Hepatoprotective Effect of Alpha Lipoic Acid Against Bromobenzene-Induced Liver Damage in Rats

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Abstract

The hepatic lesion produced as a result of oxidative stress is of wide occurrence. In this study, the effect of the antioxidant alpha lipoic acid (ALA) on liver necrosis induced by bromobenzene (BB), which is known to produce oxidative stress, in rats had been studied. Treatment of animals with ALA for one week before the induction of chemical liver injury with BB had been carried out. Various biochemical changes associated with liver damage and oxidative stress were measured. The activities of isocitrate dehydrogenase (ICDH), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) as indices of liver function were measured. Hepatic reduced glutathione (GSH) content, malondialdehyde (MDA), and nitric oxide (NO) were measured as oxidative stress markers as well as hepatic total protein (TP). Histopathological studies had been carried out to ascertain the cause of hepatic cell death and to provide further evidence to support the hepatoprotective effect of ALA and correlate it with any biochemical changes. Administration of BB produced a significant rise in the serum activities of ICDH and ALT together with a marked reduction in hepatic GSH content and a significant elevation of liver MDA and NO. Histopathological examination revealed loss of cell boundaries, marked centrilobular necrosis and disappearance of nuclei. Congestion and inflammatory cells around central vein were also observed. Pretreatment with ALA significantly reduced serum ALT activity and normalizes that of ICDH. Hepatic GSH content was significantly increased, while MDA and NO were normalized together with preservation of the hepatocyte architecture suggesting a clear hepatoprotective role of ALA against BB-induced liver damage.

Key Words: Alpha lipoic acid – Bromobenzene – Hepatic necrosis – Oxidative stress – Hepatoprotection.

Introduction

HEPATIC injuries such as necrosis, which is often produced on exposure of the tissue to viruses or many chemical agents [1] constitute a major health hazard. The liver is often a primary target for chemical-induced toxicity due to its abundance of xenobiotic- metabolizing enzymes and its high capacity for both phase I and phase II biotransformations. Although the biotransformation sequence generally provides a detoxification pathway, there is a possibility that the reactions catalyzed by CYP450 enzymes may generate metabolites that are not only more toxic, but also more reactive than the original xenobiotic. Ultimately, it is the balance between bioactivation, detoxification and defense mechanisms that determine whether a reactive metabolite may elicit a toxic effect [2]. Bromobenzene (BB) which is mainly used as an additive to motor oil, an intermediate in the synthesis of organic chemicals, in pesticides manufacturing, and an additive in the rubber industry is a documented hepatotoxicant that leads to hepatic necrosis [3,4]. BB may also be formed during chlorination of drinking water [5] and is resistant to biodegradation after its leakage to the environment. Therefore, it may pollute ambient air, food and water [6], however, the main source of human exposure to BB includes occupational environment as well as consumer exposure to motor oil [7].

BB is first metabolized in the liver to an electrophilic intermediate which readily conjugates with reduced glutathione (GSH), leading to its depletion. GSH is capable of acting as a nucleophilic scavenger and a cofactor in GSH peroxidase mediated destruction of peroxides and hydroperoxides. Thus its depletion leads to oxidative tissue injury [8]. Alpha lipoic acid (ALA) or thioctic acid has been recognized for its prominent antioxidant effects, where it scavenges reactive oxygen species (ROS) through its two thiol groups (−SH); regenerate vitamins E, C, and glutathione, and complex metal ions [9]. It has been widely used in the treatment of liver cirrhosis, mushroom poisoning,
The aim of the present study was to investigate the possible hepatoprotective effect of ALA, by virtue of its antioxidant mechanisms, against liver injury induced with BB.

**Materials and Methods**

**Drugs and chemicals**

Alpha lipoic acid was obtained from EVA pharmaceutical company, Egypt. Bromobenzene was purchased from Riedel-de Haen, Germany. Other chemicals and organic solvents were of Analar grade. The drugs used were freshly prepared prior to administration.

**Animals**

Adult male albino rats each weighing 150-200 g were obtained from the animal house at the National Research Center (Giza, Egypt). The animals were kept under suitable laboratory conditions throughout the period of investigation. They were fed standard pellet chow, provided by the animal house at the National Research Center, and allowed free access to water.

**Induction of hepatic necrosis with bromobenzene and drug treatment**

Animals were randomly allocated into four groups (eight animals each): The first group received orally normal saline for one week followed, after an overnight fast, by corn oil by oral gavage and served as normal control. The second group received orally ALA 150 mg/kg po dissolved in 0.5% KOH in normal saline for the same period followed, after an overnight fast, by corn oil. The third group received normal saline for one week followed by a single dose of BB (10 mmol/kg (≈ 1ml/kg) of 20% BB in corn oil) by oral gavage and served as BB control. The dose used in the present study is within the range reported in the literature to produce acute hepatotoxicity and necrosis. The last group received ALA for one week followed by a single necrogenic dose of BB. Two hours later, the groups that were pretreated with ALA received another dose of ALA, while the control groups received normal saline.

**Processing of tissue and biochemical estimations**

Twenty four hours after the last treatment, animals were sacrificed for blood sampling and liver dissection.

**Collection of blood samples and estimation of serum enzymes**

Blood samples were withdrawn from the retro-orbital vein of each animal, under light anesthesia by diethyl ether, according to the method of Cocchetto and Bjornsson.

Blood was allowed to coagulate and then centrifuged at 3000 rpm for 15 min. The obtained serum was used to estimate the activities of isocitrate dehydrogenase (ICDH), and aminotransferases; alanine aminotransferase (ALT) and aspartate aminotransferase (AST).

ICDH activity was estimated according to the method of Ellis and Goldberg, where the production rate of the reduced form of nicotinamide adenine dinucleotide phosphate (NADP) during reaction with isocitrate was calculated by spectrophotometric measurement of the change in absorbance at 340 nm at 1 min intervals and was expressed as IU/L.

Aminotransferases activities were estimated using diagnostic kits based on the methods described by Reitman and Frankel. For ALT, serum was mixed and incubated with α-ketoglutarate-alanine buffer, and for AST with α-ketoglutarate-aspartate buffer. Dinitrophenylhydrazine was then added followed by NaOH. The activity was determined spectrophotometrically by measuring the absorbance at 505 nm and expressed as U/ml.

**Preparation of liver samples**

Immediately after blood sampling, animals were sacrificed by cervical dislocation and the liver tissues were rapidly removed, washed in ice-cooled saline, plotted dry and weighed.

The left lobe of each liver was dissected and placed in 10% formalin in saline, to be used for histopathological examination.

A weighed part of each liver was homogenized with ice-cooled saline using a homogenizer (MPW-120, Poland), to prepare 20% w/v homogenate. The homogenate was then centrifuged at 4000 rpm for 5 min. at 4°C in a cooling centrifuge (Laborzentrifugen, 2k15, Sigma, Germany) to remove cell debris the aliquot was divided into four parts; the first was used for the assessment of reduced glutathione (GSH), the second for measurement of thiobarbituric acid reactive substances (TBARS) as malondialdehyde (MDA), the third to estimate the level of nitric oxide (NO), and the fourth part was used for the estimation of the total protein (TP) content.
Determination of reduced glutathione (GSH)

Liver GSH (µg/g protein) was determined spectrophotometrically at 412 nm using Ellman’s reagent after precipitation of protein in tissue homogenate with trichloroacetic acid [15].

Determination of lipid peroxides

Liver MDA was quantified by the thiobarbituric acid (TBA) test [16]. TBARS formed in tissue primarily consist of MDA, which form a pink or red adduct with 2 molecules of TBA (MDA-TBA 2) in acidic medium at high temperature. The pink adduct was extracted in n-butanol and measured spectrophotometrically at 535 and 520 nm the difference in absorbance between the two readings was taken as the level of TBARS in the sample and was expressed as nmol/g protein.

Determination of nitric oxide (NO)

Most of the biologically produced nitric oxide (NO) is oxidized to nitrate (NO 3 - and nitrite (NO 2 -), totally designated as NO x. Determination of NO x in liver tissue was carried out according to the method of Miranda et al. [17] after deproteinization of the sample with absolute ethanol. The assay is based on reduction of any nitrate to nitrite by vanadium chloride (VCl 3 ). The total nitrite (intrinsic + nitrite obtained from VCl 3 reduction of nitrate) is then detected colourimetrically as an azo dye product of the Griess reaction at 540 nm and was expressed as µmol/mg protein.

Determination of total proteins

Total protein in rat liver homogenate was determined spectrophotometrically at 595 nm using Bradford reagent [18] and was expressed as mg/g tissue.

Histopathological studies

For histopathological studies, few-millimeter-thick midsections of the left lobes of the livers excised from each group were processed for light microscopy. The processing involved fixing of the tissue specimens in a 10% neutral buffered formalin solution, preparing the blocks in paraffin, cutting sections 5-6 µm thickness, and staining the sections with haematoxylineosin stain. The sections were scanned and analyzed by an expert pathologist who was not aware of sample assignment to experimental groups.

Statistical analysis

In the present study, all results were expressed as mean ± standard error of the mean.

Data were analyzed by one-way analysis of variance (ANOVA). When the variation among groups was proved significant, the least significant difference (LSD) test was performed to compare significance between groups.

Difference was considered significant when p < 0.05.

Results

Effect of ALA on serum enzymes in liver necrosis

There were no significant change in the serum enzymatic activity of any of the tested enzymes in the group treated with ALA compared to the normal control group. Administration of a single dose of BB significantly elevated ICDH and ALT by about 63% and 49% respectively compared to the normal control values without significantly affecting AST activity. Pretreatment with ALA before the necrogenic dose of BB normalizes ICDH activity and significantly reduced ALT activity by about 29% compared to the BB intoxicated group (Table 1).

Effect of ALA on hepatic GSH content

Bromobenzene significantly reduced hepatic GSH content by about 53% compared to the normal control group. Pretreatment with ALA significantly elevated GSH by about 21% compared to the BB intoxicated group (Fig. 1).

Effect of ALA on hepatic MDA level

Hepatic MDA level, an index of lipid peroxidation, was significantly reduced by about 41 % in the group treated with ALA compared to the normal control. Administration of BB severely increased MDA content by about 182% compared to the normal control group, while pretreatment with ALA normalizes it (Fig. 2).

Effect of ALA on hepatic NO x

Hepatic NO x, measured as nitrite, increased significantly by about 35% compared to the normal control after the administration of BB. Pretreatment with ALA normalizes the hepatic nitrite content (Fig. 3).

Effect of ALA on hepatic TP

Administration of BB did not significantly alter hepatic TP content. However, the groups treated with ALA either alone or with BB afterwards showed a significant elevation in TP compared to the normal control (Fig. 4).
Effect of ALA on liver histology

The liver of control rat Fig. (5-A) appeared to be formed of the classical hepatic lobules. Each lobule showed radially arranged hepatocytes forming cords around the central veins. Blood sinusoids were seen separating the cords of the liver cells. In the group treated with ALA Fig. (5-B) the liver cells exhibited an almost normal architecture. Administration of BB Fig. (5-C) produced loss of cell boundaries, marked centrilobular necrosis and disappearance of nuclei. Congestion and inflammatory cells around central vein were also observed. Pretreatment with ALA preserves the structure and architecture of the hepatocyte from BB-induced damage but hemorrhage in the central vein was observed as shown in Fig. (5-D).

Table (1): Serum levels of enzymes in rats treated with alpha lipoic acid followed by a necrogenic dose of bromobenzene.

<table>
<thead>
<tr>
<th></th>
<th>ICDH (IU/L)</th>
<th>ALT (U/ml)</th>
<th>AST (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>3.4±0.4</td>
<td>42.2±1.8</td>
<td>132±2.93</td>
</tr>
<tr>
<td>ALA</td>
<td>3.75±0.3</td>
<td>43.02±1.8</td>
<td>122.27±5.1</td>
</tr>
<tr>
<td>BB</td>
<td>9.35±0.51*</td>
<td>82.16±1.19*</td>
<td>153.71±0.76</td>
</tr>
<tr>
<td>ALA + BB</td>
<td>2.89±0.4#</td>
<td>58.24±1.19#</td>
<td>144.74±4.64</td>
</tr>
</tbody>
</table>

Each value represents the mean of 8 experiments ± SEM
* Significantly different from the control group at \( p<0.05 \)
# Significantly different from BB at \( p<0.05 \)
Fig. (5): Effect of alpha lipoic acid on liver architecture in rats treated with bromobenzene. Photographs show the sections from normal (A), ALA-treated (B), BB-treated (C), ALA-treated followed by BB (D).

**Discussion**

Hepatic lesions produced as a result of oxidative stress are of wide occurrences in both clinical and experimental animal studies. A large number of chemicals inflict liver damage, like necrosis, by producing oxidative stress in different animal species. Often the injury is mediated by free radicals or by the depletion of endogenous pool of antioxidants such as GSH [19]. The compound used in this study to induce liver damage, bromobenzene, is metabolized into electrophilic product which readily conjugates with GSH, resulting in an extensive GSH depletion, thereby altering the reduced status of the tissue, and causing its injury [20].

The necrotic dose of BB given to rats significantly elevated the serum activities of ICDH and ALT, without affecting that of AST. This result is in harmony with Wong et al. and Chung et al. who claimed that ALT is more reliable than AST in detecting centrilobular necrosis [20,21]. However, Sanz et al. reported that serum ALT and AST cannot detect centrilobular necrosis as efficient as ICDH, because these enzymes are predominantly located in the periportal area whereas ICDH is mainly located in the centrilobular area (zone 3) and hence is a better marker for centrilobular necrosis caused by BB [22]. The suggestion that ICDH is a better biomarker of hepatic centrilobular necrosis is in agreement with our results, which indicated that BB caused a much higher elevation in serum activity of ICDH than in ALT activity compared to normal control animals. ALA could effectively alleviate liver injury caused by BB as indicated by the decrease in the activity levels of ICDH and ALT. The changes reported here were not due to changes in liver size as BB did not affect total protein in liver.

GSH is a unique cellular tripeptide capable of acting as a nucleophilic scavenger and a cofactor in GSH peroxidase mediated destruction of peroxides and hydroperoxides. It is essential to maintain the reduced status of the cell/tissue, and its severe depletion is reported to lead to oxidative tissue
injury [8]. Results of the present study revealed that a single oral toxic dose of BB (10 mmol/kg), administered to male-albino rats, elicited a significant decrease in hepatic GSH level by almost 50% from that of normal control animals. This result was in accordance with the findings of some investigators who found that administration of a high dose of BB caused severe hepatocellular injury and massive depletion of hepatic GSH within 24 hours [11,23-25]. This depletion was explained on the basis that BB is metabolized in the liver by CYP450 to the electrophilic intermediate 3,4-bromobenzene epoxide which binds with GSH yielding glutathionyl conjugate resulting in GSH depletion [3,4,11,23,26]. ALA administered alone, in a dose of 150 mg/kg, produced a significant improvement in GSH level, this result is consistent with many investigators who confirmed the ability of ALA to increase the level of GSH in liver of aged rats [27], xenobiotic-induced liver injury [28] and in the liver of diabetic rats [29]. The influence of ALA on the cellular status of GSH depends on the fact that a-lipoic acid/dihydrolipoic acid (ALA/DHLA) couple has a lower redox potential with respect to GSH/GSH couple, moreover ALA is reduced intracellularly to DHLA which reduces cystine to cysteine which is taken more rapidly by amino acid transporters and contributes to GSH synthesis [30-32].

The significant increase in hepatic level of MDA, a biomarker of lipid peroxidation, strongly suggested an oxidative stress-mediated hepatotoxicity following the depletion of hepatic GSH. This finding is further supported by other investigators who confirmed that BB-induced depletion of GSH was accompanied by a high level of lipid peroxides which, in turn, trigger secondary events such as mitochondrial dysfunction with associated energy imbalance and altered intracellular calcium level, which all are signs of cellular necrosis [20,25,33,34]. The ability of ALA to counteract BB-induced elevation in MDA is consistent with the findings of other investigators who reported that ALA can inhibit the process of lipid peroxidation due to its dithiol nature that renders ALA reactive against a number of ROS such as hydroxyl radical, superoxide anion, and alkoxyl radicals, moreover, ALA is able to regenerate other oxidized antioxidants such as recycling vitamin E peroxyl radical (tocopheroxyl radical), oxidized vitamin C (dehydrascorbate) and hence prevents the symptoms of both vitamin C and vitamin E deficiency in rats [30,32,35].

Results of the present study showed a remarkable increase in hepatic NO level, 24 hours after BB administration, which strongly suggested the presence of cellular injury, partially mediated by ROS. Nishida et al. further assured this finding, that cellular oxidative injury activates Kupffer cells, which in response release tumor-necrosis factor-α (TNF-α), this cytokine provokes a pro-inflammatory response by inducing leukocyte adhesion to the sinusoidal endothelium, affecting vascular permeability through upregulation of cellular adhesion molecules, and downregulation of constitutive nitric oxide synthase (cNOS), creating a pathogenic status called sinusoidal endothelial cell (SEC) dysfunction. Since cNOS is responsible for protecting the hepatic microvasculature, its inhibition is consistent with expression of the inducible form of NOS (iNOS) to maintain normal blood flow in the hepatic sinusoids by overproduction of NO, in attempt to alleviate SEC dysfunction caused by neutrophil infiltration [36]. Furthermore, according to Iwakiri and Groszmann ROS mediated injury decreases the availability of NO, as superoxide anion (O2·−) reacts with NO to yield peroxynitrite (ONOO−) a reactive nitrosative molecule which has a powerful oxidant activity that adds to the process of oxidative damage [37]. The reduction in hepatic NOx observed in this study is in accordance with those of Moini et al. and Suntres, who documented the direct effect of ALA in scavenging RNS such as peroxynitrite due to its dithiol nature, hence protect against peroxynitrite-induced inactivation of α1-antiproteinase and inhibiting nitration of L-tyrosine, and the indirect effect of ALA in inhibiting NO production from activated macrophages in different hepatic injury models [32,35]. The latter had been justified by Suntres and Suzuki et al. on the basis that involvement of reactive oxygen species (ROS) in signal transduction pathways leading to NF-kappa B (NF-kB) activation, a transcription factor required for the expression of iNOS, and as long as oxidative stress upregulates NF-kB, the antioxidants down-regulate NF-kB, resulting in reducing the iNOS expression and further decrease in NO production [35,38].

Histopathological examination, of rat liver (left lobe) intoxicated with a single dose of BB (10 mmol/kg) for 24 h, demonstrated marked morphological changes in the hepatocytes compared with the control group. It is evident that bromobenzene exerted an extensive hepatocellular centrilobular necrosis, cell swelling and vacuolization. The liver also showed distorted architecture and distorted central vein. Histopathological changes in the liver revealed also dilation of hepatic sinusoids, congestion in the lobules, some hemorrhage foci in hepatic parenchyma and infiltration of inflammatory cells around the necrotic hepatocytes. This observation
was similar to that of Brodie et al. who reported extensive centrilobular necrosis of hepatic parenchymal cells in rats killed 24 h after administration of BB, justified by the fact that BB is mainly activated by microsomal enzymes (CYP450) mainly located in the centrilobular area [28]. Furthermore, Zampaglione et al. supported the finding that a dose of BB similar to ours i.e. 10 mmol/kg is sufficient to cause intense centrilobular necrosis [11]. So the histopathological examination ascertained our previous biochemical analyses, that indicated an oxidative damage induced by BB, that can provoke a proinflammatory immune response as a clear evidence of cell death.

Since the hepatotoxic effect of BB is mediated through reactive metabolites that deplete hepatic GSH, elevate MDA, hepatic NO, and both serum ICDH and ALT activities; this mechanism strongly provides a clear evidence to oxidative stress-mediated toxicity.

In conclusion, ALA is an effective hepatoprotective agent against acute liver injury induced by bromobenzene which is a well known environmental pollutant. This protection is evidenced by its ability to improve liver function tests, preserve hepatic GSH, reduce hepatic lipid peroxidation and NO production, and its significant preservation of the liver architecture.

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References


