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Original Contribution

# Encapsulation of the flavonoid 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

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ABSTRACT

Chronic arsenic exposure causes oxidative stress and mitochondrial dysfunction in the liver and brain. The ideal treatment would be to chelate arsenic and prevent oxidative stress. *meso*-2,3-Dimercaptosuccinic acid (DMSA) is used to chelate arsenic but its hydrophilicity makes it membrane-impermeative. Conversely, quercetin (QC) is a good antioxidant with limited clinical application because of its hydrophobic nature and limited bioavailability, and it is not possible to solubilize these two compounds in a single nontoxic solvent. Nanocapsules have emerged as a potent drug delivery system and make it feasible to incorporate both hydrophilic and lipophilic compounds. Nanoencapsulated formulations with QC and DMSA either alone or coencapsulated in polylactide-co-glycolide [N(QC + DMSA)] were synthesized to explore their therapeutic application in a rat model of chronic arsenic toxicity. These treatments were compared to administration of quercetin or DMSA alone using conventional delivery methods. Both nanoencapsulated quercetin and nanoencapsulated DMSA were more effective at decreasing oxidative injury in liver or brain compared to conventional delivery methods, but coencapsulation of quercetin and DMSA into nanoparticles had a marked synergistic effect, decreasing liver and brain arsenic levels from 9.5 and 4.8 μg/g to 2.2 and 1.5 μg/g, respectively. Likewise, administration of coencapsulated quercetin and DMSA virtually normalized changes in mitochondrial function, formation of reactive oxygen species, and liver injury. We conclude that coencapsulation of quercetin and DMSA may provide a more effective therapeutic strategy in the management of arsenic toxicity and also presents a novel way of combining hydrophilic and hydrophobic drugs into a single delivery system.



Inorganic arsenic is a major environmental contaminant in parts of Asia, South America, India, and Europe as well as some regions in the United States and Canada. Chronic exposure of humans to a 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 [1]. Arsenic is recognized as a potent carcinogen in humans [2]. Chronic exposure to arsenic produces damaging effects on the liver [3] and nervous system [4]. The toxic manifestations of arsenic are partly due to oxidative stress because arsenic has a high affinity for thiol groups of functional proteins. Reports have established the fact that free radicals are generated during arsenic toxicity [5]. 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 [6]. Arsenic-mediated ROS generation causes a massive depletion of antioxidant enzymes and damage to lipid bilayers and DNA [7]. Corsini et al. [8] 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 pores in the inner membrane rendering the mitochondrial inner membrane permeable to solutes smaller than 1.5 kDa, thus preventing oxidative phosphorylation [9]. The most conventional approach by medical practitioners to the treatment of arsenic toxicity is the use of synthetic chelators such as DMSA (*meso*-2,3-dimercaptosuccinic acid). DMSA

*Abbreviations:* AFM, atomic force microscope; QC, quercetin; PLGA, polylactide-co-glycolide; DMAB, didodecyldimethylammonium bromide; DCIP, 2,6-dichloroindophenol; DMSA, *meso*-2,3-dimercaptosuccinic acid; NQC, nanocapsulated quercetin; NDMSA, nanocapsulated DMSA; N(QC + DMSA), nanocapsulated quercetin with DMSA; SDH, succinate dehydrogenase; ROS, reactive oxygen species; GSH, reduced glutathione; DPH, diphenylhexatriene; CM-$H_2$DCFDA, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester; BBB, blood–brain barrier; BrdU, bromolated deoxyuridine; HE, hematoxylin and eosin.

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contains two sulfhydryl (−SH) groups and is an effective chelator of toxic metals such as lead or arsenic.

Although DMSA is a partially effective chelator of arsenic and reduces the arsenic accumulation in tissues, it has untoward side effects such as gastrointestinal disorders over the long term [10,11]. 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 was not effective in producing any clinical or biochemical benefits, which calls into question our current therapeutic approach [12]. Importantly, the tissue distribution of DMSA seems to be restricted to extracellular fluid [13]. Because arsenic exposure leads to oxidative stress [14], a complementary approach could be the use of antioxidants as well as an arsenic chelator as combined therapy [15]. Quercetin (QC), a polyphenolic flavonoid, is a good antioxidant and protects cells from reactive oxygen species [16]. However, the clinical use of quercetin is limited because of its hydrophobic nature, with low and slow bioavailability [17,18]. Hence an improved delivery mode for quercetin may be an effective approach to delivering quercetin to tissues.

Nanocapsules are a novel mode of drug delivery and currently appear to be nontoxic, biodegradable, and nonimmunogenic, with a sustained drug-releasing ability in biological systems. Quercetin in liposomes and nanocapsules is effective at preventing liver and brain injury caused by arsenic toxicity [19,20]. In this study we have evaluated the efficacy of coencapsulated quercetin and DMSA and compared it to nanoencapsulated quercetin, nanoencapsulated DMSA, and the conventional delivery methods for each agent in a rat model of arsenic toxicity. In brief, we found that the coencapsulated form of quercetin and DMSA offered the best protection, and recovery from arsenic toxicity improved all parameters of oxidative stress and improved liver histology. We conclude that coencapsulation of quercetin and DMSA presents a novel strategy for combining fat-soluble and water-soluble drugs, which gives the best of both worlds.

## Materials and methods

### *Chemicals*

Polylactide-co-glycolide (PLGA; Resomer RG 85:50H), 2,6-dichloroindophenol (DCIP), phenazine methosulfate (PMS), succinic acid, didodecyldimethylammonium bromide (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 [21] was used to make quercetin and/or DMSA nanocapsules. QC was dissolved in ethyl acetate and 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 DMAB and DMSA. The resulting organic/water emulsion was stirred at room temperature for 3 h before being homogenized 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 a Sorval RC 5B Plus using the Sorval T-865 rotor for 1 h. The pellet of nanocapsules was washed with phosphate-buffered saline (PBS) twice and resuspended 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 in the Aquastic mode using cantilevers having a resonance frequency of 146–236 kHz, tip height 10–15 μm, and tip length 225 μm. 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 analyzed using Picoscan 5.33 software from Molecular Imaging Corp. [22].

### *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-h light/dark cycle) for 7 days before the commencement of the treatment, during which they received food (purchased from Hindustan Lever Ltd, Maharashtra, India) and drinking water. The 50% acute lethal dose ($ALD_{50}$) of sodium arsenite ($NaAsO_2$) via the oral route in rats was found to be 49 mg/kg body wt. The rats were divided into seven groups and each group contained five animals. Animals in group A were kept as untreated normals, and group B animals were considered arsenic-treated experimental controls, and one-fifth the $ALD_{50}$ of sodium arsenite (i.e., approximately 10 mg/kg body wt) 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 of $NaAsO_2$ administration the animals in group C were fed free QC (8.98 μmol/kg body wt of QC in 0.2% Tween 80 aqueous solution). Group D received a DMSA solution orally (8.98 μmol/kg body wt of DMSA in water); groups E and F were fed a nanocapsulated formulation of QC or DMSA (containing 8.98 μmol/kg body wt of either QC or DMSA), respectively, for 8 weeks. Group G received coencapsulated QC:DMSA in equimolar proportions (8.98 μmol total) in nanocapsulated form. The animals were treated with drugs twice a week for 4 weeks. All animals were fed a normal diet and drinking water without any treatment for the next 2 weeks. At the end of 26 weeks, rats of the each group were anesthetized with ether and sacrificed and the brain and liver were dissected out promptly and washed with cold physiological saline and immediately used for mitochondria and submitochondrial particle preparation.

### *Measurement of arsenic levels in liver and brain of rats*

Total arsenic accumulation in liver and brain homogenates was measured by a flow injection atomic absorption spectrometer (Spectra AA 30/40; Varian, Palo Alto, CA, USA) fitted with a graphite furnace [23].

### *Liver and brain mitochondria isolation*

Liver and brain mitochondria of experimental animals were isolated using differential centrifugation following the method of Navarro and Boveris [24].

### *Mitochondrial ROS measurement*

Intracellular ROS levels were measured in liver and brain mitochondria [25]. Fluorescence was measured through a spectrofluorimeter (LS 3B; PerkinElmer, USA) by using 499 nm as excitation and 520 nm as emission wavelength. 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 dienes. Mitochondrial membrane was extracted twice in a chloroform:methanol mixture (2:1, v/v). The pooled extract was evaporated to dryness under nitrogen