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Protective effects of alpha-lipoic acid against benzene induced toxicity in experimental rats

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Abstract. – OBJECTIVE: The aim of the present study was to examine the potential protective effects of Alpha-Lipoic Acid (ALA) against benzene induced alteration in CYP2E1 gene function, DNA damage as well as the oxidant-antioxidant status.

MATERIALS AND METHODS: Fifty adult male Wister rats were used for this study. Rats were randomized and divided into four groups: Group I was designated as control group and received a vehicle of olive oil; Group II was received alpha lipoic acid in a dose of (20 mg/kg, i.p.); Group III received only I.M. injection of benzene in a dose of 2 ml/kg. Group IV received both alpha lipoic acid in a dose of (20 mg/kg, i.p.) and I.M. injection of benzene in a dose of 2 ml/kg. The animals were treated on each alternate day for 30 days then all rats were sacrificed and both blood and liver tissue samples were taken to measure serum malondialdehyde (MDA) Level; serum level of reduced glutathione (GSH); level of serum 8-hydroxy-2'-deoxyguanosine; hepatic microsomal cytochrome P450 2E1 activity and gene expression.

RESULTS: Benzene significantly increased serum malondialdehyde (MDA), 8-hydroxy-2'-deoxyguanosine (8-OHdG) and both CYP2E1 activity and gene expression but significantly decreased level of reduced glutathione (GSH). These changes are reversed upon administration of ALA.

CONCLUSIONS: The current study provided evidence that the linkage between CYP2E1-dependent oxidative stress, DNA damage, and GSH homeostasis could contribute to the toxic actions of benzene which can be counteracted by ALA by its suppression action on CYP2E1, inhibition of lipid peroxidation and oxidative DNA damage as well as maintenance of intracellular antioxidants status.

Key Words:
Alpha lipoic acid, Benzene, Cytochrome P4502E1, Oxidative stress.

Abbreviations

ALA = alpha lipoic acid; CYP2E1 = cytochrome P4502E1; ROS = reactive oxygen species; MDA = Malondialdehyde; 8-OHdG = 8-hydroxy-2'-deoxyguanosine; GSH = reduced glutathione; DHLA = dihydrolipoic acid; TBA = thiobarbituric acid; 4-NC = 4-Nitrocatechol; RNase = ribonuclease; DEPC-H_2O = diethyl pyrocarbonate-treated water; Nrf2 = nuclear factor erythroid 2-related factor; NF-κB = nuclear factor kappa B

Introduction

Benzene is widely used for manufacturing gasoline and plastics, and is present in cigarette smoke[1]. Benzene, being lipid soluble, it tends to accumulate in tissues with high lipid content and about 50% of the absorbed dose may be eliminated unchanged through exhalation, while the remainder is metabolized in the liver, primarily by cytochrome P4502E1 (CYP2E1) systems to form reactive metabolites such as benzene oxide, catechol and hydroquinone[2].

Benzene toxicity has been linked to the ability of its reactive intermediates to produce reactive oxygen species (ROS) and to bind to cellular macromolecules to induce damage[3]. These reactive species act as signaling molecules affecting the regulation of gene expression, cell growth and cell death; they also enhance lipid perioxidation[4]. Under physiological conditions, specific metabolizing and scavenging systems tightly control the concentrations of ROS formed by cells and tissues. However, the imbalance between the production of ROS and clearance of ROS by components of the antioxidant defense system causes a state of oxidative stress[5].

The cytochrome P450 enzymes are a superfamily of hemoproteins, which catalyze the oxidation of various endogenous substrates and xenobiotics[6]. CYP2E1is the classical ethanol-inducible cytochrome P450 isozyme, which can metabolically activate numerous procarcinogens and hepatotoxic substrates in the liver such as benzene, ethanol, carbon tetrachloride and acetaminophen to more toxic products[7]. CYP2E1 exhibits enhanced NADPH oxidase activity and is

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very reactive in catalysis of lipid peroxidation and production of ROS in higher amounts relative to other P450 isoforms[8].

ROS are capable of causing damage to various cellular constituents, such as nucleic acids, proteins and lipids. When the oxidative DNA damage overwhelms the repair capacity of the cell; the mutagenic and genotoxic DNA damage products accumulate causing genomic instability and thus exponentially contribute to an increasing cancer risk[9]. Previous studies depicted that measurement of the promutagenic DNA lesion, 8-hydroxy-deoxyguanosine (8-OHdG), might be a useful marker in estimating oxidative DNA damage[10].

Alpha-lipoic acid (ALA) is a sulfur-containing coenzyme which is essential for the function of different enzymes involved in mitochondria's oxidative metabolism[11]. ALA is soluble in both lipid and aqueous environments, and is readily absorbed from the diet, transported to cells and reduced to dihydrolipoic acid (DHLA)[12]. ALA and DHLA have potent antioxidant activities via scavenging of oxygen free radicals, redox interaction with other antioxidants, metal ion chelation, repair of oxidatively damaged proteins and inhibition of lipid peroxidation[13]. However, the studies on the effects of ALA on benzene induced toxicity are still inconclusive.

In view of these considerations, the present work was undertaken to examine the potential protective effects of ALA against benzene induced alteration in CYP2E1 gene function (evaluated by mRNA expression and enzyme activity), DNA damage as well as the oxidant-antioxidant status.

Materials and Methods

Chemicals

Biochemicals and substrates were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA).

Animals

Experimental animals and diets: Fifty adult male Wister rats, weighed 120-150 g, were used for this study. They were housed in wire mesh cages at an ambient temperature of 22°C, on a 12 h light/dark cycle, and allowed *ad libitum* to water and a standard pellet diet through the whole period of the experiment. The experiment was conducted in accordance to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978).

Experimental design: After being acclimatized for 1 week, the rats were randomized and divided into four groups: Group I (10 rats) was designated as control group and received a vehicle of olive oil in a dose of 0.2 ml; Group II (10 rats) was received alpha lipoic acid in a dose of (20 mg/kg, i.p.)[14]; Group III (15 rats) received only I.M. injection of benzene (dissolved in olive oil) in a dose of 2 ml/kg. Group IV (15 rats) received both alpha lipoic acid in a dose of (20 mg/kg, i.p.) and I.M. injection of benzene in a dose of 2 ml/kg. The animals were treated on each alternate day for 30 days. At the end of the experiment (30 days), and following 16 hours of fasting, all rats were sacrificed by decapitation and both blood and liver tissue samples were taken.

Blood and Tissue Sampling

Blood sampling: It was collected into a dry sterile centrifuge tube, allowed to clot at room temperature for 30 minutes and, then, centrifuged at 1000 × g for 20 minutes at 4°C; serum was separated and stored at –70°C until the time of analysis.

Tissue sampling: After decapitation, livers were immediately perfused in situ with ice-cold 0.9% (w/v) NaCI solution and divided into two parts: one part was used for microsome preparation as described elsewhere[15]; the livers were homogenized with the homogenizing medium that consisted of isotonic sucrose containing 0.05 M Tris-HCI, pH 7.5, 0.005 M $MgCI_2$, 0.025 M KCI and 0.008 M $CaCI_2$. After clarifying the homogenate at 10,000 × g for 15 min, 4 or 5 ml of the post-mitochondrial supernatant was diluted with 25 ml of 0.0125 M sucrose solution containing 0.008 M $CaCI_2$, and 0.005 M $MgCI_2$. The entire solution was stirred occasionally for a few minutes and then centrifuged at 1500 × g, for 10 min. The reddish pellet obtained was dispersed and re-centrifuged twice at 1500 × g max for 10 min in dilute (0.0125 M) sucrose and finally homogenized in 0.05 M Tris-HCI buffer containing 0.005 $MgCI_2$, and 0.025M KCI. Treating the rat liver under identical conditions described above with the exception of the addition of calcium chloride resulted in no pellet sedimenting at 1500 × g for 10 min. On the other hand, normal microsomes which had been isolated at 105,000 g

could be quantitatively sedimented at 1500 × g in the presence of 0.008 M $CaCl_2$, under the similar experimental conditions described above according to Kamath et al[15]. Another part of the liver was used for determination of CYP2E1expression as mentioned below.

Biochemical Analysis

Serum malondialdehyde (MDA) level: MDA levels in the serum were determined by a spectrophotometric method as described by Ohkawa et al[16], it was based on spectrophotometric measurement of the color occurring at 532 nm during the reaction of MDA with thiobarbituric acid (TBA).

Reduced glutathione (GSH) level: Serum GSH levels were measured using a commercial kit supplied by (Biodiagnostic, Cairo, Egypt) according to the manufacturer's instructions. It was based on spectrophotometric measurement of the color occurring at 412 nm during the reaction to 5-5 dithiobis-2-nitrobenzoic acid (DTNB).

Serum 8-hydroxy-2'-deoxyguanosine level: The levels of serum 8-OHdG were determined by an enzyme-linked immunosorbent assay (ELISA) using a commercially available kit (Cat# ADI-EKS-350, Enzo Life Sciences Inernational, Plymouth Meeting, PA 19462-1202, USA) according to the manufacturer's instructions. Concentrations were calculated using a standard curve generated with specific standards provided by the manufacturer.

Hepatic microsomal cytochrome P450 2E1 activity: CYP2E1 activity was measured spectrophotometrically by the rate of oxidation of p-nitrophenol to p-nitrocatechol according to the method of[17]. Briefly; 1 ml of the assay mixture (consisted of: 200 µl of rat liver microsome, 100 µl of 1 mM NADPH, 100 µl of 10 mM Ascorbic acid, 500 µl of 100 mM Potassium Phosphate buffer (pH 6.8) and 100 µl of 100 µM P-nitrophenol) was incubated for 10 minutes at 37ºC; then 0.5 ml of 0.7 N Perchloric acid was added to terminate the reaction. Following centrifugation at 1300 × g for 5 minutes, 1 ml of supernatant was mixed with 100 µl of 10 N NaOH. The absorbance of 4-Nitrocatechol (4-NC) was recorded with a HITACHI U-2000 spectrophotometer at 546 nm and, then, its concentration was calculated using an extinction coefficient of 9.53 mM^{-1} cm^{-1} for p-nitrocatechol). The protein concentration was estimated by the method of Bradford using bovine serum albumin as a standard[18]. Results are expressed as nmol 4-Nitrocatechol formed/min per mg of microsomal protein.

Gene Expression of Cytochrome P450 2E1

Preparation of Total Liver RNA

Total RNA was prepared from liver tissue according to the manufacturer's instructions (Promega, Madison, WI, USA). Approximately 100-200 mg of liver tissue was homogenized and subjected to RNA extraction to disrupt cells followed by denaturation of nucleoprotein complexes, inactivation of endogenous ribonuclease (RNase) activity, and finally removal of proteins and DNA. The resulting RNA was reconstituted in diethyl pyrocarbonate-treated water (DEPC-H_2O), checked for purity and integrity spectrophotometrically at 260 nm and by gel electrophoresis and used for RT-PCR.

RT-PCR for CYP2E1

Reverse transcription of 10 µg of RNA to the corresponding amount of cDNA was carried out according to the manufacturer's instructions (Promega, Madison, WI, USA), and the material was stored at –20°C until use. Amplification of cDNA and the selection of the primer sequences were done according to[19]. The PCR reactions of cDNA were performed in a final volume of 50 µL consisting of 5 µL of cDNA, 5 µL of 10X PCR buffer, 200 µM of each deoxynucleotide triphosphate, 2.5 mM $MgCI_2$, 1.5 U AmpliTaq DNA polymerase (Promega, Madison, WI, USA) and 50 pmol each of the following: CYP2E1 specific forward and reverse primers (Life Technologies, Carlsbad, CA, USA): forward, 5`-TGC-CATCAAGGATAGGCAAG-3`; reverse, 5`-AAT-GCTGCAAAATGGCACAC-3`. PCR reactions were carried out in a Perkin-Elmer 9600 thermal cycler starting with heating at 95°C for 4 min and then using melting, annealing, and extension cycling conditions of 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min respectively. All amplifications were carried out for 30 cycles followed by final extension time at 75°C for 4 min. All reactions were conducted with β-actin primers (790 bp) as internal controls (5`primer, 5`TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA 3`; 3`primer, 3`CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG 5`, Stratagene). An aliquot of 20 µL from each amplified cDNA product (356 bp) was separated on 2% agarose

gel (Sigma), and visualized by UV light illuminator using ethidium bromide staining and photographed by Polaroid film. The intensity of bands was quantified by gel proanalyzer image analyzer version III where the area of a selected band was blotted against calibration curve of a single image using multiple standard bands and the results were analyzed by computer software (Biorad, Warrington, UK)[19].

Statistical Analysis

Results are expressed as mean ± standard deviation (SD). The intergroup variation was measured by one-way analysis of variance (ANOVA) followed by the Tukey's test. The statistical evaluation of the data was performed using Graph Pad Prism 4.03 (GraphPad Software, San Diego, CA, USA). p values less than 0.05 were considered statistically significant.

Results

There was a significant increase in serum MDA, 8-OHdG and CYP2E1activity and gene expression but significant decrease in GSH in benzene group as compared with control, which are all reversed upon administration of ALA, with no significant differences between benzene co-administrated with ALA group and control group regarding serum MDA, 8-OHdG and both CYP2E1 activity and gene expression (Table I, Figure 1).

Discussion

Despite extensive investigations, mechanisms underlying benzene induced toxicity and leukemogenicity are not yet fully elucidated. Therefore, this study aimed to investigate the protective effects of the antioxidant, ALA against benzene-induced toxicity by determining its effect on CYP2E1gene function and on some oxidative stress markers.

Our data revealed a significant increase in activity and mRNA expression level of hepatic CYP2E1 in benzene injected group compared to control group. These results came in accordance with that of Thomas et al and McHale et al[20,21] who reported dose-dependent changes in CYP2E1 gene expression associated with occupational exposure to benzene. Likewise, González-Jasso et al[22] noted that benzene administration resulted in a clear, dose-dependent induction of rat CYP2E1 and increased the content of CYP2E1mRNA and protein in both liver and peripheral lymphocytes. This inductive effect could be probably achieved by transcriptional or post-transcriptional regulation. Moreover, benzene was found to stabilize CYP2E1 by inhibiting protein degradation[23]. CYP2E1, in turn, may then be responsible for the increased formation of toxic metabolites of benzene such as hydroquinone which might then potentiate the toxic effect of benzene[24]. Being an effective generator of ROS that can result in cell damage[25], CYP2E1 can be suggested as a major contributor to benzene induced oxidative stress and liver injury.

Intriguingly, pretreatment with ALA resulted in a significant decrease in activity and mRNA expression level of hepatic CYP2E1 than their levels in benzene-injected group. This effect could be attributed to the ability of ALA to decrease the activity of NAPDH CYP2E1 reductase which is essential for proper catalytic function of CYP2E1[26]. Given that oxidative stress has a crucial role in CYP2E1 induction as evidenced by studies showing that ethanol mediated induction

Table I. Effectiveness of ALA against benzene induced alterations in studied parameters.

	Control group (n = 10) mean ± SD	ALA group (n = 10) mean ± SD	Benzene group (n = 15) man ± SD	Benzene + ALA group (n = 15) mean ± SD	F	p
Serum MDA nmol/ml	3.1 ± 0.2	2.9 ± 0.3	5.2 ± 1.1*	3.5 ± 0.6#	28.9	< 0.001
Serum GSH mg/dl	3.2 ± 0.1	3.3 ± 0.2	1.8 ± 0.5*	2.8 ± 0.3*,#	54.1	< 0.001
Serum 8-OHdG ng/ml	4.2 ± 1.6	4.1 ± 0.7	9.8 ± 2.9*	5.2 ± 1.4#	26.1	< 0.001
Cytochrome P450 2E1 (nmol/min/mg microsomal protein)	0.19 ± 0.08	0.25 ± 0.09	0.56 ± 0.12*	0.26 ± 0.08#	40.9	< 0.001

*$p < 0.05$ versus control group; #$p < 0.05$versus benzene group.

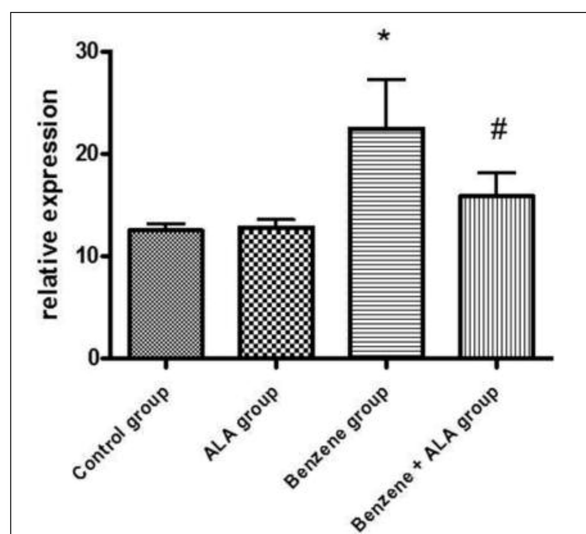

Figure 1. The effect of ALA on hepatic CYP2E1 mRNA expression in rats. The mRNA levels were normalized to those of β-actin. RT-PCR analysis reveals that the mRNA expression of CYP2E1was significantly increased in benzene group. In contrast, co-administration of ALA with benzene reduced the expression of CYP2E1. Data are expressed as the mean ± SD. $^{*}p < 0.05$ versus control group; $^{\#}p < 0.05$ versus benzene group.

of CYP2E1 was significantly abrogated by antioxidants such as vitamin C; it is tempting to speculate that the cytoprotective role of ALA against benzene induced activation of CYP2E1 might also be credited to its ability to suppress the activation of NADPH oxidase and to reduce NADPH-induced generation of ROS[27].

Noteworthy, ROS produce cellular injury and necrosis via several mechanisms including peroxidation of lipids, protein and DNA[5]. Our data demonstrated that benzene administration caused a significant increase in lipid peroxidation estimated by serum levels of MDA as compared to control group. This is in agreement with previously published data reporting that benzene-injected rats showed increased serum MDA and decreased total antioxidant activity[28].

Besides, it has been stated that the released free radicals causing peroxidation of membrane lipids, result in rupture of the lysosomal membranes, the release of lysosomal enzymes, necrosis of the cell and destruction of parenchymal tissue. All these processes culminate in an increase in serum MDA levels[29].

Moreover, MDA serum levels were significantly decreased in ALA pretreated group compared to benzene only injected group; this finding is not without precedence as previous studies, both *in vivo* and *in vitro*, have shown strong antioxidant activity of ALA against lipid peroxidation[30]. Well in line, reduced lipid peroxidation following ALA administration has been observed to be associated with a protective effect against oleic acid[31], drugs[32] or toxins-induced[33] peroxidative and cellular damage in experimental rats; suggesting oxidative stress preventive role for ALA. Additional studies on diabetic rats have documented a preventive role for ALA against the increased MDA concentration in rat plasma, liver and kidneys[34].

Nevertheless, increased oxidants associated with benzene toxicity could results in cell death through two different pathways. One pathway involves lysosomal rupture secondary to peroxidative damage to lysosomal membranes as a result of Fenton-type reactions catalyzed by intra-lysosomal iron[35]. The alternative pathway involves loss of mitochondrial-cytochrome c content due to enhanced outer mitochondrial membrane permeability and loss of mitochondrial trans-membrane potential. This, in turn, activates caspase cascade and apoptosis[36]. ALA proved to be beneficial in alleviating oxidative stress mediated apoptosis possibly by ROS scavenging, enhancing the redox status, by chelating intralysosomal iron and, consequently, preventing lysosomal rupture[37].

On the other hand, GSH and redox-modulating enzymes play a prominent role in detoxification of ROS and many pollutants by either direct interaction with ROS, or by reducing erroneous disulfide linkages in proteins, thus protecting cells from oxidative damage[38]. Therefore, we further studied the non-enzymatic antioxidant defense by estimating the circulating levels of GSH, where there was a significant reduction in GSH serum levels in benzene injected group compared to control group. In this context, Uzma et al[39] stated that GSH depletion is one of the important consequences of toxic injury; which might be attributed to reduced activity of GSH-dependent enzyme, GSH-peroxidase, leading to damage of lipids, proteins and DNA by the ROS. Relevant to this notion is the finding that the tripeptide glutathione is a vital defense mechanism against oxidative stress and its cellular level is a critical factor determining whether a cell survives or succumbs to death mechanisms induced by toxins[40].

Moreover, ALA pretreatment caused restoration of GSH levels as evident from the significant rise in their serum levels in ALA pretreated group compared to the benzene-injected group. This finding is consistent with earlier reports

documented that ALA administration ameliorated oxidative stress induced either by certain drugs or under various physiological and pathophysiological conditions in rats[13] through restoration of non-enzymatic antioxidants such as GSH, maintaining normal activities of the antioxidants enzymes such as catalase and glutathione peroxidase[30]; thus substantiating the antioxidant, cytoprotective effect of ALA.

In harmony with this finding, Chen et al[41] showed that ALA could enhance *de novo* synthesis of GSH. This increase in intracellular GSH synthesis is accomplished by the regulating effect of ALA on glutathione synthesis; increasing the expression of gamma-glutamylcysteine ligase, increasing cysteine availability and cellular uptake. Likewise, ALA can recycle and prolong the metabolic lifespan of endogenous antioxidants, such as GSH, vitamin C and coenzyme Q10; which in turn can regenerate oxidized vitamin E, forming an antioxidant network that protects cells from oxidative damage[42]. Moreover, Al-Rasheed et al[43] reported that ALA decreased liver damage due to zinc oxide nano-particles by synergistically increasing cellular levels of GSH.

Since oxidation of benzene is a prerequisite of cellular toxicity activated by CYP2E1, DNA oxidative lesions induced with benzene might be used as a phenotypic marker[44]. In nuclear and mitochondrial DNA, (8-OHdG) is one of the predominant forms of free radical-induced oxidative lesions and has, therefore, been widely used as a biomarker for oxidative stress associated with chemical exposure[10]. Along this line, we investigated the effects of benzene administration on serum level of 8-OHdG, where there was a significant increase in serum 8-OHdG level in the benzene injected group compared to control group. This was in according with Goethel et al[45] who underscored the concept that benzene is genotoxic by reporting that 8-OHdG levels were significantly higher in workers exposed to benzene than those without known occupational exposure. The high biological relevance of 8-OHdG is due to its ability to induce G → T transversions, which are among the most frequent somatic mutations found in human cancers[46].

Concomitantly, ALA pretreatment caused a significant decrease in 8-OHdG level compared to the benzene-injected group. This is in accord with studies reported that lipoic acid has protective effects as an antioxidant against oxidative damage to DNA induced by alcohol in the developing hippocampus and cerebellum of rat[47].

Taken together, it can be concluded that ALA modulated antioxidant defense and decreased oxidative tissue damage and thus exerts a protective effect against benzene-induced toxicity. Several mechanisms have been proposed to explain this protective efficacy. One plausible mechanism is the ability of ALA to promote the translocation of nuclear factor erythroid 2-related factor (Nrf2) to the nucleus[48]; which is responsible for induction of the expression of detoxifying phase II enzyme encoding genes and antioxidant genes including heme oxygenase-1 and (NADPH) quinine-oxidoreductase resulting in a cytoprotective effect against oxidative stress[49]. Additionally, ALA was found to modulate the signaling transduction of several pathways such as nuclear factor kappa B (NF-κB), insulin and nitric oxide synthesis[11]. It can inhibit the redox sensitive NF-κB signaling; resulting in reduction of production of free radicals and cytotoxic cytokines[50].

Conclusion

The current study provides evidence that the linkage between CYP2E1-dependent oxidative stress, DNA damage, and GSH homeostasis could contribute to the toxic actions of benzene which can be counteracted by ALA probably through its suppressive actions on CYP2E1, lipid peroxidation and oxidative DNA damage as well as maintaining intracellular antioxidants status.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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