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Abstract

The patient of chronic arsenic toxicity shows oxidative stress. To overcome the oxidative stress, several anti-oxidants such as β -carotene, ascorbic acid, α -tocopherol, zinc and selenium had been suggested in the treatment of chronic arsenic toxicity. In the present study universal anti-oxidant (both water and lipid soluble anti-oxidant) α -lipoic acid was used to examine the effectiveness of reducing the amount of arsenic from arsenic-loaded isolated liver tissues of rat. Isolated liver tissues of Long Evans Norwegian rats were cut into small pieces and incubated first in presence or absence of arsenic and then with different concentrations of α -lipoic acid during the second incubation. α -Lipoic acid decreases the amount of arsenic and malondialdehyde (MDA) in liver tissues as well as increases the reduced glutathione (GSH) level in dose dependent manner. These results suggest that α -lipoic acid remove arsenic from arsenic-loaded isolated liver tissues of rat.

Introduction

About half of the total populations (more than 50 millions) of Bangladesh, at present, are consuming arsenic through drinking and cooking (Misbahuddin, 2003; Mudur, 2000). Among them more than 40,000 people have already developed the signs and symptoms of chronic arsenic toxicity. The basic treatment is to stop drinking arsenic contaminated water and allow the patient to use arsenic-safe water (Smith et al., 2000). Some authors suggest the use of β -carotene, vitamin A, ascorbic acid, α -tocopherol, zinc, selenium and spirulina for the treatment of chronic arsenic toxicity (Ahmad et al., 1998; Misbahuddin et al., 2006). These are anti-oxidants in nature. Although some are water-soluble anti-oxidants and some are lipid soluble. Duration of treatment ranges from 4-12 months. Prolong duration of treatment affects the patients' compliance as well as treatment cost.

Our body also contains α -lipoic acid, which is a short

chain fatty acid with sulfhydryl (-SH) groups that has potent anti-oxidant property (Packer et al., 1995). Anti-oxidant properties of α -lipoic acid are due to its ability to scavenge hydroxy radicals, hypochlorous acid and singlet oxygen (Biewenga et al., 1997). α -Lipoic acid is present in our diet such as spinach, broccoli and tomatoes. Naturally occurring Renantiomer of α -lipoic acid is an essential cofactor in α -keto-acid dehydrogenase complexes and the glycine cleavage system (Jones et al., 2002). It is readily absorbed from the gut and the mean peak plasma concentration of α -lipoic acid following a single oral administration of 200 mg was 3.1 μ M. The mean plasma half-life of α -lipoic acid was about 30 min (Teichert et al., 1998). Within the tissue, it is rapidly converted into dihydrolipoic acid (DHLA). Both α -lipoic acid and DHLA may chelate or bind metal ions and facilitating their removal from the cell (Ou et al., 1995). Exogenous administration of α -lipoic acid has been found to be effective in many pathological condition associated with oxidative stress,



diabetic neuropathy (Zeigler et al., 1999), metal toxicity (Muller and Menzel, 1990), hypertension (El Midaoui and Champlain, 2002), diabetic complication and cataracts (Packer, 1994). Recently it has been found that α -lipoic acid suppressed the free radicals initiated by arsenic in different parts of rat brain regions (Shila et al., 2005a). α -Lipoic acid also causes an increase in intracellular GSH *in vitro* as well as *in vivo* (Busse et al., 1992). Simultaneous administration of lipoate (α -lipoic acid) to arsenic-treated rats has been shown to decrease arsenic content and increase glutathione status remarkably in discrete brain areas (Shila et al., 2005b). Glutathione and glutathione related enzyme play an important role in the cell against the effect of reactive oxygen species (ROS). GSH also stimulates the arsenic detoxification process by modulating arsenic speciation (Scott et al., 1993). Therefore, this study was designed to evaluate the effect of α -lipoic acid on the removal of arsenic from the arsenic-loaded isolated liver tissue of rat.

Materials and Methods

Chemicals and reagents

Arsenic trioxide (As_2O_3), reduced glutathione (GSH), 5,5-dithio-bis-2-nitro-benzoic acid (DTNB) and thiobarbituric acid were purchased from Sigma Chemical company (St. Louis, MO, USA). Chemicals and reagents to measure lactate dehydrogenase (LDH) and total protein were from Human Gmbh (Germany). α -Lipoic acid was a gift from Opsonin Pharma Limited, Bangladesh. All other reagents and solvents were highest analytical grade available.

Preparation of isolated liver tissues

The study was carried out on isolated liver tissues of Long Evans Norwegian adult healthy male rats weighing about 150-180 g. The rats were housed in standard plastic cages in a clean rodent room where a 12 hours light/dark cycle was maintained. On the day of experiment, rats were sacrificed under chloroform anesthesia and the abdomen was opened by giving a midline incision. The liver was dissected out and immersed immediately into the physiological solution (NaCl 150 mM, KCl 5.6 mM, $NaHCO_3$ 25 mM, NaH_2PO_4 2.5 mM, Glucose 10 mM), placed in ice bath. The liver tissues were chopped into small pieces (approximately 2 mm in size).

Research design

Isolated liver tissues of rat were incubated with in presence or absence of arsenic (50 μ g) at 37°C for 45 min. After the first incubation, tissues were washed

twice with physiological solution. The purpose of this incubation was to load the liver tissues with arsenic. Then during the second incubation (at 37°C for another 45 min), liver tissues were treated with different concentrations of α -lipoic acid (1, 10, 100 μ M).

Several test tubes were taken and each test tube contains 250 mg small pieces of liver tissues immersed in 5 mL of physiological solution. The test tubes were marked as groups: Group I- control; Group II- arsenic (50 μ g); Group III- arsenic (50 μ g) + α -lipoic acid (1 μ M); Group IV- arsenic (50 μ g) + α -lipoic acid (10 μ M); Group V- arsenic (50 μ g) + α -lipoic acid (100 μ M). Number of the test tubes (samples) in each group was six. After second incubation, tissues were homogenized and used for the estimation of total arsenic, total protein, malondialdehyde (MDA), and GSH level.

Estimation of arsenic

The amount of total arsenic was measured using Atomic absorption spectrophotometer with hydride generator (Buck Scientific, USA). In brief (Wang et al., 1994): The sample, at first, digested with nitric acid (3 ml), sulfuric acid (2 mL) and perchloric acid (2 mL) for 2 hours by Bunsen burner. Following digestion, each sample was introduced into the hydride generator by continuous flow of 10% hydrochloric acid, 3% sulfuric acid and 1.5% sodium borohydride into a gas-liquid separator. The arsine vapor produced by arsenic and the hydrogen gas (produced by sodium borohydride and acid) was carried out by flowing argon gas into quartz T-tube. The tube was heated in an air-acetylene flame (2300°C), serve as atomization cell. The current of the Hollow Cathode Lamp for arsenic was 10 mA. The wavelength and spectral bandwidth were 193.7 and 0.7 nm respectively.

Estimation of MDA

The extent of lipid peroxidation was estimated by using the thiobarbituric acid method to determine MDA levels described by Wilber et al. (1949). Briefly, 1 mL of tissues homogenate was reacted with 4.5 mL of 5.5% trichloroacetic acid. The mixture was vortexed and centrifuged at 4,000 x g for 10 min. 1 mL of 0.7% thiobarbituric acid was added to supernatant and heated at 100°C for 10 min, forming a pink colored solution. After cooling of the mixtures, absorbance was measured by Spectrophotometer (UV-Vis 1201; Shimadzu, Japan) at 532 nm. The results were expressed as nmol MDA per mg of protein.

Estimation of GSH

GSH level was assayed by the method of Ellman, (1959). In brief, 1 mL of tissues homogenate was added to 1 mL of 5% trichloroacetic acid and the mixture was

Table I

Effect of α -lipoic acid on the removal of arsenic from arsenic-loaded isolated liver tissues of rat					
Group	n	Incubation of liver tissues at 37°C for 45 min		Tissues concentration of arsenic after second incubation ($\mu\text{g/g}$ protein; mean \pm SEM)	p value
		First incubation	Second incubation		
I	6	None	None	1.1 \pm 0.1	
II	6	Arsenic (50 μg)	None	91.9 \pm 2.0	
III	6	Arsenic (50 μg)	lipoic acid (1 μM)	67.7 \pm 2.5	p<0.001
IV	6	Arsenic (50 μg)	lipoic acid (10 μM)	50.1 \pm 1.7	p<0.001
V	6	Arsenic (50 μg)	lipoic acid (100 μM)	27.2 \pm 1.7	p<0.001

n = Number of samples in each group; None means tissues were incubated with physiological solution without arsenic

Table II

Effect of α -lipoic acid on the levels of GSH and MDA in arsenic-loaded isolated liver tissues of rat						
Parameter	Control	Arsenic (50 μg)	Arsenic (50 μg) + lipoic acid (1 μM)	Arsenic (50 μg) + lipoic acid (10 μM)	Arsenic (50 μg) + lipoic acid (100 μM)	p value
GSH	2.8 \pm 0.1	1.4 \pm 0.04	1.7 \pm 0.1	2.1 \pm 0.1	2.5 \pm 0.1	p<0.001 ^a
MDA	3.1 \pm 0.1	9.3 \pm 0.1	6.8 \pm 0.2	5.2 \pm 0.2	3.9 \pm 0.2	p<0.001

Values were expressed as mean \pm SEM of six samples in each group; GSH refers to reduced glutathione level expressed as μg sulphhydryl groups/mg protein; MDA refers to malondialdehyde level expressed as nmol/mg protein. ^aSignificance based on Dunnett's multiple comparison tests for comparison of treated groups with standard (only arsenic-treated) group

vortexes and centrifuged at 4,000 \times g for 5 min. To 250 μL of supernatant, 2 mL Na_2HPO_4 (4.3%) and 250 μL of DTNB (0.04%) were added. The mixture was allowed to stand for approximately 15 min, and forming a yellow substance. The absorbance was measured at 412 nm using a spectrophotometer.

Cell viability test

The tissues were incubated with different strengths of arsenic (25, 50, 100, 200 μg) at 37°C for 45 min to determine the concentration of arsenic that would not induce tissues damage. Cytotoxicity was determined by the release of LDH into the medium. After the incubation of tissues with arsenic, the supernatant (medium) were removed and analyzed for LDH content using Human Gmbh diagnostic LDH assay based on the technique of Schumann et al. (2002).

Estimation of protein

Protein concentration of tissues was estimated by 'Biuret' method described by Weichselbaum (1949). Bovine serum albumin (8 g/dL) was used as standard.

Statistical analysis

Statistical analyses were carried out using Statistical Package for Social Science (SPSS), version 9.0, USA. The values were expressed as mean \pm SEM for results obtained with six samples in each group and the significant of differences between values was

determined by one-way analysis of variance (ANOVA) F-test coupled with the Dunnett's multiple comparison test. Statistical significance was determined by p value less than 0.05.

Results

The amount of total arsenic in arsenic-loaded isolated liver tissues of rat after second incubation was 91.9 \pm 2.0 $\mu\text{g/g}$ of protein (Table I). But when the arsenic-loaded tissues were incubated with 1, 10, and 100 μM concentration of α -lipoic acid during the second incubation, the amounts of total arsenic in tissues were decreased to 67.7 \pm 2.5, 50.1 \pm 1.7, and 27.2 \pm 1.7 $\mu\text{g/g}$ of protein respectively. The removals of accumulated arsenic from tissues were 26.3, 45.5 and 70.5% respectively. These effects of removing arsenic were dose dependent and statistically significant (p<0.001).

As shown in Table II, the mean (\pm SEM) concentration of GSH in arsenic-untreated tissues was 2.8 \pm 0.1 $\mu\text{g}/\text{mg}$ of protein and following incubation of tissues with 50 μg of arsenic, the concentration of GSH decreased to 1.4 \pm 0.04 $\mu\text{g}/\text{mg}$ of protein which was statistically significant (p<0.001) in comparison to the control group. In arsenic plus α -lipoic acid (1 μM) treated group, GSH concentration averaged 1.7 \pm 0.1 $\mu\text{g}/\text{mg}$ of protein. With increasing the doses of α -lipoic acid (10 and 100 μM), GSH concentration significantly (p<0.001)

increased to 2.1 ± 0.1 and 2.5 ± 0.1 $\mu\text{g}/\text{mg}$ of protein respectively. This result also indicate that GSH level significantly ($p < 0.001$) decreased in arsenic-treated groups by 52% compared to control while administration of α -lipoic acid in arsenic-loaded isolated liver tissues significantly ($p < 0.001$) restored the GSH level by 25, 47 and 79% at 1, 10 and 100 μM concentration respectively. The mean (\pm SEM) concentration of MDA in arsenic-untreated group was 3.1 ± 0.1 nmol/mg protein. When the tissues were treated with 50 μg of arsenic, the concentration of MDA increased to 9.3 ± 0.1 nmol/mg protein which was statistically significant ($p < 0.001$) in comparison to the control group. Treatment of arsenic-loaded isolated liver tissues with different concentrations of α -lipoic acid (1, 10, and 100 μM) significantly ($p < 0.001$) decreased the MDA concentration thus bring back the changes to near normalcy (6.8 ± 0.2 , 5.2 ± 0.2 , 3.9 ± 0.2 nmol/mg protein respectively). The increased production of MDA by exposure to arsenic and its recovery by different concentrations of α -lipoic acid treatment were shown in Table II. Results indicate that MDA production, an indicator of lipid peroxidation, was increased ($p < 0.001$) in arsenic-treated groups and it was 201% compared to control while administration of different concentrations of α -lipoic acid (1, 10, and 100 μM) in arsenic-loaded tissues could significantly ($p < 0.001$) reduce the production of MDA by 40, 66 and 88% respectively.

The effect of different concentrations of arsenic (25, 50, 100 and 200 μg) on cell viability was examined. The LDH activity after incubation of tissues with 100 and 200 μg of arsenic were 58.5 ± 0.9 and 139.2 ± 3.1 U/I respectively, whereas, the LDH activity was not detected in tissues loaded with 25 and 50 μg of arsenic. These result suggested that 25 and 50 μg of arsenic preserve cell viability.

Discussion

Oxidative stress due to enhanced production of free radicals has been incriminated as one of the several mechanisms involved in arsenic-induced toxic effects in different organs. In view of the anti-oxidant properties of α -lipoic acid, the present work was conducted to evaluate its effects on the removal of arsenic from arsenic-loaded isolated liver tissues of rat.

In this study, viability of the tissues was determined by the activity of LDH. The activity of LDH was not showed in a concentration of 25 and 50 μg of arsenic. These data not only suggested that the preparations were viable but also suggested that arsenic exposure at

least at the 50 μg concentrations used in this experiments was not induce tissue damage.

In the present work, a substantial increase in the level of arsenic was observed in the tissues treated with arsenic. However, treatment with different concentrations of α -lipoic acid remarkably brings down the level of arsenic ($p < 0.001$) in a dose-dependent manner.

Arsenic binds to the SH group of dihydrolipoate and inhibits pyruvate dehydrogenase consequently prevents oxidation of dihydrolipoate to lipoate, which is needed in the formation of acetyl-CoA from pyruvate (Miller et al., 2002). Dose-dependent protection offered by α -lipoic acid might be attributed to the ability of α -lipoic acid to protect the SH groups in the reduced form or compete with mitochondrial lipoamide for availability of arsenic, which thus prevents the binding of arsenic to proteins in isolated liver tissues of rat.

Arsenic exposure in this experiment resulted in a significant ($p < 0.001$) reductions in the level of the GSH and associated with increases in lipid peroxidations in comparison to control group. Arsenic content causes extensive oxidation of intramitochondrial NADPH by inhibition of α -ketoacid dehydrogenase (Shi et al., 2004). The shortage of NADPH production during arsenic exposure would suppress the reduction of GSSG subsequently decrease the GSH content. The increase in the levels of MDA was due to the increased release of iron that was believed to be involved in the Fenton type of reaction. Arsenic is shown to stimulate the release of iron from ferritin and through the activation of heme oxygenase the rate limiting enzyme in heme degradation (Ahmad et al., 2000). The free iron is considered as a potent enhancer of ROS formation, as exemplified by the reduction of H_2O_2 with the generation of highly aggressive hydroxyl radical (Farber, 1994). Therefore, this may be the possible reason for elevation of the levels of MDA with a concomitant fall in the GSH content. The inverse relationship between lipid peroxidation and GSH levels in the liver on arsenic exposure was suggested by Ramos et al. (1995).

However, treatment with different concentrations of α -lipoic acid significantly ($p < 0.001$) increase the levels of GSH. Enhancement of intracellular availability of cysteine, by passing the rate limiting cystine transport system may be the underlying mechanism of α -lipoic acid-induced elevation of GSH levels observed in this study. This finding was in agreement with previous *in vitro* research work (Busse et al., 1992).

In the present study, the decrease levels of MDA observed in the α -lipoic acid treatment group may be

attributed to its capacity to regenerate the reduced glutathione pool and/or may be directly react with ROS. These findings were consistent with the results of previous studies (Shila et al., 2005b).

Conclusion

The finding of the present study suggests that exposure of liver tissues with arsenic was associated with a depletion of GSH and increased lipid peroxidation. α -Lipoic acid administration offered a significant removal of arsenic, which was associated with its anti-oxidant activity that combine free radical scavenging and metal chelating properties. However, further studies with α -lipoic acid need to be carried out *in vivo* to ascertain their therapeutic efficacy in modifying chronic arsenic toxicity.

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Ethical Issue

The study was approved by the Ethical Committee of Bangabandhu Sheikh Mujib Medical University.

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