Neuroprotective Role of Quercetin against Arsenic Induced Oxidative Stress in Rat Brain

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Abstract
Arsenic, a poisonous metalloid, is ubiquitous in the environment, and affects nearly all organ systems of animals including humans. The present study was conducted to understand the protective role of quercetin, a natural flavonoid on arsenic-induced oxidative damage in rat brain. Forty male Sprague Dawley rats, aged between 7 to 8 weeks and weighing 150-200 g were divided into four groups viz. normal control, arsenic treated, quercetin treated and arsenic + quercetin treated. Rats in normal control group were given normal food and drinking water. Rats in arsenic treated group were given arsenic daily in the form of sodium arsenite (NaAsO2) in drinking water at a dose of 100 mg/l. Rats in the quercetin treated group were given quercetin, dissolved in distilled water, orally everyday through intubation gavage at a dose of 50 mg/kg body weight. Rats in the combined arsenic + quercetin treated group were given arsenic and quercetin in a similar manner as was given to rats belonging to arsenic and quercetin treated group. After 2 months of treatment, antioxidant defense status and changes in the brain histo-architecture were assessed. A significant increase in the levels of lipid peroxidation and a decrease in reduced glutathione levels were observed in the brain of arsenic treated rats, when compared to the normal controls. Further, decrease in the activities of antioxidant defense enzymes such as superoxide dismutase, glutathione peroxidase, glutathione-s-transferase and glutathione reductase, as well as the activity of enzyme nitric oxide synthase were detected in arsenic treated group, which however were restored to normal levels upon simultaneous treatment with quercetin. Brain tissue of arsenic treated rats also showed changes at the histo-architectural level which were normalized on simultaneous treatment with quercetin. The study, therefore, reveals that quercetin shall prove to be beneficial in containing the neuro-toxic effects of arsenic.

Keywords: Arsenic; Quercetin; Cerebellum; Oxidative stress

Introduction
Human exposure to arsenic is primarily a result of inhalation of metal particles in air, ingestion of contaminated food and through drinking water. Intake of inorganic arsenic over a long duration can lead to chronic arsenic poisoning (arsenicosis). It is known now that inorganic arsenic has toxic effects at both high and low levels of exposure. Chronic exposure to arsenic-contaminated water and food can have adverse effects on various organs that may lead to development of cancer in skin, liver, lung and bladder [1-5]. It is believed that damage due to arsenic is closely associated with oxidative stress induced by arsenic. As reported in earlier studies, arsenic is known to cause generation of free radicals, like reactive oxygen species (ROS) and reactive nitrogen species (RNS), as well as metabolic intermediates like dimethyl arsenic (DMA) peroxyl radical [6-11].

Brain is one of the critical organs particularly susceptible to the damaging effects of ROS, as it has a limited capacity to detoxify ROS owing to low glutathione-producing capacity of neuronal cells [12], and also because of its high metabolic rate as well as relatively reduced capacity for cellular regeneration as compared to other organs. In addition, arsenic can easily cross the blood–brain barrier [13,14]; therefore making the brain highly susceptible to arsenic exposure and also to the resultant ROS induced oxidative stress.

Quercetin, a member of the flavonoids family, is ubiquitously present in food items including vegetables, fruits, tea and wine. It is a powerful antioxidant, which can prevent oxidative injury and cell death by chelating metal ions, scavenging oxygen radicals, and protecting against lipid peroxidation (LPO) [15]. With its chelating effect, quercetin scavenges free radicals, thereby preventing oxidative damage to DNA and also to cell membranes thus stabilizing lipid membranes by preventing LPO by free radicals [16,17]. Additionally, quercetin is also known to decrease ROS production, increases both Mn SOD (manganese superoxide dismutase) activity and glutathione levels and also suppresses over-expression of the inducible form of nitric oxide synthase (iNOS).

An important property of quercetin which could be relevant with regard to its protective role in arsenic induced neurotoxicity is that, it is able to cross the blood brain barrier [18]. Therefore, quercetin could be a potent nutrient that can access the brain and may protect it from disorders associated with oxidative stress [19]. Since, brain damage due to arsenic is expected to be caused mainly by oxidative stress, so role of quercetin in such a condition needs to be explored. Therefore, the present study was designed to evaluate the protective role of quercetin, if any, during arsenic induced neurotoxicity.

Material and Methods
Chemicals
All the chemicals and reagents used in the present study were of reagent grade. Sodium arsenite (NaAsO2) and quercetin were purchased from Sigma-Aldrich (USA). All other chemicals such as nicotinamide adenine dinucleotide phosphate (NADPH), bovine serum albumin (BSA), trichloroacetic acid (TCA), thiobarbituric acid (TBA), ethylene diamine tetra-acetic acid (EDTA), Triton X-100, 5’,5’-Dithiobis-(2-Nitrobenzoic Acid) (DTNB), 1-chloro-2, 4-dinitrobenzene (CDNB), reduced glutathione (GSH), nitro blue tetrazolium chloride (NBT), glutathione reductase (GR) and hydrogen peroxide (H2O2), were purchased from Sisco Research Labs (India) and Hi-media chemicals (India).

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Animals

Healthy male Sprague Dawley (S.D.) rats in the weight range of 150-200 g were obtained from the central animal house of Punjab University, Chandigarh, India. The animals were housed in polypropylene cages under a hygienic bed of husk (regularly changed) in a well-ventilated animal room. Throughout the treatment period, the animals were provided with standard animal palliated feed obtained from Ashirwad Industries, Kharar, Punjab, India and were also provided with water ad libitum. Before treatment, the rats were acclimatized to experimental conditions for a period of one week. All the procedures were done in accordance with ethical guidelines for care and use of laboratory animals, which were approved by Institutional Animal Ethics Committee (IAEC), Punjab University Chandigarh, India.

Grouping of animals

Rats of age 7-8 weeks were segregated into following four groups and each group consisted of 10 animals. Animals in group I served as normal controls, and was given standard laboratory feed and water ad libitum throughout the period of experimentation. Group II animals were given arsenic daily in the form of NaAsO₂ in drinking water at a dose of 100 mg/kg body weight [19]. The animals in Group III were given quercetin orally every day in drinking water through intubation gavage at a dose of 50 mg/kg body weight [19]. The animals in Group IV were given a combined treatment of arsenic as well as quercetin in a similar manner as was given to Group II and Group III animals, respectively. The study was carried out for a total duration of 2 months.

Sample preparation

After 2 months of treatment period, the animals from all the treatment groups were sacrificed using overdose of ether anesthesia. After dissection, the brains were immediately removed, washed in ice-cold isotonic saline and a small portion of the brain (cerebellum) was fixed in formalin saline (10%) for histological examination using light microscope. The remaining cerebellar tissue was used to prepare the tissue homogenate. The homogenates (10% w/v) were prepared in ice-cold 10 mM phosphate-buffered saline (PBS), 0.15 M sodium chloride (NaCl), pH 7.4. The homogenates were centrifuged at 2000 x g for 15 min, and the supernatants were used for the estimation of the remaining oxidative stress parameters namely, glutathione peroxidase (GPx), glutathione transferase (GST), glutathione reductase (GR), superoxide dismutase (SOD), iNOS and catalase.

Lipid peroxidation (LPO)

Lipid peroxidation was assayed by using the method of Wills, 1966 [21]. Briefly, 0.5 ml of tissue homogenate was diluted to 1.0 ml using Tris-HCl buffer (0.1 M, pH 7.4). The reaction mixture was incubated at 37°C for 2 hrs with constant shaking. At the end of the incubation, 1.0 ml of TCA (10 %, w/v) was added and then after thorough mixing, the reaction mixture was centrifuged at 800 rotations per min (rpm) for 10 min. To 1.5 ml supernatant, 1.5 ml TBA (0.67 % w/v) was added. Color was developed by placing the test tubes at 100°C for 10 min in a boiling water bath. The samples were cooled and diluted with 1.0 ml distilled water. The optical density was recorded at a wavelength of 535 nm. The results were expressed as nano moles of malonaldehyde (MDA) formed/min/mg of protein.

Glutathione peroxidase (GPx)

GPx activity was measured by using method described by Paglia and Valentine’s [22]. The reaction mixture contained 2.65 ml of 50 mMmold/l phosphate buffer (pH 7), 0.1 ml of 150 mM GSH solution, 0.1 ml GR (10 mg/ml), 0.1 ml of 3 mM NADPH-Na salt, 0.1 ml 50 mMol/l H₂O₂ solution and 0.02 ml of tissue homogenate. The GPx activity was monitored as a decrease in absorbance due to the consumption of NADPH, which absorbs at 340 nm. The GPx activity was expressed as nano moles of NADPH consumed/min/mg protein using an extinction coefficient of 6.22 x 10⁻³ M⁻¹ cm⁻¹.

Reduced glutathione (GSH)

GSH levels were determined according to the method of Ellman et al. [23]. 0.1 ml of TCA was added to 500 µl of tissue homogenate. After mixing the contents, the precipitated proteins were separated by centrifugation at 2000 x g for 15 min. 500 µl of the supernatant obtained, was diluted in a test tube to 1.0 ml with sodium phosphate buffer. To this, 2.0 ml of freshly prepared DTNB was added. The yellow color developed was read immediately at 412 nm. The GSH contents were expressed in term of µmol of GSH/g tissue.

Glutathione reductase (GR)

GR activity was measured by continuous rate determination method using a UV spectrophotometer [24]. To 3 ml cuvette, 2.6 ml of phosphate buffer, 0.15 ml of NADPH and 0.15 ml of oxidised glutathione (GSSG) were added. The reaction was initiated by the addition 0.2 ml of sample to the cuvette and the decrease in absorbance at 340 nm was followed for 5 min at 25°C. The enzyme activity was calculated using the molar co-efficient for NADPH of 6.22 x 10⁻³ M⁻¹ cm⁻¹ and was expressed as µmoles of NADPH oxidised/min/mg of protein.

Glutathione-s-transferase (GST)

The enzyme activity of GST was determined by using the method of Habig et al. [25]. The sample buffer was prepared by mixing 650 µl of phosphate buffer, 25 µl CDNB and 25 µl GSH. To the above sample buffer, 10 µl of tissue homogenate was added and the absorbance was measured at 340 nm for 3 min. The activity was expressed as µmoles of CDNB conjugate formed min⁻¹ mg⁻¹ of protein by using the extinction coefficient of 9.6 µM⁻¹ cm⁻¹.

Superoxide dismutase activity (SOD)

Superoxide dismutase activity was estimated following the method of Kono [26] wherein, the reduction of NBT was inhibited by SOD and measured at 560 nm. The reaction was initiated by addition of hydroxylamine hydrochloride to the reaction mixture containing NBT and PMF of brain homogenate. The results were expressed as units per mg of protein with one unit of enzyme defined as the amount of SOD required to inhibit the rate of reaction by 50%.

Catalase (CAT)

Catalase activity was assessed by following the method of Luck H [27], whereby the breakdown of H₂O₂ was measured at 240 nm. Briefly, the assay mixture consisted of 3ml of H₂O₂ phosphate buffer (0.0125 M; H₂O₂) and 0.05 ml PMF of brain homogenate. The change in absorption was observed at 240 nm as a result of H₂O₂ decomposition. The amount of H₂O₂ decomposed was calculated on the basis of molar co-efficient of H₂O₂ (0.0394 M⁻¹ cm⁻¹) and the results were expressed as µmoles of H₂O₂ decomposed/min/mg of protein.
Nitric oxide synthase (NOS)

Nitric oxide synthase activity was determined in terms of nitric oxide (NO) production. The estimation was carried out by using the method of Radassi et al. [28]. NO production was estimated by measuring nitrite, a stable metabolic product of NO, using Griess reagent. To 0.10 ml of tissue homogenates, 0.100 ml of Griess reagent was added into the ELISA plate. The ELISA plate was then incubated in dark at 37°C for 30 min. Pink color thus obtained was read at 540 nm on an ELISA plate reader. The amount of nitrite produced was determined by a standard curve prepared by using sodium nitrite as reference standard. Results were expressed as nM of nitrite/g tissue.

Protein determination

Protein content was determined by the method of Lowry et al. [29] by using bovine serum albumin as protein standard.

Histological study

For various histological studies, small sections of cerebellum from each of the normal control and treated animals were taken, washed in ice-cold 0.9% NaCl and fixed in the buffered formalin for about 24 to 28 hours. After fixation, the tissues were dehydrated by using ascending grades of alcohol. Dehydrated tissue were embedded in paraffin wax (58-60°C) after subjecting them to different treatments as described in the procedure by Pearse [30]. Tissue sections of 5-7 microns thickness were cut using microtome and were stained using H&E stain [31] and viewed under light microscope for histological changes.

Statistical Analysis

The results of all the experiments conducted during the current study are depicted in the form of tables and figures represented as Mean ± SD. The results of different treatments have been compared with normal controls. Additionally, the results obtained from arsenic treatment group were compared with combined arsenic+ quercetin treatment group. The statistical significance of the values has been determined by using analysis of variance (ANOVA), followed by Newman Keuls’s test. All statistical analyses were performed using SPSS 14 software. P values of less than and equal to 0.001, 0.01, and 0.05 were considered to indicate statistically significant differences.

Results

Arsenic treatment for a total duration of two months significantly increased (p ≤ 0.01) the levels LPO in cerebellum as compared to normal control groups (Table 1). When arsenic treated rats were co-treated with quercetin, the MDA levels were observed to be within the normal range. Quercetin treatment when given to normal rats did not reveal any significant effect on lipid peroxidation.

A significant increase (p ≤ 0.001) in the enzyme activity of catalase in cerebellum was observed after arsenic treatment when compared with normal control group (Table 2). Quercetin alone and combined arsenic + quercetin did not show any significant change in the enzyme activity of catalase when compared to normal control group. However, when catalase activity in combined arsenic + quercetin was compared with arsenic treated rats, a significant decrease (p ≤ 0.001) was observed; thereby indicating that simultaneous treatment with quercetin brought the enzyme activity of catalase within normal limits. The activities of antioxidant enzymes SOD and GPx were found to be significantly decreased (p ≤ 0.001 in SOD; p ≤ 0.05 in GPx) in brain after arsenic treatment when compared to normal controls (Table 2). Quercetin alone did not show any significant change in both SOD and GPx activities when compared to normal rats, however when co-administration to arsenic treated animals, a significantly increase (p ≤ 0.001) in the SOD enzyme activity was observed when compared with arsenic treated group, although the activity was still significantly lower in comparison to normal group. Further, no significant change in the GPx activity was observed after combined arsenic + quercetin administration when compared to arsenic treated group.

As shown in Table 3, the enzyme activities of GST and GR were also decreased significantly (p ≤ 0.001 in GST and p ≤ 0.01 in GR) after arsenic treatment when compared with normal control group. Combined arsenic + quercetin treatment, did not cause any significant change in the activities of GST or GR when compared to arsenic treated group. Both the enzyme activities in the combined treatment group were significantly lower than control levels. Also quercetin alone did not show any significant change in the activities of GST and GR when compared to normal rats. The GSH content was found to be significantly decreased (p ≤ 0.01) after arsenic treatment when compared to normal control rats (Table 3). Quercetin alone and combined arsenic + quercetin did not show any significant change in GSH levels when compared to normal controls. However, combined arsenic + quercetin treatment significantly increased (p ≤ 0.05) the levels of GSH when compared to arsenic treated animals.

A significant reduction (p ≤ 0.001) in the NOS activity was observed after arsenic treatment when compared to normal control group (Table 4). Quercetin alone did not show any significant change in the NOS activity when compared to normal rats. Interestingly, the combined arsenic + quercetin treatment significantly increased (p ≤ 0.01) the NOS activity in brain when compared to arsenic treated rats.

As shown in the Figure 1, the tissue sections obtained from normal control rats showed normally nucleated neurons, glial cells and pyramidal cells arranged in several layers. Arsenic treated sections showed marked alterations in the histo-architecture of glial cells, which were enlarged showing nuclear pyknosis and moderate cytoplasmatic vacuolation. Quercetin co-administration to arsenic treated rats, showed an appreciable improvement in the overall histo-architecture.

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Discussion

Enhanced oxidative stress has been suggested to be an important mechanism in arsenic-induced neurotoxicity [32]. In this study, we observed that chronic arsenic administration caused oxidative damage in the brain of arsenic treated rats which was assessed in terms of the activity of various antioxidant enzymes, level of LPO and by histological examination of stained brain tissue sections.

Lipid peroxidation is a vital marker for toxicity induced by various xenobiotics and is thought to be a consequence of oxidative stress initiated when the dynamic balance between peroxidant and antioxidant mechanism is impaired. Its significance lies in the fact that increases in the peroxidation of membrane lipids by the action of free radicals results in the loss of membrane integrity and function [33].

It has been demonstrated previously that arsenic treatment to animals increased the levels of LPO [34], even at low doses leading to apoptosis and necrosis of brain cells [35,36], suggesting cellular injury by action of free radicals. Therefore, increase in LPO observed in the present study following arsenic treatment to rats, could be a consequence of increased free radical production and/or consequent suppression in the activity of antioxidant defense enzymes and glutathione levels. In the present study, quercetin supplementation to arsenic treated rats proved to be beneficial as the levels of LPO in the combined group were found to be reduced when compared to arsenic alone treated group, thus demonstrating the protective role of quercetin in activating the antioxidant defense system in rat brain by preventing lipid peroxidation in cellular membranes.

Antioxidant enzymes such as SOD, catalase and GPx are the primary antioxidant enzymes, which help in degrading the toxic oxidative intermediates and are considered to be the first line of cellular defense against oxidative damage. Catalase is a haemo protein which reduces hydrogen peroxide to molecular oxygen and water. SOD is an antioxidant metallo enzyme that reduces superoxide radicals to water and molecular oxygen. SOD catalyzes the dismutation of superoxide radicals by scavenging free superoxide anions (O$_2^-$). GPx uses GSH as a reducing substrate, and catalyzes the reduction of organic hydro peroxides [37-39].

Our results show that the activities of SOD and GPx in cerebellum region of brain decreased significantly following arsenic treatment. Decreased activity of SOD reflects suppression of the scavenging ability of SOD, which may be a result of accumulation of superoxide anion radical in the brain tissue post arsenic treatment. The suppressing ability of arsenite on SOD activity was also demonstrated by Ramanathan et al. [40] in liver and kidney of male Wistar rats. Another report by Yamanaka et al. [41] suggested that free radical species are generated by the reaction of molecular oxygen with dimethyl arsine, a metabolite of dimethyl arsenic acid. They considered one of these radicals to be superoxide anion radical which is produced by one electron reduction of molecular oxygen by dimethyl arsine [42]. Similarly, catalase a detoxifying agent responds well to excess amount of H$_2$O$_2$ generated by the action of SOD. Catalase shares its function with GPx. Both these enzymes detoxify H$_2$O$_2$ but vary in their substrate affinities. As a matter of fact, H$_2$O$_2$ molecules are preferably metabolized by GPx because of its lower km for H$_2$O$_2$ than catalase, which is activated at comparatively higher conc. of H$_2$O$_2$. In the present study, catalase activity was found to be increased in brain tissue of rats following arsenic exposure, whereas GPx activity was suppressed suggesting that H$_2$O$_2$ production although increased following arsenic treatment, but was not high enough to suppress catalase activity, since only GPx activity was found to be suppressed and catalase was still activated.

Quercetin co-treatment along with arsenic normalized SOD and catalase activity in the combined treatment group. However, quercetin co-treatment could not normalize GPx activity probably because...
of direct effect of arsenic on its protein structure causing complete inhibition or loss of the enzyme activity. These results suggest that co-treatment with quercetin, helped maintain the balance between ROS production and antioxidant defense, as can be inferred from normalized antioxidant enzyme activity values in the combined treatment group. There are reports supporting the protective role of quercetin, against oxidative stress [43-45] whereby, quercetin, when administered at a dose of 50 mg/kg to rats, significantly increased SOD activity and decrease MDA level.

GST, on the other hand, is an important water-phase antioxidant and an essential cofactor for antioxidant enzymes. It has facile electron-donating capacity, linked to its sulfhydryl (-SH) group that provides protection against mitochondrial damage by endogenous oxygen radicals. In the present study, a significant decrease in GST activity was observed following arsenic treatment when compared to normal rats. The decrease in GST levels can be correlated to elevated levels of ROS in the arsenic treated rats, as GST would be consumed in converting the unstable free radical species to stable ones. The other reason for GST depletion could be the direct toxic effect of arsenic, since arsenic is known to form complexes with GST (arsenic-GSH). These complexes are known to cross the blood–brain barrier easily [46] leading to oxidative damage and consequently apoptotic cell death. A decrease in GSH is known to trigger the activation of neuronal 1,2-lipoxygenase, which leads to the production of peroxides, an influx of calcium, and ultimately cell death [47].

GR is a crucial enzyme that reduces GSSG to the sulfhydryl form (GSH) by the NADPH-dependent mechanism. Decrease in GR activity after arsenic treatment when compared to normal rats as observed in the present study, which correlates with the decreased level of GSH, thereby indicating that arsenic toxicity suppressed the activity of GR, leading to decrements in GST levels in brain and finally imbalance in redox status [48]. Quercetin supplementation alone did not show any significant change but prevented the decline in GR activity in combined treatment group. The neuroprotective activity of quercetin has been attributed to its antioxidant properties whereby quercetin is reported to trigger the activity of GR, which increases the overall GSH content, thus strengthening the antioxidant defense system [49].

GST is a natural antioxidant which catalyzes the conjugation of glutathione with various electrophiles and free toxic species produced from various toxins. Under oxidative stress, the excessive ROS induces an increase in GST levels, and then GST metabolizes the toxic products of lipid peroxidation, and other molecules [50]. In our study, a significant decrease in the GST activity was observed after arsenic treatment, which usually occurs under extreme oxidative stress conditions [6]. The reduced activity of GST is the consequence of oxidative modifications of proteins, or low glutathione levels. GST utilizes glutathione as a cofactor and therefore the decrease observed in its activity after acute arsenic exposure is suggested to come from the paucity of glutathione. However, quercetin supplementation alone, statistically did not show any significant change but prevented the decline in GST activity in the combined treatment group, thereby suggesting an increase in the antioxidant versus oxidant ratio which led to up-regulation of GST activity.

Nitric oxide is a signaling molecule in multicellular eukaryotic organisms, where it coordinates the function and interactions between cells of the cardiovascular, neuro, and immune system [51]. These cells have the ability to synthesize NO with the enzyme NOS using arginine and O₂ as substrates [52]. Nitric oxide and L-citrulline are the principal products of NOS enzyme activity [53]. In our study, a significant decrease in iNOS enzyme activity was observed after arsenic treatment when compared to normal rats. It is known that the impairment of NO production is due to lower NOS protein levels, which results from decreased expression and/or increased degradation of the proteins. Previous studies reported a decrease in Ca-dependent NOS activity measured in vivo and in vitro in arsenic-exposed rats (37 ppm drinking water) with a decrease in nitric oxide levels and an increase in ROS generation and lipid peroxidation [54,55]. Quercetin supplementation to arsenic treated rats was able to increase the NOS enzyme activity in brain. Quercetin, a well-known O₂⁻ scavenger is also a NO scavenger [56], and therefore apparently less decrease as observed in the activity of NOS enzyme when quercetin is simultaneously administered along with arsenic, could be seen as a feedback mechanism to replenish the loss of NO induced by the scavenging action of quercetin.

Enhanced oxidative stress in the brain after arsenic exposure was reflected in histological observations where the tissue sections obtained from normal control rats showed normally nucleated neurons, glial cells and pyramidal cells arranged in several layers, whereas arsenic treated sections showed marked alterations in the histo-architecture of glial cells (enlarged glial cells), nuclear pyknosis and moderate cytoplasmic vacuolization; thereby suggesting that exposure to arsenic induces neuronal damage mediated by excessive generation of ROS. Similar results have been observed by Flora et al. [57] following arsenic exposure to rats. However, quercetin co-administration with arsenic showed an appreciable improvement in the overall histo-architecture of brain showing normal sized glial cells, pyramidal cells with mild pyknosis and cytoplasmatic vacuolization.

Conclusion

The present study, therefore, concludes that quercetin as a prophylactic intervention could ameliorate the neurotoxicity action of arsenic by reducing oxidative stress.

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