CHAPTER-7

Encapsulation of the flavanoid Quercetin with an arsenic chelator into nanocapsules enables the simultaneous delivery of hydrophobic and hydrophilic drugs with a synergistic effect against chronic arsenic accumulation and oxidative stress.
Encapsulation of the flavanoid Quercetin with an arsenic chelator into nanocapsules enables the simultaneous delivery of hydrophobic and hydrophilic drugs with a synergistic effect against chronic arsenic accumulation and oxidative stress

INTRODUCTION

Inorganic arsenic is a major environmental contaminant in parts of Asia, South America, India, Europe as well as some states in the USA or Canada. Chronic exposure of humans to high concentration of arsenic in drinking water is associated with skin lesions, peripheral vascular disease, hypertension, Blackfoot disease, liver fibrosis and a high risk of cancer. (National Research Council 2001) Arsenic is recognized as a potent carcinogen in humans. (Valko et al., 2006) Chronic exposure to arsenic produces damaging effects on the liver (Das et al., 2005) and nervous system. (Chattopadhyay et al., 2002) The toxic manifestations of arsenic are partly due to oxidative stress since Arsenic has a high affinity for thiol groups of functional proteins. Reports have established the fact that free radicals are generated during arsenic toxicity. (Yamanaka et al., 2001) Superoxide anion, arising through metabolic processes, is considered to be the primary reactive oxygen species (ROS), and can either directly or prevalently through enzyme or metal catalyzed processes further interact with other molecules to generate secondary ROS. (Fridovich, 1986) Arsenic mediated ROS generation causes a massive depletion of antioxidant enzymes, damage to lipid bilayer and DNA. (Chen et al., 2001) Corsini et al., 1999 demonstrated mitochondria to be the main source of intracellular ROS produced by arsenite. Oxidative stress generated by arsenicals in conjunction with calcium loading also alters mitochondrial permeability, creating nonspecific pore in the inner membrane rendering the mitochondrial inner membrane permeable to solutes smaller than 1.5 kDa thus preventing oxidative phosphorylation. (Larochette et al., 1999) The most conventional approach by medical practitioners for the treatment of arsenic toxicity is the use of synthetic chelators such as DMSA (meso-2,3-Dimercaptosuccinic acid). DMSA contains two sulfhydryl (-SH) groups and is an effective chelator of toxic metals such as lead or arsenic.

While DMSA is a partially effective chelator of arsenic and reduces the arsenic accumulation in tissues, it has untoward side effects like gastrointestinal disorders over the long term use. (Smith, 2000; Mann and Travers 1991) In a double blind, randomized
controlled study in selected patients with arsenic exposure in West Bengal (India) with oral administration of DMSA, it was observed that DMSA not effective in producing any clinical or biochemical benefits, which calls into question our current therapeutic approach. (Guha Mazumder et al., 1998). Importantly, the tissue distribution of DMSA seems to be restricted to extracellular fluid. (Kalia and Flora 2005) Since arsenic exposure leads to oxidative stress (Shi et al., 2004) a complementary approach could be the use of antioxidants as well as an arsenic chelator as combined therapy. Quercetin, (QC) a polyphenolic flavanoid is a good anti-oxidant and protects cells from reactive oxygen species (Kim and Jang 2009). However, the clinical use of quercetin is limited due to its hydrophobic nature with low and slow bioavailability (Hollman et al., 1997; Biasutto et al., 2010). Hence an improved delivery mode of quercetin may be an effective approach to deliver quercetin to tissues.

Nanocapsules are a novel mode of drug delivery and which currently appear to be nontoxic, biodegradable, non immunogenic, and with sustained drug releasing ability in biological systems. Quercetin in liposomes and nanocapsules are effective in preventing liver and brain injury caused by arsenic toxicity (Ghosh et al., 2009; Ghosh et al 2010) In the current study we have evaluated the efficacy of co-encapsulated quercetin and DMSA and compared it to nano-encapsulated quercetin, nano-encapsulated DMSA, as well as the conventional delivery methods for each agent in a rat model of arsenic toxicity. In brief, we found that the co-encapsulated form of quercetin and DMSA offer the best protection and recovery from arsenic toxicity improved all parameters of oxidative stress and improved liver histology. We conclude co-encapsulation of quercetin and DMSA presents a novel strategy of combining both fat soluble and water soluble drugs which gives the best of both worlds.

MATERIALS AND METHODS

Materials

PLGA (Resomer RG 85:50H), 2,6-dichloroindophenol (DCIP), Phenazine methosulfate (PMS), Succinic acid and Didodecyldimethylammoniumbromide (DMAB) Quercetin and DMSA were purchased from Sigma Aldrich (St. Louis, MO, USA) Ethyl acetate (AR Grade) was purchased from Rankem Fine Chemicals (New Delhi, India). Chloroform and Methanol were purchased from E. Merck. All other reagents were of analytical grade.
Nanocapsulated QC and DMSA preparation

A modified emulsion-diffusion-evaporation method (Hariharan et al., 2006) was used to make Quercetin and/or DMSA nanocapsules. QC was dissolved in ethyl acetate while DMSA in water. In brief, 36 mg of PLGA was dissolved in 2.5 ml of ethyl acetate at room temperature. The organic solution of PLGA and drug in ethyl acetate was then emulsified with 5 ml of an aqueous phase containing didodecyldimethylammonium bromide (DMAB) and DMSA. The resulting o/w emulsion was stirred at room temperature for 3 h before homogenizing at 15,000 rpm for 5 min with a high speed homogenizer (Polytron PT4000; Polytron Kinematica, Lucerne, Switzerland). The organic solvent was removed by constant stirring on a water bath set at 40°C. The suspension was ultracentrifuged at 105,000 g in Sorval RC 5B Plus using the rotor Sorval T-865 for 1 h. The pellet of nanocapsules was washed with PBS twice and re-suspended in 2 ml PBS.

Nanocapsule characterization using atomic force microscopy

The atomic force microscopy (AFM) observations were performed with an Agilent Technologies, 5500 Pico Plus AFM system. All the images were obtained with the Aquastic mode using cantilevers having resonance frequency 146–236 kHz, Tip height 10–15 μm and Tip length 225μm. Mica was chosen as a solid substrate and used immediately after cleavage in a clean atmosphere. During the characterization experiment, the probe and cantilever were immersed completely in the water solution. The nanocapsule suspensions on mica were dried in air (65% humidity) for 30 min. Images were captured and analysed using Picoscan 5.33 software of Molecular Imaging Corporation (Ruozi et al., 2007).

Experimental design

Female Wister rats, each weighing approximately 100-120 g were acclimatized to conditions in the laboratory (20-22°C, 60-80% relative humidity, 12-hr. light/dark cycle) for 7 days prior to the commencement of the treatment during which they received food (purchased from Hindustan Lever...
Limited, Maharashtra, India) and drinking water. Acute lethal dose 50 (ALD$_{50}$) via oral route in rats was found to be 49 mg/kg body weight of sodium arsenite. The rats were divided into seven groups and each group was made with 5 animals. Animals in Group A were kept as untreated normal whereas group B animals were considered as Arsenic treated experimental control and one-fifth of ALD$_{50}$ of sodium arsenite (i.e. approximately 10 mg/kg body weight) was given daily for 16 weeks by oral gavage. All animals except Group A were treated identically for 16 weeks, and then various treatments were started for 8 weeks. After 16 weeks NaAsO$_2$ administration animals in Group C were fed with free QC (8.98 μmole/kg body weight of QC in 0.2% tween 80 aqueous solution). Group D received DMSA solution orally (8.98 μmole/kg body weight of DMSA in water); Group E and F were fed nanocapsulated formulation of QC and DMSA (containing 8.98 μmole/kg body weight of either QC or DMSA) respectively for 8 weeks. Group G received coencapsulated QC: DMSA in equimolar proportion (8.98 μmole total) in nanocapsulated form. Animals were drug treated twice a week for 4 weeks. All animals were kept with normal diet and drinking water without any treatment for next 2 weeks. At the end of twenty six weeks, rats of the each group were anesthetized with ether, sacrificed and brain and liver dissected out promptly and washed with cold physiological saline and immediately used for mitochondria and sub mitochondrial particle preparation.

**Measurement of arsenic level in liver and brain of rats**

Total arsenic accumulation in liver and brain homogenate were measured by flow injection atomic absorption spectrometer (Spectra AA 30/40; Varian, Inc., Palo Alto, CA) fitted with a graphite furnace. (Mandal et al., 2007)

**Liver and Brain Mitochondria isolation**

Liver and brain mitochondria of experimental animals were isolated using differential centrifugation the method of Navarro and Boveris 2004.

**Mitochondrial ROS measurement**

Intracellular ROS level was measured in liver and brain mitochondria (Betainder et al., 2002). Fluorescence was measured through a spectrofluorometer (LS 3B, Perkin Elmer, USA) by using 499 nm as excitation and 520 nm as emission wavelengths. The data were normalized to normal values, and the normal was expressed as a value of 100%.
Lipid peroxidation assay

Lipid peroxidation in the mitochondrial membrane was determined by measuring the amount of conjugated diene. 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 were assayed at 234 nm, and the results were expressed as micromoles of Lipohydroperoxide per milligram of protein by using a ε of 2.52x10⁴ l/mol/cm. (Recknagel and Glende 1984). Proteins were measured as per Lowry et al., 1951.

Mitochondrial membrane fluidity

The fluorescence depolarisation, associated with the hydrophobic fluorescence probe diphenyl hexatriene (DPH), was used to monitor the changes in the fluidity of the lipid matrix accompanying the gel to liquid crystalline phase transition. The fluorescence anisotropy was calculated by using the equation, \[ r = \frac{(I_\parallel - I_\perp)}{(I_\parallel + 2I_\perp)} \] where \( I_\parallel \) and \( I_\perp \) are the fluorescence intensities in parallel and perpendicular to the direction of polarization of the excited light. The micro viscosity parameters \( [(r_0/r) - 1]^{-1} \) were calculated in each case, knowing the maximal limiting fluorescence anisotropy \( r_0 \) which for DPH is 0.362. (Mandal et al., 2007)

Reduced glutathione (GSH) assay

GSH level of a part of tissue homogenate was determined by the method of Davila et al. 1991 (Davila et al., 1991) with the help of a spectrophotometer and using tetrachloroacetic acid with EDTA as protein precipitating reagent.

Succinate Dehydrogenase activity

The enzyme Succinate Dehydrogenase activity was assayed at 30°C by the PMS-mediated reduction of DCIP in a final volume of 1 ml containing 0.01 ml of appropriately diluted enzyme, 0.78 ml of 50 mM Tris hydrochloride (pH 8.2), 0.1 ml of 1.5 mM DCIP containing 10 mM KCN, and 0.1 ml of PMS (1 mg/ml). The reaction was initiated with 0.01 ml of 0.5 M sodium succinate (pH 8.0), and the decrease in the absorbance of DCIP was measured at 600 nm in a Reyleigh UV 2601 spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme that reduced 1 μmol of DCIP per min with an extinction coefficient of 21 mM⁻¹ cm⁻¹. Specific activity is expressed as units per milligram of protein.
NADH oxidase activity assay
NADH oxidase activity was measured (Elingold et al., 2008) at 30 °C in an incubation mixture (final volume 1.5 ml) containing 50mM Na$_2$HPO$_4$-KH$_2$PO$_4$ (pH 7.4). The reaction was started by adding 0.125mM NADH to a preparation containing submitochondrial particles (0.2 mg/ml). Enzyme activity was followed by the oxidation of NADH at 340 nm ($\varepsilon = 6.22$mM$^{-1}$ cm$^{-1}$).

Mitochondrial membrane potential
Mitochondrial membrane potential was measured by rhodamine 123 fluorescence (Elingold et al., 2008). The reaction mixture contained: mitochondria (1–2 mg protein/ml), 0.5mM ADP, 5mM l-malate, 5mM l-glutamate, 10mM succinate and 3μM rotenone in respiration buffer containing 240mM sucrose, 34mM KCl, 5mM MgCl$_2$, 1mM EDTA, 9mM HCl-Tris, and 6mM Na$_2$HPO$_4$–KH$_2$PO$_4$ (pH 7.4). Rhodamine 123 (1μM) was added to mitochondria at 37 °C and after 10 min incubation, the reaction mixture was centrifuged at 6800xg. Rhodamine 123 accumulation in mitochondria was determined by the difference between the fluorescence in the supernatant and in a solution of rhodamine 123 (1 μM). Fluorescence was measured in a Perkin Elmer LS3B spectrofluorometer, at 498 nm excitation and 525 nm emission.

In situ DNA fragmentation assay
Formalin fixed, paraffin embedded glass slide mounted liver and brain sections of experimental rats were stained with Anti-BrdU; FITC antibody using the Apo- BrdU In situ DNA fragmentation assay kit (Biovision K401-60) as per the manufacturer’s protocol.

Histological examination of liver and brain of experimental animals
Liver and brain portions of experimental animals were removed quickly at the time of sacrifice and further fixed overnight in 4% paraformaldehyde at 4 °C. Postfixed portions were embedded in paraffin, and 5 μm sections were cut on a microtome. The sections were stained with Hematoxylin-Eosin (HE), Reticulin and Congo Red and examined under a light microscope.

p53 and cytochrome c immunoblotting
Liver and brain tissues were homogenized (1:10, w/v) in 10mM Tris-HCl, pH 7.4, 150mM NaCl, containing freshly added protease and phosphatase inhibitors and cytosolic fractions were prepared by centrifugation at 15,000 g for 10 min at 4°C. SDS/PAGE was performed by subjecting 30 μg total protein under reducing conditions on 10% polyacrylamide gels.
followed by electrophoretic transfer to Polyvinylidene Fluoride (PVDF) membrane (Millipore) at 15 V for 20 min by using Semi dry (Bio-Rad) transblot apparatus. Membrane was blocked in 4% BSA in PBS (overnight) at 4°C, followed by incubation with the primary rabbit anti Cytochrome C and p53 antibodies separately for 3 h and then in secondary alkaline phosphatase conjugated anti rabbit goat IgG antibody (1:1000) for 1 h 30 min. Bands were visualized by the development of colour using Sigma premixed BCIP/NBT substrate solution. Their pixel density was analysed with Image J software system.

**Statistical analysis**

The mean and standard deviation were calculated for all data. Significant differences between means were evaluated by an analysis of variances. A difference was considered significant when $p<0.05$.

**RESULTS**

**Table 1: Nanocapsule composition, size and their percentage encapsulation**

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>% Encapsulation</th>
<th>Mean particle size (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank PLGA Nanocapsules</td>
<td>-</td>
<td>8 ± 2.3</td>
</tr>
<tr>
<td>PLGA – Quercetin Nanocapsules</td>
<td>68 ± 3.2</td>
<td>13.28 ± 7.8</td>
</tr>
<tr>
<td>PLGA – DMSA Nanocapsules</td>
<td>57 ± 4.6</td>
<td>22.33 ± 5</td>
</tr>
<tr>
<td>Co encapsulated PLGA- Quercetin:</td>
<td>62 ± 8.5</td>
<td>43.66 ± 27</td>
</tr>
<tr>
<td>DMSA Nanocapsules</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Chapter-7 Co-encapsulated QC and DMSA nanocapsules against chronic As toxicity

Fig 1: AFM images of nanocapsules

Nanocapsule characterisation

The nanocapsules obtained were in the size range 8-44nm (Table 1) The blank nanocapsules had least mean size whereas the N(QC+DMSA) nanocapsules showed largest size amongst the four types of particles studied under AFM. (Fig 1) The order of particle size is NQC<NDMSA<N(QC+DMSA). The AFM images revealed that nanocapsules have more spherical shape with narrow size distribution. Encapsulation of QC and DMSA in their respective nanocapsules was 68% and 57% respectively while that of combined QC and DMSA was 62%. Encapsulation of drug increased the size of nanocapsules. Percentage encapsulation of a drug delivery system determines the amount of drug and excipient a dose incorporates. Percentage encapsulation was found higher in QC than DMSA or the co encapsulated particles. Percentage encapsulation information was used to calculate the amount of nanocapsule suspension to be administered to the animals.

Arsenic accumulation in rat liver and brain

Chronic sodium arsenite exposure (10 mg/kg b. wt.) daily for 16 weeks resulted in a deposition of arsenic in liver and brain of rats at the level of 9.5 and 4.8 μg /g tissue. (Fig. 2) Arsenic content was not detected by atomic absorption spectrophotometer in normal rats. For a 200-250g (a body weight achieved through the treatment regimen) rat having a liver weight of 8.52- 8.6 g, amount of arsenic accumulation in the organ is 77.04μg or 1.02 μmoles of arsenic. Since 2 nmoles of arsenic can be bound to 4 nmoles DMSA, 1.02
μmoles of arsenic can be bound by 2.04 μmoles of DMSA. Since we are applying 8.98μmole/ kg body weight i.e. for a 200 g rat it comes out to 1.8 μmoles for a single dose. A biweekly treatment for 4 weeks results in administration of approximately 14.4 μmoles DMSA. This amount is capable of chelating 7.2 μmoles of arsenic. But application of DMSA at this dose could not chelate significant amount of arsenic from the organs. The elevated arsenic level in experimental animals was found to reduce moderately by the treatment with NQC (7.4μg and 3.3μg/g of liver and brain respectively) and NDMSA (7.2μg and 2.9μg/g of liver and brain respectively). However synergistic action was found by the application of N(QC+DMSA) post arsenic exposure where arsenic level was found to be 2.2 and 1.5 μg/g in liver and brain respectively.

![Fig-2](image-url)  
Fig.2: Arsenic content in liver and brain of experimental rats. NaAsO₂ treated control groups were compared with normal animals and drug treated groups were compared with NaAsO₂ control group. Values are mean ± SEM for 5 rats. * indicates p<0.001, ‡ indicates p<0.05.

**ROS level in rat liver and brain mitochondria**

The fluorescence intensity produced by H₂DCFDA on oxidation to H₂DCF is proportional to the amount of ROS produced by the mitochondrion. Chronic exposure of experimental animals to inorganic arsenic causes a 2.5 fold increase in mitochondrial ROS generation in liver and brain. Animals treated with NQC and NDMSA showed a significant decrease in mitochondrial ROS level (p< 0.05). N(QC+ DMSA) offered synergistic benefit (p<0.001) against Arsenic induced ROS generation in mitochondria. (Fig. 3a)
Conjugated diene generation by chronic NaAsO₂ exposure in rat liver and brain mitochondria

Conjugated diene generated as a result of lipid peroxidation caused by arsenic induced oxidative stress was studied. Arsenite exposed control animals showed a significant increase (p<0.001) in mitochondrial diene level both in liver and brain than normal animals. NQC could decrease diene level to 13.36 and 8.12 µmol/mg protein (p<0.01) in both the organs and NDMSA could bring the level to 13.57 and 8.06 µmol/mg protein (p<0.01) in liver and brain respectively. However maximal protection was achieved through N(QC+DMSA) treatment post arsenic exposure depicting synergistic behaviour (p<0.001) of the formulation. (Fig. 3b)

Regulation of GSH by Arsenic

Rats exposed to Arsenic showed a marked reduction in liver and brain mitochondrial GSH level from 15.12 to 6.65 µg/mg protein and 11.06 to 4.42 µg/mg protein respectively (Fig 3c). No significant protection was observed in the case of rats treated with free QC and DMSA or blank nanocapsules. However NQC and NDMSA treatment increased the GSH levels as compared to Arsenic treated (p<0.01) but a synergistic effect showing higher level of significance (p<0.001) was observed in case of rats treated with N(QC+DMSA).

Mitochondrial respiratory complex enzyme activities

The mitochondrial respiratory complex I has a NADH oxidase activity while the complex II is a Succinate Dehydrogenase. NaAsO₂ treated rats showed a decreased activity of SDH.
with an increase in NADH oxidase than their normal counterparts. (Table 2) Only NQC or NDMSA treatment was unable to bring the levels close to the control values. However these levels were found to be closer to the normal values when rats were fed with N(QC+DMSA) post arsenic administration.

**Table 2: Effect of chronic Arsenic (Sodium Arsenite) exposure on rat liver and brain SDH and NADH oxidase activity and its regulation by NPQC, NPDMa and NP (QC+DMSA)**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Succinate Dehydrogenase (μl DCIP reduced/min/mg pr)</th>
<th>NADH Oxidase (nmole oxidized NADH/min/mg pr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver</td>
<td>Brain</td>
</tr>
<tr>
<td>Normal</td>
<td>1.62 ±0.04</td>
<td>1.55 ±0.03</td>
</tr>
<tr>
<td>Na-arsenate treated (A)</td>
<td>0.71 ±0.03*</td>
<td>0.53 ±0.06*</td>
</tr>
<tr>
<td>A + QC</td>
<td>0.70 ±0.04</td>
<td>0.51 ±0.05</td>
</tr>
<tr>
<td>A + DMSA</td>
<td>0.76 ±0.05</td>
<td>0.54 ±0.05</td>
</tr>
<tr>
<td>A + NQC</td>
<td>0.93 ±0.06*</td>
<td>0.78 ±0.07*</td>
</tr>
<tr>
<td>A + NDMSA</td>
<td>0.95 ±0.06*</td>
<td>0.82 ±0.06*</td>
</tr>
<tr>
<td>A + N(QC+DMSA)</td>
<td>1.32 ±0.05*</td>
<td>1.21 ±0.08*</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SE. NaAsO₂ treated group was compared with normal and experimental groups were compared with NaAsO₂ treated group. * indicate P<0.001, # indicate p< 0.05

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Table 3: Effect of chronic Arsenic (Na-arsenite) exposure on rat liver and brain mitochondrial membrane microviscosity \([(r_o/r -1)^{-1}]\) and its regulation by NQC, NDMSA and N(QC+DMSA)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mitochondrial Membrane Microviscosity ([(r_o/r -1)^{-1}])</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver</td>
</tr>
<tr>
<td>Normal</td>
<td>0.69 ± 0.08</td>
</tr>
<tr>
<td>Na-arsenite treated (A)</td>
<td>0.22 ± 0.01*</td>
</tr>
<tr>
<td>A + QC</td>
<td>0.28 ± 0.01</td>
</tr>
<tr>
<td>A + DMSA</td>
<td>0.25 ± 0.02</td>
</tr>
<tr>
<td>A + NQC</td>
<td>0.38 ± 0.07#</td>
</tr>
<tr>
<td>A + NDMSA</td>
<td>0.41 ± 0.07#</td>
</tr>
<tr>
<td>A + N(QC+DMSA)</td>
<td>0.64 ± 0.09*</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SE. NaAsO₂ treated group was compared with normal and experimental groups were compared with NaAsO₂ treated group. * indicate P<0.001, # indicate p<0.05

Arsenic induced mitochondrial membrane microviscosity changes

Arsenic induced a decrease of mitochondrial membrane microviscosity from 0.692 to 0.217 in liver and 0.582 to 0.200 in brain of experimental rats. (Table 3) NQC and NDMSA treatment reduced the alteration in mitochondrial membrane microviscosity of hepatic and neuronal cells (p<0.05), whereas N(QC+DMSA) treatment protected mitochondrial membrane of both liver and brain cells completely (p<0.001) from any decrease mediated by Arsenic consumption.

Mitochondrial membrane potential dissipation

The effect of Arsenic on mitochondrial membrane potential was investigated in rat liver and brain mitochondria. 10 mg/kg b.wt. of NaAsO₂ consumption for 16 weeks decreased mitochondrial membrane potential from 0.4 to 0.153 in liver and 0.35 to 0.136 in brain using succinate as substrate. (Fig 4) On the other hand animals treated with N(QC+DMSA) post Arsenic exposure showed a significant increase in mitochondrial
membrane potential (p < 0.001). Considerable repression in membrane dissipation (p < 0.05) was achieved through NQC and NDMSA treatment in liver mitochondria. However, the same did not produce any significant effect in brain mitochondria.

Fig. 4: Mitochondrial membrane potential values in liver and brain mitochondria of experimental rats.

NaAsO_2 treated control groups were compared with normal animals and drug treated groups were compared with NaAsO_2 control group. Values are mean ± SEM for 5 rats. * indicates p<0.001, † indicates p<0.05 and ‡ indicates p<0.01.

Fig. 5: Correlation between arsenic accumulation in liver and brain tissue and mitochondrial membrane potential in the respective organs in experimental rats.
DNA fragmentation assay

We observed increased BrdU positive cells indicating apoptosis in liver and brain of NaAsO₂ treated control animals in comparison to the normal group. (Fig 6) N(QC+DMSA) showed visibly less apoptotic cells in both liver and brain sections when compared to NaAsO₂ treated group.

Fig. 6: Representative photomicrographs (20X) of BrdU positive cells observed by double staining with BrdU (under FITC filter) and PI staining of liver and hippocampal slices of normal, NaAsO₂ treated and NaAsO₂ + N(QC+DMSA) treated animals.

Histopathology of liver and brain

Histopathological examination revealed NaAsO₂ induced changes in liver and brain of experimental animals. Liver sections show loss of liver architecture, dilated and congested central veins with depletion of portal tract. Interportal bridging fibrosis (Fig 7b) and fatty deposition with dividing nucleus (Fig 7c) were prominent negative changes observed in
the liver of NaAsO₂ exposed control animals. Liver sections were stained with Reticulin stain to confirm fibrosis. (Fig 7j) N(QC+DMSA) treated animals show normal portal tract (Fig 7d) and lack of fatty deposition (Fig 7e) with HE and visibly less black regions on reticulin staining. (Fig 7k)

Brain sections of NaAsO₂ exposed animals show glial tissue degeneration and degenerative astrocytes and a pink stain deposition with HE. (Fig 7g) When investigated with Congo Red (image not shown) staining showed no apple green autofluorescence, depicting the depositions not to be amyloid fibrils and confirmed them to be necrotic lesions with few acute inflammatory cell infiltrations. N(QC+DMSA) showed marked improvement with no signs of deposition and necrotic lesions. However normal orientation of neurons was not observed. (Fig 7h)

![Liver (HE staining) Brain (HE staining) Liver (Reticulin staining)](image)

Fig. 7: Representative photomicrographs of HE stained liver and brain sections of experimental animals. 8a showing liver section of normal rats having proper arrangement of hepatic cords (10x) 8b showing interportal bridging fibrosis (10x) and fatty deposition with dividing nucleus (8c, 40x) in liver sections of NaAsO₂ exposed rat. N(QC+DMSA) treated animals show normal portal tract (8d, 10x) and lack of fatty deposition. (8e, 40x) Hippocampal slice of brain of normal rat showing regular neuronal arrangement. (8f, 10x) Brain sections of NaAsO₂ exposed animals show glial tissue degeneration and degenerative astrocytes and a pink stain deposition with HE. (8g, 10x) N(QC+DMSA) showed marked improvement with no signs of deposition and necrotic lesions. (8h, 10x)
Cyt c release in the cytosol AND p53 translocation to mitochondria

Release of cyt c, from the mitochondrial membrane to the cytosol is a key event in cell apoptotic signalling following cell death. Rats when treated with 10 mg/kg b.wt of NaAsO₂ showed increase of cyt c release in liver and brain cytosol respectively above the level of normal group of rats as detected by Western blot. N(QC+DMSA) treatment following As exposure hold down the release with respect to the NaAsO₂ treated control group of rat liver and brain cytosol. However neither NQC nor NDMSA could produce any significant difference in cyt c expression in liver and brain cytosol when compared to NaAsO₂ treated control. (Fig 8)

p53 immunoreactivity was evident as a band in mitochondrial fractions from both liver and brain (Fig 8). Expression of p53 in mitochondrial fraction indicates its translocation of mitochondria from the cytosol. P53 protein in mitochondria of liver and brain of NaAsO₂ treated animals was strongly expressed. NQC and NDMSA gave visibly less dense immunoreactive bands than NaAsO₂ treated control. N(QC+DMSA) treated group showed significantly less accumulation of p53 protein.

Fig.8: Western Blot analysis of cytochrome c protein expression in cytosolic fraction and p53 expression in mitochondrial fraction of liver (I) and brain (II) tissues of experimental rats: Histogram showing representative pixel intensities (arbitrary units of densitometric analysis using Image J software) of the immunoblot performed with different individual rats.
DISCUSSION

Quercetin acts as an antioxidant against arsenic generated oxidative stress (Mishra and Flora 2008). Our previous works revealed that enhancement of the antioxidant activity of QC was achieved through vesicular entrapment showing no nanoparticle toxicity on the system. (Ghosh et al., 2009; Ghosh et al., 2010) Chelation therapy using water soluble analogues of dimercaprol like DMSA and DMPS are the commonest form of therapy against arsenic poisoning. Therefore it was important to explore, the therapeutic efficacy of QC in combination with the potent arsenic chelator, DMSA, in a nanocapsulated drug delivery system against a chronic case of arsenic toxicity in rats. Our present report, for the first time to our knowledge, demonstrates the synergistic potency of oral application of the flavonoid QC with DMSA in a nanocapsulated form against p53 mediated hepatic and cerebral cell death in response to chronic arsenic consumption.

Efficiency of vesicular formulations is governed by their size and other physicochemical properties. The size and size distribution of nanoparticles are important in determining their stability drug release and cellular uptake efficiency. (Win and Feng 2005) Larger sized particles tend to aggregate. The size range achieved as observed under Atomic Force Microscope (Fig 1) shows no aggregation and all being below 100nm makes the delivery system suitable for drug delivery to target organs, most importantly brain, which is the ultimate challenge in drug delivery.

Efficiency of vesicular formulations was reflected in their ability to remove arsenic deposition in liver and brain of experimental rats. Treatment with NQC and NDMSA resulted in a significant decrease in arsenic content in liver and brain as compared with the nontreated arsenic control group. Interestingly synergistic action was found by the application of N(QC+DMSA) post arsenic exposure. (Fig 2)

Mitochondria, the seat of energy transduction when damaged results in a fall in ATP supply ultimately leading to cell death and subsequent organ failure. Arsenic is a direct poison to cellular respiration with an established evidence of oxidative stress in liver. (Pi et al., 2002) Fluorescence intensity produced by H$_2$DCFDA on oxidation to H$_2$DCF is proportional to the amount of ROS produced by the mitochondrion. Chronic exposure of experimental animals to inorganic arsenic causes a 2.5 fold increase in mitochondrial ROS generation in liver and brain. Our findings are in direct conjunction with the previous reports (Ghosh et al., 2010) and have shown a downregulation of mitochondrial ROS production by the application of NQC, NDMSA or N(QC+DMSA) in
brain and liver with N(QC + DMSA) offering synergistic benefit. (Fig 3a) Conjugated
diene, a hallmark in the ROS induced damage was also found to be significantly higher
\((p<0.001)\) in liver and brain mitochondria of chronically exposed rats. (Fig 3b) Maximal
protection was achieved through N(QC+DMSA) treatment post arsenic exposure depicting
synergistic behaviour \((p<0.001)\) of the formulation. Lower ROS level in nanocapsule
treated animals chronically exposed to arsenic explains the lesser amount of conjugated
showed arsenic may induce formation of ROS by alteration of GSH concentration. GSH,
the endogeneous antioxidant, is the central player in maintaining the cellular redox balance.
Free radicals produced by arsenite exposure oxidize GSH to GSSG. Depletion of GSH
content impairs a cell’s ability to counterbalance ROS and succumb to oxidative stress
mediated damage. Elevation of mitochondrial GSH content by coencapsulated QC: DMSA
nanoparticles (Fig 3c) give a positive indication in the reduction of oxidative burden in
liver and brain of rats chronically introduced to arsenic.

Ubiquinone, an electron carrier in the respiratory chain is affected due to arsenic
and induces the formation of ROS. (Corsini et al., 1999) An increased activity of NADH
oxidase suggests a higher ROS production. The impaired electron transport system in
mitochondria is reflected from the release of cytochrome \(c\) from mitochondria to cytosol
(Fig 8) A decreased activity of SDH (Table 2) reflects a lower proton accumulation
leading to the dissipation of mitochondrial membrane potential. (Fig 4) The fall in
mitochondrial membrane potential was also accompanied by an increase in mitochondrial
membrane fluidity. (Table 3) Arsenic causes a decrease in membrane microviscosity of
liver and brain mitochondria which might be attributed to the accumulation of lipids and
protein oxidative damage due to cellular arsenic consumption. Lipid peroxidation
products, especially conjugated diene, may play an important role in mediating the
decreased mitochondrial membrane microviscosity causing hyperpermeable membrane
thus allowing cyt \(c\) to pass into the cytosol. A positive correlation was observed between
arsenic accumulation in tissues with the dissipation of mitochondrial potential (Fig 5)
giving a direct evidence in the effect of cumulative arsenic accumulation in tissues
followed by mitochondrial depolarization and its regulation by N(QC+DMSA).

While cyt \(c\) release in the cytosol may lead a cell towards both apoptosis and
necrosis (Bustamante et al., 2005) a clear indication of apoptosis was noticed by Bashir et
al., 2006 in rats treated with NaAsO\(_2\) for 60 days. Lau et al., 2004 also observed exposure
to a long term high concentration of arsenic causes apoptosis while a low concentration drive the cells towards carcinogenesis. We observed increased BrdU positive cells indicating apoptosis in liver and brain of NaAsO$_2$ treated control animals in comparison to the normal group. (Fig 6) N(QC+DMSA) showed visibly less apoptotic cells in both liver and brain sections when compared to NaAsO$_2$ treated group. Previous experimental evidences also suggest that free radical damage induced by arsenic occurs through activation of oxidative sensitive signalling pathways. (Kamat et al., 2005) Persistent generation of ROS during chronic arsenic exposure (Pi et al., 2002) causes accumulation of stress induced protein p53 in the cytosol. p53, often referred to as the “guardian of the genome” plays a master role in controlling cell death and apoptosis. p53 immunoreactivity was evident as a band in mitochondrial fractions from both liver and brain (Fig 8). Increased expression of p53 on sodium arsenite exposure is well documented. (Shi et al., 2004) Expression of p53 in mitochondrial fraction indicates its translocation of mitochondria from the cytosol. P53 protein in mitochondria of liver and brain of NaAsO$_2$ treated animals was strongly expressed. NQC and NDMSA gave visibly less dense immunoreactive bands than NaAsO$_2$ treated control. N(QC+DMSA) treated group showed less accumulation of p53 protein. A p53 fraction translocating to mitochondria implies the onset of p53-dependent apoptosis.

While DMSA have been generally accepted among clinicians as a plausible management of many human metal intoxication, combined treatment with lipophilic and hydrophilic chelators, has a minimal clinical role. (Andersen and Aaseth 2002) Flavonoidal antioxidants like QC pose to be a useful tool in curing chronic arsenicosis but the therapeutic challenge remains to fix the correct dose and proper time of administration. Among the two compounds DMSA is hydrophilic and the other i.e. QC is hydrophobic in nature. It is not expected to solubilize these two compounds in a single non toxic solvent because of their different degree of solubility at that particular solvent. Thus these two compounds are not expected to reach at a definite area of a biological system simultaneously. Usually a nanocapsulated drug delivery system has an edge over others in reaching the cells at a much higher concentration than the corresponding “free” form (Reddy and Labhasetwar 2009). This property is particularly interesting in treating the CNS as these particles can circumvent the BBB. Having the feasibility to incorporate both hydrophobic and hydrophilic compounds, this colloidal carrier has the ability to cross both the GI tract mucosal barrier and the BBB to enhance drug bioavailability via particle
uptake mechanisms, and, therefore, it appears to have significant promise in delivering therapeutic molecules. (Lockman et al., 2002) Thus a nanocapsulated drug delivery system is the apt alternative over conventional treatment modalities. Hence nanocapsulated QC or DMSA showed a better result than the corresponding free drugs in removing the arsenic store in liver and brain tissues (Fig 3). Interestingly N(QC+DMSA) [a combination of lipophilic and hydrophilic compounds in equimolar concentration] showed more effective activity in all the parameters related to chronic arsenic induced mitochondrial damage in liver and brain than NQC or NDMSA taken alone. More interestingly, it is evident from the results that N(QC+DMSA) is having a synergistic effect rather than simple additive enhancement.

The fact that nanocapsulated QC was found more potent in reducing the oxidative stress than nanocapsulated DMSA can be attributed to the superior antioxidant property usually exhibited by polyphenols. DMSA being an efficient chelator might have helped in reducing the arsenic burden from the tissues which in turn have contributed to the generation of lesser amount of oxidative stress in the respective organs. When the coencapsulated nanoparticulated form was applied increased intracellular concentration of QC disarmed ROS and might have spared GSH getting oxidised to GSSG. GSH might have contributed to form the soluble methylated derivatives with arsenic which might have got excreted through urine. (Styblo and Thomas 1997) Thus apart from the role of DMSA in reducing the arsenic load in tissues, QC also might have an indirect role in decreasing arsenic burden in tissues in addition to its antioxidant effect. This explains the synergistic benefits of N(QC + DMSA) in reducing the arsenic content in liver and brain than NQC or NDMSA alone.

Our study evidently demonstrate that a combination of QC and DMSA in nanocapsulated drug delivery system is most effective in reducing the arsenic burden in liver and brain, protect liver from arsenic induced fibrosis, safeguarded cells from ROS induced damage, restored mitochondrial integrity and downregulated ROS induced signaling pathway that lead the cells towards p53 dependent apoptosis. This demonstration of using the nanoparticulated formulation of N(QC+DMSA) and evaluating its efficacy in conferring protection to neuronal and hepatic mitochondria in experimental model of chronic Arsenic consumption in rats provide a “lead” in the search for orally deliverable curatives for “silent killer” arsenic.