Hepatoprotective Activity of Liposomal Flavonoid against Arsenite-Induced Liver Fibrosis

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ABSTRACT

Arsenic, the environmental metalloid toxicant, is known to induce oxidative damage to liver and produce hepatic fibrosis. The theme of our study was to optimize and evaluate the therapeutic efficacy of galactosylated liposomal flavonoidal antioxidant, quercetin (QC), in combating arsenic-induced hepatic fibrogenesis. The rats of the hepatic damage group were injected s.c. a single dose of sodium arsenite (NaAsO₂) (100.06 μM/kg b. wt. in 0.5 ml of physiological saline). Hepatocytes and stellate cells were separated. Mitochondrial membranes were isolated from all those separated cells. Oxidative damage was monitored at different isolated subcellular parts of different hepatic cells. Liver fibrosis was also induced by the injection of NaAsO₂. Galactosylated liposomal QC injection before NaAsO₂ treatment checked fibrogenesis completely by protecting the liver from oxidative attack in cellular and subcellular levels. The maximal protections from hepatocellular and fatty metamorphosis, necrosis, Kupffer cell hyperplasia, fibrosis, and in the deposition of collagen contents were observed and confirmed by our histopathological and histochemical analysis when rats were treated with galactosylated liposomal QC before NaAsO₂ injection. Application of galactosylated liposomal QC may be a potent therapeutic approach for NaAsO₂-induced fibrogenesis through a complete protection against oxidative attack in cellular and subcellular parts of rat liver.

Arabidopsis thaliana, a price in a form of hepatic fibrosis that causes portal hypertension (Guha Mazumder et al., 1998). However, although mechanism(s) by which arsenic induces hepatic fibrosis remains poorly understood, the common theme that was emerged is the role of reactive oxygen species (ROS) in the pathogenesis of arsenic-induced hepatic fibrosis. Hepatocyte necrosis itself may activate lipocytes. Such activation could be mediated by the lipid peroxides formed after the membrane of the hepatocyte is injured (Greenwel et al., 2000). The critical event in hepatic fibrosis is the activation of lipocytes (stellate, fat-storing, or Ito cell), the main source of extracellular matrix in fibrosis formation (Du et al., 1999).

A complementary approach that delineates the NaAsO₂-induced contribution of ROS includes the use of antioxidants, superoxide dismutase (SOD), and catalase. However, introduction of either SOD or catalase can only suppress a little part of toxic oxygen species that is generated by the induction of NaAsO₂ (Kessel et al., 2002).

Nonenzymatic supplementation as a protective strategy has also resulted in conflicting outcomes in in vivo experiments. Administration of D-α-tocopherol to rats provided no protection against ROS in liver diseases. Therefore, the present challenge is to counter NaAsO₂-induced oxidative attack and to isolate a nontoxic antioxidant that can be selectively targeted to liver, which provides an even distribution in intrahepatic membrane.

Liposomes are accepted as potent drug carriers not only for their biocompatible nature but also for their negative biological responses in the biological system. Furthermore, because of the presence of galactosyl receptor on the surface of hepatocytes, galactosylated liposomes are effective in the site-specific drug delivery to hepatic tissue with a homogenous...
intrahepatic membrane distribution of its intercalated components (Sinha et al., 2000). The mammalian liver consists primarily of hepatocytes and stellate cells. The membrane composition of liposome is crucial for its targeting and function. It was observed that galactosylated liposome-entrapped material was largely taken up by hepatocytes. The high endocytic activity of sinusoids lining cells makes them most competent to internalize colloidal particles like liposomes. So, natural targeting of liposomes takes place by those cells, and it is reasonable to assume that galactosylated liposome administration increases intracellular accumulation of vesicular content in hepatocytes (Manajit et al., 2005).

Quercetin (QC), the most abundant flavonoid in nature, presents in large amounts in vegetable, fruits, tea, and olive oil, and because it contains a number of phenolic hydroxyl groups, it exhibits its therapeutic potential against many diseases, including ischemic heart diseases, atherosclerosis, liver fibrosis, renal injury, and chronically biliary obstruction (Peres et al., 2000; Lee et al., 2003; Sing et al., 2004; Tokyo et al., 2006). In light of the above observations, it seems reasonable to expect that administration of QC in galactosylated liposomes might contribute to reduce NaAsO₂-induced oxidative damage in cellular or subcellular parts of hepatic tissue and provide a protective mechanism against liver fibrosis.

Despite the fact that rats, unlike other mammals, retain the dimethyl arsenic acid, one of the intermediate metabolites of NaAsO₂ in the blood, studies were performed in this report on rats because this species is known to be superior to others in forming monomethyl arsenous acid (Csanyik and Gregus, 2002). Monomethyl arsenous acid is most toxic among other NaAsO₂ metabolic intermediates to rat as well as human hepatocytes (Styblo et al., 2000). The purpose of our in vivo studies was to ascertain whether treatment with QC intercalated in galactosylated liposomes exerts any hepatoprotective effect against NaAsO₂-induced fibrosis in liver.

**Materials and Methods**

**Materials**

Phosphatidylethanolamine (PE), cholesterol, dicetyl phosphate (DCP), p-amino phenyl α-D-galactoside, glutaraldehyde, collagenase, diphenyl hexatriene (DPH), Triton X-100, and glutathione reductase were purchased from Sigma Chemicals (St. Louis, MO). Sodium arsenite (Merck, Darmstadt, Germany) was used for experimental purposes. Chloramine-T, Fast Green FCF, and Sirius Rose BB were acquired from Loba Chemie Pvt. Ltd. (Mumbai, India) and Fluka (Buchs, Switzerland), respectively, whereas chloroform and methanol were from E. Merck (India) Ltd. (Mumbai, India). Quercetin was purified and isolated from *Fagopyrum esculentum* (buckwheat). All other reagents were of analytical grade.

**Preparation of Liposomal QC and Coupling of p-Aminophenyl α-D-Galacto-Pyranoside with Liposomes Intercalated with QC**

Multilamellar liposomes were prepared with PE, cholesterol, DCP, and QC in molar ratio 7:1:1:1 (Budai and Szoqvi, 2001). In short, PE, cholesterol, DCP, and QC were dissolved in chloroform and methanol mixture (2:1, v/v) in a round-bottom flask. The lipid film was made by drying the organic solvents and was desiccated overnight. The thin film was swollen in phosphate-buffered saline, pH 7.2, for 1 h and sonicated in a probe-type sonicator for 30 s. Liposomal QC was coupled with p-aminophenyl α-D-galacto-pyranoside by using glutaraldehyde as the coupling agent (Mitra et al., 2005).

**Animal and Experimental Design**

Adult male Swiss albino rats, each weighing 120 to 150 g, were acclimatized to conditions in the laboratory (26–28°C, 60–80% relative humidity, 12-h light/dark cycle) for 10 days before the commencement of the treatment, during which they received food (purchased from Hindustan Lever Limited, Maharashtra, India) and drinking water. Rats were divided into six groups with five animals in each group. Rats in the normal group were injected s.c. with a single dose of physiological saline (0.5 ml) at multiple sites. Rats in the NaAsO₂-treated group were injected s.c. with NaAsO₂ (100.06 μM/kg body weight in 0.5 ml of physiological saline) at five different sites of its abdominal skin by making a 10-s time gap between one injection to its next. Free drug (0.5-ml suspension of 0.2% Tween 80 aqueous solution containing 0.898 μM QC) was injected into the tail vein of a third group of rats 2 h before NaAsO₂ treatment. Liposomes with QC (0.5-ml suspension containing 0.898 μM QC), empty galactosylated liposome, or galactosylated liposome intercalated with QC (0.5-ml suspension containing 0.222, 0.445, 0.898, or 1.335 μM QC) was injected into the tail vein of rats in those different experimental groups 2 h before NaAsO₂ treatment. QC uptake by liver was estimated in the other groups of rats after 2 h of injection of free QC, liposomal QC, or galactosylated liposomal QC. For LD₅₀ determinations, a single dose of NaAsO₂ (84.67–130.8 μM/kg body weight) was injected to each group of rats s.c.

Twenty-four hours after NaAsO₂ administration, the rats of all groups were anesthetized with ether, and blood was collected from heart. Serum aspartate transaminase (AST), alkaline phosphatase (AP) (Karim et al., 2001; Tyagi et al., 2005), and serum urea and creatinine were determined using a standard kit. Animals were sacrificed immediately after collecting blood. The liver was promptly removed, and a part of the organ was immediately fixed in Bouin's fixative and processed for histological examination. All the rats used in this study received proper care in compliance with the Animal Ethics Committee, India. Liver histochemistry for collagen and liver tissue histology were studied by microscopic examination (Lin et al., 2005).

Perfusion of liver and separation of subcellular fractions were done (Sinha et al., 2000). Mitochondria were separated and purified with Percoll gradient. The purity and yield of the mitochondrial membrane fractions were assayed using standard subcellular markers (Tirmenstein and Nelson, 1989).

**Biochemical Analysis and Enzyme Assays**

**Lipid Peroxidation Assay.** Lipid peroxidation in the mitochondrial membrane was determined by measuring the amount of conjugated diene. The mitochondrial membrane was extracted twice in a chloroform-methanol mixture (2:1, v/v). The pooled extract was evaporated to dryness under nitrogen atmosphere at 25°C and redissolved in cyclohexane. Lipids in cyclohexane solvent was assayed at 234 nm, and the results were expressed as micromoles of lipohydroperoxide per milligram of protein by using an ε₉₀ of 2.52 × 10⁴ l/mol/cm (Mandal et al., 2002).

**Quantitation of Quercetin Level in Liver.** Liver homogenates of normal and experimental rats were diluted with an equal volume of absolute ethanol containing 1 μg/ml butylated hydroxyanisole. One milliliter of 0.1-heptane was added, and the whole suspension was vortexed. This sample was centrifuged at 1000 rpm for 5 min at 4°C. The heptane layer was removed, and another 1 ml of fresh n-heptane was added. The sample was centrifuged as earlier until 3 volumes of heptane were added. These volumes were combined and dried under nitrogen atmosphere. The residue was dissolved in 0.2 ml of methanol, and 20 μl was injected onto a high-performance liquid chromatography column, and quantitation of quercetin in liver homogenate was performed spectrophotometrically at ASPET Journals on April 14, 2017.
Different Enzyme Activities. Rat liver was perfused with the perfusion buffer (125 mM NaCl, 2.47 mM KH2PO4, 15 mM Na2HPO4, 5 mM NH4Cl, 34.01 mM CaCl2, 2H2O, 5 mM glucose, and 0.2% bovine serum albumin, pH 7.2) containing collagenase and CaCl2 at 37°C through the portal vein of the animal for 10 min at a rate of 10 to 15 ml/min. The tissue was chopped, homogenized in ice-cold perfusion buffer containing bovine serum albumin (2 g/l), and filtered through a nylon gauge (110 mesh). The cell suspension was centrifuged at 50g for 4 min. The pellet was resuspended, washed, and collected as hepatocytes. The supernatant was then centrifuged at 500g for 9 min to precipitate stellate cells. The isolated stellate cells were further purified with Percoll gradient. The cells were resuspended in 9.3 ml of perfusion buffer and mixed with 12.7 ml of Percoll containing 1/10 volume of 10-fold concentrated perfusion buffer. After centrifugation at 20,000g for 10 min at 4°C, the cell layer above the 1.07 g volume was carefully recovered. Thus, stellate cells were collected and re-suspended in buffer. The hepatocytes and stellate cell suspensions were homogenized at 4°C with 10 up-and-down strokes in a glass homogenizer fitted with a Teflon pestle. The homogenates were centrifuged at 105,000g for 60 min to get cytosolic fractions.

Catalase Activity. The cytosolic fractions, thus obtained, were used for enzyme activities. Catalase activity was assayed (Moragon et al., 2005). The reaction mixture contained sodium phosphate buffer (0.05 M, pH 7.0), 50 mM H2O2, and 50 μL of enzyme extract in a 3-ml volume. The activity was assayed by monitoring the decrease in absorbance at 340 nm as a consequence of H2O2 consumption and enzyme activity expressed as amount of H2O2 decomposed per minute per milligram of protein.

Glutathione Peroxidase Activity. The glutathione peroxidase (GPx) activity was determined (Sarkar and Das, 2006). The cytosol containing the enzyme source was mixed with 0.25 M potassium phosphate buffer, 25 mM EDTA, glutathione reductase, 40 mM glutathione (GSH), and 20 mM NADPH. The mixture was mixed and then incubated for 2 min at 37°C. The reaction was initiated by adding 1-butylnitroperoxide at the final concentration of 0.3 mM. The mixture was stirred, and the absorbance was read immediately at 340 nm at 1-ml intervals for 4 min. The absorbance change during the 2- to 4-min interval was used to calculate enzyme activity. The activity was determined and expressed as micromoles of NADPH oxidized per minute per milligram of protein.

Glutathione Reductase Activity. Glutathione reductase was assayed (Castro et al., 1990). A 3-ml reaction mixture contained 100 mM phosphate buffer, pH 7, 1 mM oxidized glutathione, 1 mM EDTA, 0.1 mM NADPH, and 25 to 50 μL of enzyme extract. The reaction was started by adding the enzyme extract. The rate of NADPH oxidation was followed by monitoring the decrease in absorbance at 340 nm with a recording spectrophotometer. The activity was expressed as micromoles of NADPH oxidation per minute per milligram of protein.

Glutathione S-Transferase Activity. Glutathione S-transferase activity was determined in a total volume of 1.0 ml, containing 100 mM potassium phosphate buffer, pH 6.5, and 2 mM each of GSH and 1-chloro-2,4-dinitrobenzene (final concentration). The rate of formation of chloroethylglutathione (a GSH-1-chloro-2,4-dinitrobenzene conjugate) by enzyme extract was quantified at 340 nm using the extinction coefficient of 9.6 l/mmol/cm (Maiti and Chatterjee, 2000), and the activity was expressed as nanomoles per minute per milligram of protein.

Glucose-6-Phosphate Dehydrogenase Activity. The glucose-6-phosphate dehydrogenase (G6PDH) activity was determined using a Sigma Diagnostics Kit that is based on a modification of a spectrophotometric method (Maiti and Chatterjee, 2000). G6PDH catalyzes the first step in the pentose phosphate pathway, oxidizing glucose 6-phosphate to 6-phosphogluconate and reducing NADP to NADPH. The rate of formation of NADPH is proportional to G6PDH activity and was measured spectrophotometrically as an increase in absorbance at 340 nm. One unit of G6PDH activity was defined as 1 μM NADPH produced/min.

Fluorescence Depolarization Measurements of the Fluidity of Mitochondrial Membrane

The fluorescence depolarization, associated with the hydrophobic fluorescence probe DPH, was used to monitor the changes in the fluidity of the lipid matrix accompanying the gel to liquid crystalline phase transition. Mitochondrial membrane fractions of hepatic cells were incubated at 37°C by the addition of DPH dissolved in tetra-hydrofuran (DPH/lipid molar ratio, 1:500). The excitation and the emission maxima were kept at 365 and 430 nm, respectively. The fluorescence anisotropy was calculated by using the equation:

\[ r = \frac{I_{II} - I_{I}}{I_{II} + 2I_{I}} \]  

where \( I_{II} \) and \( I_{I} \) are the fluorescence intensities in parallel and perpendicular to the direction of polarization of the excited light. The microviscosity parameters \( \langle r_o \rangle \), which for DPH is 0.362 (Sarkar et al., 2002).

Estimation of Hepatic 4-Hydroxyproline

The liver was cut into small pieces and homogenized in sufficient deionized water to yield 10% homogenate (w/v). Aliquots (2 ml) of the homogenate were added to an equal volume of 12 N HCl and hydrolyzed in Teflon-capped vials at 105°C for 18 h. 4-Hydroxyproline (4-HP) levels from those hydrolysates were measured (Sarin et al., 1999). The absorbance at 558 nm was read, and values were plotted against a standard graph using known amounts of 4-HP.

Estimation of Total Hepatic Collagen

The technique (Sarin et al., 1999) was used that is based on selective binding of Sirius Rose BB and Fast Green FCF to collagen and noncollagen components, respectively. When the sections are stained with both dyes dissolved in aqueous saturated picric acid, they are eluted readily with NaOH; simultaneously, the absorbance obtained at 550 and 625 nm can be used to determine the amount of collagen and total protein, respectively.

Arsenic Analysis

Subcellular fractions were digested with acid mixture containing nitric acid, sulfuric acid, and perchloric acid in the ratio of 6:1:1 over a regulated heater. After the digestion, the acid mixture was evaporated with occasional addition of triple distilled water, and the solution thus obtained was employed for estimation of arsenic content. Estimation was carried out using the atomic absorption spectrophotometer (Spectra AA 30/40; Varian, Inc., Palo Alto, CA) fitted with a graphite furnace (Flora et al., 1995).

Statistical Analysis

Statistical analysis was performed with Student’s t test. Linear correlations between two data were calculated by means of Pearson’s correlation coefficient r. In all instances, P < 0.05 was considered as the minimum level of significance.

Results

Acute NaAsO2-Induced Mortality in Rats. A single injection of NaAsO2 produced a dose-dependent mortality in rats (Fig. 1). Arsenite induced mortality at a dose of arsenic higher than 84.67 μM/kg b. wt., and no rats survived the dose of 130.8 μM/kg. The LD50 value for arsenic-induced lethality in the rats was 107.75 μM/kg.

Effect of QC in Galactosylated Liposome on Arsenic Deposition in Mitochondria of Different Hepatic Cells. A single dose of NaAsO2 injection (s.c.) (100.06 μM/kg b. wt.) resulted in a deposition of arsenic in mitochondria of hepatocytes and stellate cells at the level of 264 ± 24 and 252 ±
18 μg/g mitochondrial protein. The elevated arsenic level in experimental animals was found to be reduced maximally by the treatment with QC in galactosylated liposomes (Fig. 2, a and b), where arsenic content was not detected by atomic absorption spectrophotometer in normal rats.

**Correlation of Mitochondrial Arsenic Content of Hepatocytes and Stellate Cells with Lipid Peroxidation.** Figure 3a shows positive correlation ($r = 0.97, P < 0.001$) between hepatocytic mitochondrial arsenic level and its conjugated diene level. The increased stellate mitochondrial arsenic level was also found to be associated with its increased lipid peroxidation as was evident from a significant positive correlation ($r = 0.93, P < 0.001$) (Fig. 3b) between its arsenic and conjugated diene content.

**Effect of QC in Galactosylated Liposomes on NaAsO$_2$-Induced Antioxidant Enzymes, Protection in Hepatocytes, and Stellate Cells.** A single injection of NaAsO$_2$ (100.06 μM/kg b. wt.) resulted in significant depletion of antioxidant level both in hepatocytes and stellate cells (Table 1). No significant protection was observed in the case of rats treated with free QC before arsenic treatment. Liposomal QC treatment prevented thiol depletion in stellate cells completely, but QC in galactosylated liposomes treatment prevented the depletion completely both in hepatocytes and stellate cells.

**Effect of QC in Galactosylated Liposome Treatment on NaAsO$_2$-Induced Hepatic 4-HP and Collagen Contents in Rat Liver.** A single injection of NaAsO$_2$ (100.06 μM/kg b. wt.) induced an appreciable increase in hepatic collagen protein and 4-HP (Table 2). These increments of collagen and 4-HP were not reduced more by the treatment of free QC, whereas these levels were decreased appreciably by the treatment of liposomal QC. QC in galactosylated liposome injection inhibited completely the collagen protein deposition and 4-HP increase in rat liver.

**Effect of QC-Entrapped Galactosylated Liposomes on NaAsO$_2$-Mediated Hepatocellular Toxicity.** Rats treated with a single dose of NaAsO$_2$ developed significant hepatic damage as observed from elevated hepatospecific enzymes in serum. The activity of AST and AP in serum was increased in NaAsO$_2$-intoxicated animals. The serum urea and creatinine levels were also increased upon NaAsO$_2$ administration. A single injection (i.v.) of liposome-entrapped QC exerted a significant protection compared with free QC against NaAsO$_2$-induced liver toxicity. The degree of protection was observed maximally when galactose-coated liposomal QC was injected (Table 3).

**Effect of Galactosylated Liposomal QC on NaAsO$_2$-Induced Mitochondrial Membrane Microviscosity of Hepatocytes and Stellate Cells.** Arsenite induced a decrease of mitochondrial membrane microviscosity both in hepatocytes and stellate cells of rat liver. Liposomal QC treatment prevented the alteration in mitochondrial membrane microviscosity of stellate cells, whereas QC in galactosylated liposome treatment protected mitochondrial membrane of both hepatocytes and stellate cells completely from any decrease mediated by NaAsO$_2$ treatment (Table 4).

**Quantitation of Quercetin in Liver Homogenate.** In Table 5, QC levels were expressed per milligram of protein.
TABLE 1

<table>
<thead>
<tr>
<th></th>
<th>GPx</th>
<th>G6PDH</th>
<th>Glutathione Reductase</th>
<th>Catalase</th>
<th>Glutathione-S-transferase</th>
</tr>
</thead>
<tbody>
<tr>
<td>HP</td>
<td>0.31</td>
<td>9.25</td>
<td>0.17</td>
<td>4.55</td>
<td>3.92</td>
</tr>
<tr>
<td>ST</td>
<td>0.12</td>
<td>4.76</td>
<td>0.23</td>
<td>5.95</td>
<td>0.15</td>
</tr>
<tr>
<td>HP + NaAsO2 treatment</td>
<td>0.48</td>
<td>5.95</td>
<td>0.41</td>
<td>6.05</td>
<td>0.22</td>
</tr>
<tr>
<td>ST + NaAsO2 treatment</td>
<td>0.24</td>
<td>6.82</td>
<td>0.24</td>
<td>6.35</td>
<td>0.13</td>
</tr>
<tr>
<td>A + free QC-treated</td>
<td>0.31</td>
<td>8.72</td>
<td>0.33</td>
<td>11.48</td>
<td>0.31*</td>
</tr>
<tr>
<td>A + galactosylated liposomal QC-treated</td>
<td>0.59</td>
<td>22.46</td>
<td>1.21</td>
<td>32.07</td>
<td>1.65*</td>
</tr>
<tr>
<td>A + empty liposomal QC-treated</td>
<td>1.67</td>
<td>61.73</td>
<td>1.07</td>
<td>32.07</td>
<td>1.65*</td>
</tr>
<tr>
<td>A + galactosylated liposomal QC-treated</td>
<td>1.21</td>
<td>32.07</td>
<td>1.07</td>
<td>32.07</td>
<td>1.65*</td>
</tr>
</tbody>
</table>

By determining the amount of protein present in total liver homogenate, the amount of uptake of QC and QC entrapped in different types of liposomes by liver was calculated. For liposomal QC, the uptake of QC in the liver was found to be 50.29% of the injected dose, whereas only 25.61% of the injected dose was detected in the liver when free QC was injected. For galactosylated liposomal QC, the uptake in the liver was 85.16%.

**Pathomorphology and Histochemistry of the Liver.**

Hematoxylin and eosin-stained liver sections of normal rats showed (Fig. 4a) the cords of normal hepatocytes, normal-looking sinusoids lined by Kupffer cells. Central veins were normal. The histology was within normal limits, but the positive histological changes in the areas of hepatocellular and fatty metamorphosis, few focal areas of necrosis, Kupffer cell hyperplasia, and localized fibrosis in the periportal region resulted from a single injection of NaAsO2 (Fig. 4b). The induction for mild hyperplasia of Kupffer cell and the histology within normal limits seemed in the case of NaAsO2-intoxicated animals injected with galactosylated liposomal QC (Fig. 4c).

Liver sections of normal rats stained with Van Gieson exhibited very small amounts of collagen in the periportal region (Fig. 4d), but periportal region showed mild to moderate amount of collagen tissues when injected with a single injection of NaAsO2 (Fig. 4e). The maximal protection from the deposition of collagen tissues was observed when rats were treated with galactosylated liposomal QC before NaAsO2 injection (Fig. 4f).

**Discussion**

Exposure to the metalloid arsenic has become an increasingly recognized source of illness worldwide. Arsenic has a direct toxic effect on cellular respiration in liver mitochondria with an evidence of oxidative stress and hepatic collagenesis in human (Pi et al., 2002; Das et al., 2005). Hepatic fibrosis is a disease state characterized by exuberant synthesis and deposition of collagen in the extracellular matrix. Fibrogenesis is expressed by an increase in the hepatic hydroxyproline levels (Testa et al., 1993). The present observation also indicates that fibrogenesis could be induced with an increment of 4-HP by a single injection (s.c.) of NaAsO2 (100.06 μM/kg b. wt.) (Table 2). The exact mechanism of
Liposomal Flavonoid Protects Arsenite-Induced Liver Fibrosis

Table 3: Effect of liposomal-encapsulated QC injection (i.v.) on blood serum biochemical parameters in NaAsO2-induced hepatocellular injury

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal</th>
<th>NaAsO2-Treated Group (A)</th>
<th>Experimental Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A + Empty Galactosylated Liposome-Treated</td>
<td>A + Free QC-Treated (8.9 μM/kg b. wt.)</td>
</tr>
<tr>
<td>Alkaline phosphatase (U/l)</td>
<td>280 ± 11.7</td>
<td>818 ± 36.6*</td>
<td>808 ± 16.50</td>
</tr>
<tr>
<td>Serum aspartate</td>
<td>36.86 ± 2.16</td>
<td>89.71 ± 6.42*</td>
<td>85.52 ± 4.51</td>
</tr>
<tr>
<td>transaminase (U/l)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urea (g/l)</td>
<td>0.45 ± 0.04</td>
<td>1.48 ± 0.08*</td>
<td>1.40 ± 0.02</td>
</tr>
<tr>
<td>Creatinine (mg/l)</td>
<td>13.4 ± 1.84</td>
<td>74.17 ± 5.49*</td>
<td>72.43 ± 2.67</td>
</tr>
</tbody>
</table>

* P < 0.001.

Table 4: Effect of QC in galactosylated liposome treatment on the NaAsO2-induced mitochondrial membrane microviscosity, ([r2/τ − 1]−1) of rat hepatocyte (HP) and stellate (ST) cells

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HP</th>
<th>ST</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0.677 ± 0.073</td>
<td>0.687 ± 0.024</td>
</tr>
<tr>
<td>NaAsO2-treated (A)</td>
<td>0.216 ± 0.021*</td>
<td>0.274 ± 0.019*</td>
</tr>
<tr>
<td>A + empty galactosylated liposome-treated</td>
<td>0.231 ± 0.018*</td>
<td>0.286 ± 0.027*</td>
</tr>
<tr>
<td>A + free QC-treated</td>
<td>0.340 ± 0.089*</td>
<td>0.382 ± 0.048*</td>
</tr>
<tr>
<td>A + liposomal QC-treated</td>
<td>0.312 ± 0.099</td>
<td>0.606 ± 0.052**</td>
</tr>
<tr>
<td>A + QC in galactosylated liposome-treated</td>
<td>0.661 ± 0.045**</td>
<td>0.666 ± 0.061**</td>
</tr>
</tbody>
</table>

*P < 0.001 significantly different from normal.

**P < 0.001 significantly different from NaAsO2-treated.

Arsenic toxicity was reported as an inhibitory effect on cellular respiration at the level of mitochondria (Stanton et al., 2006). In our observations, the increased arsenic deposition in hepatocytes and stellate cell mitochondria from NaAsO2-treated rats are generating more ROS than normal animals (Fig. 2; Table 1). When arsenic deposition in mitochondria is prevented by galactosylated liposomal QC treatment, NaAsO2-induced fibrogenesis is markedly reduced. Maintenance of mitochondrial membrane microviscosity, i.e., reciprocal of membrane fluidity of hepatocytes and stellate cells, could be achieved by the protective action of QC in galactosylated liposomes in cases where significant fall of mitochondrial membrane microviscosity takes place by the induction of NaAsO2 (Table 4).

Previous investigators have shown a deviation of the pro-oxidant/antioxidant balance in NaAsO2-treated rats with development of oxidative damage and a reduction in antioxidant status (Das et al., 2005). We also observed that liver injury was accompanied by the accumulation of arsenic with impaired activity and depletion of antioxidant status in NaAsO2-induced rats. Reduction of liver injury was noticed by the improvement of antioxidant status with an increased concentration of QC and a marked reduction of arsenic content in liver by QC in galactosylated liposomes.

Our results indicate that galactosylated liposomal QC prevents arsenic deposition and protects liver from NaAsO2-induced collagen deposition and fibrogenesis (Fig. 2; Table 2). Administration of QC in galactosylated liposomes to rats protects those animals from arsenic-induced liver fibrosis, whereas free QC does not. It is expected that QC in galactosylated liposomes may be more protective than free QC or liposomal QC because of the enhanced intracellular accumulation of QC by selective tissue targeted delivery of galactosylated liposomes (Table 5). We demonstrated a positive correlation between arsenic accumulation and lipid peroxidation level in liver (Fig. 3) as reported earlier (Ramos et al., 1995). A single injection (s.c.) of NaAsO2 (100.06 μM/kg) to rats resulted in impairment of antioxidant status with a marked increase in arsenic content in liver. Our observation suggests that the mechanism of the protective effect of galactosylated liposomal QC against arsenic-induced liver injury could be related primarily to the reduction of arsenic accumulation in liver.

As the levels of AST and AP increase in NaAsO2-induced hepatocellular injury, it seems that targeting of QC prevents hepatic cells against NaAsO2-induced membrane damage by decreasing the leakage of AST and AP in the circulation (Table 3). The protective role of QC in galactosylated lipo-

fibrogenesis by arsenic is not known. Arsenic induces the production of lactic acid because of an imbalance in cellular energy metabolism, and the enol acid thus utilized by cells significantly increases the intracellular proline pool and collagen synthesis by stimulating the activity of prolyl hydroxylase.

A greater number of drugs including arsenic specific antidotes have been tested to reduce hepatic damage or necrosis and to inhibit liver fibrogenesis. However, none of them is liver-specific or cell type-specific (Guha Mazumder et al., 2001). Quercetin, a polyphenolic flavonoidal compound has been suggested in preventing the development of hepatic fibrosis (Lee et al., 2003). It is also known to reduce toxicant-induced liver damage (Peres et al., 2000). The protective role of QC in galactosylated liposomes against carbon tetrachloride-induced hepato cellular damage has been shown in our previous observation (Mandal and Das, 2005). Hepatospecific liposomal QC (galactosylated liposome) with a high incorporation rate in hepatocytes has been formulated by us and tested in reducing NaAsO2-induced liver fibrogenesis and hepato cellular damage. The increased hepatoprotective effect of galactosylated liposome-encapsulated QC compared with its free form that has been shown in this report against NaAsO2-induced liver damage could be explained by the observation of another group (Datta et al., 2003). They demonstrated that the liposome-entrapped compound interacts with target cells at a much faster rate than that of free components. Moreover, by utilizing the galactosyl receptor of hepatocytes, galactosylated liposomes could be targeted to those nonphagocytic cells (Kawakami et al., 2001) with a simultaneous delivery to other types of hepatic cells of natural phagocytic activity.
sommes against NaAsO₂-induced hepatotoxicity has also been confirmed by our histopathological and histochemical analysis (Fig. 4). This approach of delivering a nontoxic herb origin polyphenolic compound QC selectively to the liver might be useful in therapeutic application to prevent NaAsO₂-induced liver fibrogenesis. Further studies are planned to perform subchronic and chronic studies to substantiate our claims that QC might be useful in therapeutic application in combating NaAsO₂-induced fibrogenesis resultant from chronic arsenic exposure.

Acknowledgments
We acknowledge Suman Bhattacharya (University of Pittsburgh) for cooperation.

References

TABLE 5
Quercetin level in liver homogenate from rats treated with QC (in Tween 80), liposomal QC, and galactosylated liposomal QC
Rats received 8.9 μM/kg b. wt. i.v. injection of both of the free QC and liposomal QC and galactosylated liposomal QC (QC1, 2.22 μM/kg b. wt.; QC2, 4.45 μM/kg b. wt.; QC3, 8.90 μM/kg b. wt.; QC4, 13.35 μM/kg b. wt.).

<table>
<thead>
<tr>
<th>Free QC</th>
<th>Liposomal QC</th>
<th>Galactosylated Liposomal QC</th>
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<tr>
<td>QC concentration in liver homogenate (nM/mg protein)</td>
<td>0.83 ± 0.17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.63 ± 0.32&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Injected QC in whole liver (%)</td>
<td>25.61</td>
<td>50.29</td>
</tr>
</tbody>
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<sup>a</sup> Values expressed as mean ± S.D. (n = 5).
<sup>b</sup> Values are significantly different (P < 0.001) from free QC.

Fig. 4. Histopathological examination of Hematoxylin and eosin-stained liver section of normal and experimental rats with magnification ×400. a, physiological saline-treated control. b, NaAsO₂ treated. c, NaAsO₂ + galactosylated liposomal QC (8.9 μM/kg b. wt.) treated. Histochemical examination of Van Gieson-stained liver section of normal and experimental rats with magnification ×400. d, physiological saline-treated control. e, NaAsO₂-treated. f, NaAsO₂ + galactosylated liposomal QC (8.9 μM/kg b. wt.) treated. ▲, fibrosis; ▲▲, fatty metamorphosis; ▲▲▲, necrosis.
Liposomal Flavonoid Protects Arsenite-Induced Liver Fibrosis


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