forming patients (Huang et al 2003). Known to increase the activities of antioxidant enzymes (Zhong et al 2003b) fucoidan administration was able to decrease the plasma MDA level which directly correlates with their effect on the antioxidant defense system.

The principal high molecular weight thiol containing compounds like proteins and their thiol groups are in equilibrium with low molecular weight thiol species such as glutathione. The maintenance of appropriate concentration of these species in their reduced state is essential for numerous cellular functions. In the present study hyperoxaluric rats had decreased levels of protein and non protein thiols probably as a consequence of oxidative stress. Himmelfarb et al (2000) suggest that increased plasma protein oxidation associated with chronic renal failure patients may be due to increased oxidative stress. Reactive species formed by oxalate can react directly with the protein or they can react with molecules such as sugars and lipids generating products that then react with the protein. Many of the reactions are mediated by free radicals usually in a site specific fashion (Jaine 2002). Depletion of cellular reduced glutathione might also result in protein thiol oxidation and alteration in renal membrane integrity has suggested by Rashed et al (2004). In the present study the increase in protein oxidation might be due to the decrease in glutathione concentration (Veena et al in press). Protein carbonyl formation also serves as an index for protein oxidation (Dalle-Donne et al 2003). Recent research indicates that protein carbonyl deriva- tives are formed during chronic renal failure as a consequence of oxidative stress (Dursun et al 2005). Oxidation of protein thiol and formation of protein carbonyls were profoundly decreased on fucoidan administration and this might be
attributed to their antioxidant property and the potential to replenish the reduced glutathione (Veena et al., in press).

Membrane ATPases play a key role in the production/maintenance of gradients and in the ion distribution in cells. They provide active ion transport across biological membranes, using energy of ATP hydrolysis. In the cell, most important transport ATPases are the Na⁺/K⁺- and Ca²⁺-ATPases located in plasma membrane, but greatest attention has been paid to Na⁺/K⁺-ATPase which performs the active transport of Na⁺ and K⁺ ions and is involved in the process of maintaining the membrane potential. The observed decrease in the Na⁺/K⁺-ATPase activity in the present study might be due to increased oxidative stress (Bergendi et al., 1999). Increasing evidence supports the point, that free radicals are involved in the inactivation of Na⁺/K⁺-ATPase and that supplementation with antioxidant results in abolishing the inhibitory effect on the enzyme (Streck et al., 2001). With respect to other ATPases, specifically the Ca²⁺-ATPase, experiments show that they are affected by oxidative inactivation, whereas antioxidants are able to prevent it (Mark et al., 1995, Selvam et al., 1995). Decrease in Ca²⁺- and Mg²⁺-ATPase in the present study might be due to the oxidation of thiol groups in the enzyme which in turn is mediated by the increased LPO. Fucoidan with their antioxidant potential was able to prevent the inactivation of renal membrane ATPases by oxidation, thereby diminishing the toxic effects of oxalate. This is in concordance with our observation where low molecular weight heparin supplementation prevented ATPase inactivation (Deepa and Varalakshmi, 2003).

Histopathological observations of hyperoxaluric renal sections showed dilated collecting systems with deposition of crystallites. Dilated tubules with epithelial edema, and mild mononuclear infiltration were also observed. Experimental evidence supports the fact that in the renal interstitium, calcium oxalate crystals induce a chronic abacterial interstitial nephritis in which inflammatory cells such as granulocytes and lymphocytes are involved. Inflammatory reactions are enhanced when serum components gain access to the crystal surface and leads to the activation of complement system, production of peroxides and toxic superoxide anion radicals (Boogaerts et al., 1983). Marked changes in hyperoxaluric renal sections were normalized with fucoidan which are evident from EG₂+fucoidan treated section with near normal glomeruli.

Conclusion

The present findings indicate that fucoidan reduces the toxic effects of oxalate on renal cells through its antioxidant property. Increased enzymuria, decreased protein and non-protein thiol along with increased plasma MDA level confirm the hyperoxalurica-induced damage. The abnormal histologic findings and decreased ATPase activity point out that oxalate damages the renal membrane, increases crystal binding in renal epithelial cells and supports the notion that oxalate toxicity may contribute to the development of stone disease by altering the properties of the renal epithelial cell membrane. Fucoidan administration reversed the abnormal biochemical changes thus proving to be a potential candidate in alleviating oxalate mediated toxicity.

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