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Influence of alpha lipoic acid on antioxidant status in D-galactosamine-induced hepatic injury

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D-Galactosamine (GalN)-induced liver injury is associated with reactive oxygen species and oxidative stress. In the present study, we evaluated the effect of alpha lipoic acid (ALA) supplementation on acute GalN-induced oxidative liver injury. Hepatotoxicity induced by single intraperitoneal injection of GalN (500 mg/kg body wt) was evident from increase in lipid peroxidation and serum marker enzymes (asparate transaminase, alanine transaminase, alkaline phosphatase, and lactate dehydrogenase). The decreased activities of enzymic antioxidants (superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase) as well as glutathione levels were the salient features observed in GalN-induced hepatotoxicity. Pretreatment with ALA (50 mg/kg body weight for 7 days) significantly precluded these changes and prevents the hepatic injury. Hence, this study clearly exemplified that ALA might be a suitable candidate against GalN-induced cellular abnormalities. *Toxicology and Industrial Health* 2008; **24**: 635–642.

Key words: *antioxidants; alpha lipoic acid; D-galactosamine; hepatotoxicity; oxidative stress*

Introduction

The liver is an organ of paramount importance, which plays an essential role in the metabolism of foreign compounds entering the body. Human beings are exposed to these compounds through environmental exposure, consumption of contaminated food, or during exposure to chemical substances in the occupational environment. All these chemicals produce a variety of toxic manifestations in liver (Rajesh and Latha, 2004). GalN-induced experimental rat model is recognized to be much like viral hepatitis in humans from both morphological and functional points of view (Keppler, *et al*., 1968). GalN has great liver specificity because hepatocytes have high levels of galactokinase and galactose-1-uridyltransferase and hence does not affect other organs (Maley, *et al*.,1968). GalN causes hepatic injury with spotty hepatocyte necrosis and marked portal and parenchymal infiltration (Keppler and Decker, 1969). GalN also causes depletion of uridine diphosphate (UDP) by increasing the formation of UDP-sugar derivatives, which results in inhibition of RNA and protein synthesis leading to cell membrane deterioration (Decker, *et al*., 1973; El-Mofty, *et al*., 1975). Free radicals are predominantly participating in pathogenesis of liver injury induced by GalN (Seckin, *et al*., 1993). Free radicals react with lipids and causes peroxidative changes that result in enhanced lipid peroxidation (LPO) (Girotti, 1985). GalN induces LPO which is an auto-oxidative process

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initiated by a variety of free radicals to which polyunsaturated fatty acids present in cell membranes are susceptible (Bindoli, 1988). ALA (Thioctic acid) is a derivative of octanoic acid, which forms an intermolecular disulfide bond that can be reduced to form two highly reactive vicinal sulfhydryl groups. As is generally known, ALA act as a cofactor which is covalently attached to the lysine residue forming an essential lipoamide, which is involved in mitochondrial energy metabolism (Pteichert, *et al*., 2003). ALA and its reduced form dihydrolipoic acid, reduce oxidative stress by scavenging a number of free radicals in both membrane and aqueous domains, through chelating transition metals in biological systems and also prevent the membrane LPO and protein damage through the redox regeneration of other antioxidants such as vitamins C and E, and there by increasing intracellular glutathione (GSH) (Packer, 1998; Evans and Goldfine, 2000). ALA is used as a modulator in several liver disorders, together with alcohol induced liver damage, mushroom poisoning, metal intoxication, and chloroform poisoning (Busamante, *et al*., 1998). ALA aggravates the hepatic GSH levels due to the presence of thiol groups (Packer, *et al*., 1995). The free thiols may be precursors or intermediates in GSH metabolism or foreign substances applied in medical treatments. (Lillig and Holmgren, 2007; Haendeler, 2006). In this light, the present study was designed to scrutinize the hepatoprotective and antioxidant potential of ALA against GalN-induced oxidative stress in rat liver.

Materials and methods

Drugs and chemicals

D-Galactosamine and Lipoic acid were purchased from Sigma Aldrich Limited (St Louis, MO, USA). All other chemicals and solvents used were of the highest purity and analytical grade.

Animal model

This study was conducted using male albino Wistar rats weighing about 120–150 g. Animals were obtained from the Animal House, Vel's College of Pharmacy, The Tamilnadu Dr M.G.R. Medical University, and Chennai, India. Animals were fed with commercially available standard rat-pelleted feed from M/s Hindustan Lever Limited, Bangalore, India. The feed and water were provided *ad libitum*. The animals were deprived of food for 24 h before experimentation but allowed free access to tap water throughout. The rats were housed under conditions of controlled temperature (25 ± 2 °C) and were adapted to 12-h light/12-h dark cycles. Experimental animals were used after obtaining prior permission and handled according to the University and Institutional legislation as regulated by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India.

Experimental protocol

The experimental animals were randomized into four groups of six rats each as follows:

- Group I: control rats received normal saline (1 mL/kg, p.o.) for 7 days.
- Group II: toxic control rats received normal saline (1 mL/kg, p.o.) for 7 days.
- Group III: drug control groups received ALA (50 mg/kg, p.o.) for 7 days.
- Group IV: rats orally pretreated with ALA (50 mg/kg, p.o.) for 7 days.

Groups II and IV also received GalN (500 mg/kg, i.p.) on 7th day.

After 24 h of GalN administration, all the animals were anesthetized and decapitated. Liver tissues were immediately excised and rinsed in ice cold physiological saline. Blood and tissue (liver) were instantly processed and used for various biochemical evaluations.

Preparation of serum and tissue homogenates

Blood was collected and serum was separated by centrifugation at 3000 rpm for 10 min for various biochemical estimations. Tissues (liver) were removed and cleared off blood and immediately transferred to ice-cold container containing 0.9 NaCl for various estimations. A known amount of tissue was weighed and homogenized in Tris-Hcl (0.1 M pH 7.4), and the homogenate was

used for the estimation of different biochemical parameters.

Biochemical parameters

Enzyme indices of cellular integrity

To evaluate the membrane damage, the activity of liver marker enzymes in serum was determined. Lactate dehydrogenase (LDH) activity was assayed by the method of King (1965a). The method is based on the ability of LDH to form pyruvate in the presence of coenzyme nicotinamide adenine dinucleotide NAD^+. The pyruvate formed is made to react with 2, 4-dinitrophenol in hydrochloric acid. The hydrazone formed turns in to an orange colored complex in alkaline medium, which is measured at 540 nm. Alanine transaminase (ALT) and aspartate transaminase (AST) were estimated by the method of King (1965b). Alkaline phosphatase (ALP) was estimated by the method of King (1965c). Their activities were expressed in terms of μmoles of pyruvate liberated m^{-1} mg^{-1} of protein. Protein content was estimated by the method of Lowry, *et al*. (1951).

Measurement of LPO

Malondialdehyde (MDA) levels were determined by the method of Buege and Aust (1978). The absorbance was measured photometrically at 532 nm and the concentrations were expressed as nmol/g tissue.

Estimation of antioxidant enzymes

Superoxide dismutase (SOD) activity was analyzed by the method of Rai, *et al*. (2006). Color intensity of the chromogenin was measured spectrophotometrically at 560 nm, and concentration of SOD was expressed as U/mg of protein.

Catalase (CAT) activity was measured by the method of Aebi (1974). The rate of decomposition of H_2O_2 was measured spectrophotometrically at 240 nm. Activity of catalase was expressed as U/mg of protein.

Glutathione peroxidase (GPx) activity was determined by the method of Lawrence and Burk (1976). This method is based on measuring the oxidation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) using hydrogen peroxide as the substrate. Absorbance was measured at 340 nm. The results were expressed as U/mg of protein.

Glutathione reductase (GR) activity was assayed by the method of Carlberg and Mannervik (1975). The enzyme activity was quantified by measuring the disappearance of NADPH at 340 nm at 30 s intervals for 3 min. The activity was expressed as nanomoles NADPH oxidized/min/mg protein.

Estimation of GSH

Reduced GSH activity was determined according to the method of Ellman, 1959. The method is based on the reduction of Ellman's reagent [5, 5'-dithio-bs-(2nitrobenzoic acid)] by SH groups to form 1 mole of 2-nitro-5-mercaptobenzoic acid per mole of SH. The nitro-mercaptobenzoic acid has an intense yellow color and can be determined spectrophotometrically at 412 nm against a reagent blank; the results were expressed as μmol/g tissue.

Statistical analysis

The results were expressed as mean ± standard deviation (SD) for six animals in each group. Differences between groups were assessed by one-way analysis of variance (ANOVA) using the SPSS 13.0 software package for Windows. Post-hoc testing was performed for inter-group comparisons using the least significance difference test; significance at $P < 0.05$ was considered to be statistically significant.

Results

In the present study, intraperitoneal administration of a single dose of GalN (500 mg/kg body weight) induced severe biochemical changes as well as oxidative injury in liver tissue. There was a significant ($P < 0.05$) rise in the levels of serum marker enzymes asparate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), and LDH in the serum of GalN administered (Group II) rats as compared with that of Group I control rats (Table 1). The administration of ALA to Group IV animals restored the levels of these enzymes to near normalcy ($P < 0.05$) as compared with those Group II GalN-injected rats. In ALA alone administered rats (Group III) versus controls, no significant changes were observed.

Intraperitoneal injection of GalN induced a significant ($P < 0.05$) increase in the level of LPO (Figure 1), which was paralleled by significant

Table 1. Effect of alpha lipoic acid and D-galactosamine on the activities of liver marker enzymes in serum

Groups	AST (IU L^{-1})	ALT (IU L^{-1})	ALP (IU L^{-1})	LDH (IU L^{-1})
Group I (Control)	78.13 ± 5.00	49.22 ± 5.22	76.19 ± 5.07	211.40 ± 5.21
Group II (GalN)	616.36 ± 45.16[a],*	553.96 ± 56.58[a],*	160.96 ± 12.44[a],*	361.04 ± 30.19[a],*
Group III (LA)	77.81 ± 5.04[NS]	49.04 ± 6.02[NS]	76.07 ± 5.50[NS]	210.52 ± 5.09[NS]
Group IV (GalN + LA)	81.52 ± 5.05[b],*	54.96± 6.15[b],*	81.59±5.96[b],*	220.42 ± 6.33[b],*

AST, aspartate transaminase; ALT, alanine transaminase; ALP, alkaline phosphatase; LDH, lactate dehydrogenase; NS, non-significant.
Results are expressed as mean ± SD for six rats. Comparisons are made between the following:
[a]Group I and Group II.
[b]Group II and Group IV.
[NS]Group I and Group III.
*Statistically significant ($P < 0.05$).

($P < 0.05$) reduction in the level of GSH (Figure 2) in the liver tissue of Group II animals as compared with normal controls. GSH plays an important role in the regulation of variety of cell functions and in cell protection from oxidative injury. Depletion of GSH results in enhanced LPO and due to this, there is an increase in GSH consumption (Seckin, *et al.*, 1993), as observed in the present study. In this study, the pretreatment with ALA (Group IV) significantly ($P < 0.05$) counteracted the GalN-induced LPO and restored the level of GSH to near normal level in Group IV rats as compared with that of Group II animals.

Activities of endogenous antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), GPx, and GR were significantly ($P < 0.05$) lower in the liver tissue of Group II GalN-injected rats as compared with that of Group I normal control rats (Table 2). The observed reduction in the activities of GPx, GR in GalN-induced oxidative condition might be due to decreased availability GSH. In the present study, the treatment of Group IV rats with ALA, significantly ($P < 0.05$) reversed all these GalN-induced alterations in the activities of antioxidant enzymes (SOD, CAT, GPx, and GR) to a near normal status. The normal rats receiving ALA alone (Group III) did not show any significant change when compared with control rats.

Discussion

GalN-induced oxidative stress in the rat liver produces the imbalance between biochemical processes leading to formation of reactive oxygen species and the cellular antioxidant cascade, causes molecular damage that can lead to a critical failure of biological functions and ultimately cell death (Sun, *et al.*, 2003; Sies, 1997; Sies, 1991). At the course of liver injury, the transport function of hepatocytes is distressed which can result in the leakage of plasma

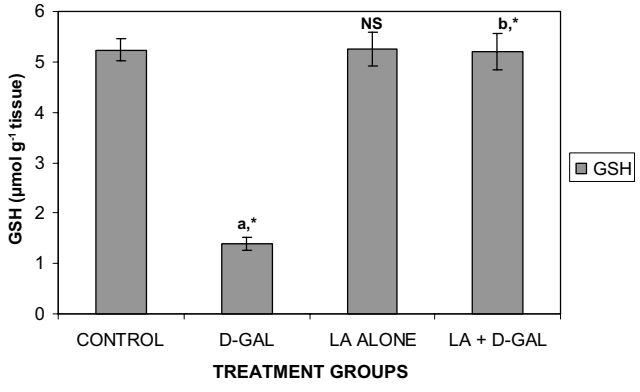

Figure 1 Levels of Malondialdehyde in the liver of the experimental animals. Results are given as mean ± SD for six rats. Comparisons are made between the following: a, Group I and Group II; b, Group II and Group IV; NS, Group I and Group III. *Statistically significant ($P < 0.05$); NS, non-significant.

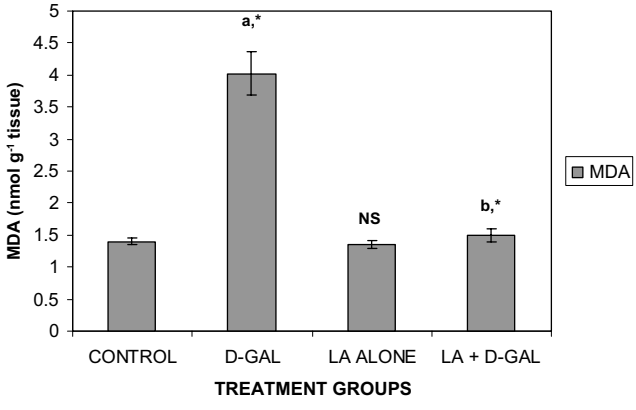

Figure 2 Levels of glutathione in the liver of the experimental animals. Results are given as mean ± SD for six rats. Comparisons are made between the following: a, Group I and Group II; b, Group II and Group IV. NS, Group I and Group III. *Statistically significant ($P < 0.05$); NS, non-significant.

Table 2. Effect of alpha lipoic acid and D-galactosamine on the activities of liver enzymic antioxidants

Groups	SOD	CAT	GPx	GR
Group I (Control)	8.54 ± 0.28	75.65 ± 4.18	17.36 ± 0.59	27.40 ± 1.19
Group II (GalN)	4.73 ± 0.40[a],*	46.51 ± 5.9[a],*	9.53 ± 0.66[a],*	18.95 ± 1.65[a],*
Group III (LA)	8.57 ± 0.45[NS]	76.00 ± 4.31[NS]	18.63 ± 0.86[NS]	28.14 ± 1.26[NS]
Group IV (GalN + LA)	7.11 ± 0.41[b],*	71.20 ± 4.60[b],*	15.80 ± 0.78[b],*	25.50 ± 1.57[b],*

Results are expressed as mean ± SD for six rats. Units: SOD, U/mg protein; CAT, U/mg protein, μmoles H_2O_2 consumed min^{-1} mg^{-1} protein; GPx, U/mg protein, μmoles GSH oxidized min^{-1} mg^{-1} protein; GR, nmoles NADPH oxidized min^{-1} mg^{-1} protein.
SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; GR, glutathione reductase; NS, non-significant.
Comparisons are made between the following:
[a]Group I and Group II.
[b]Group II and Group IV.
[NS]Group I and Group III.
*Statistically significant ($P < 0.05$).

membrane thereby causing an increase in serum marker enzymes such as AST, ALT, ALP, and LDH (Drotman and Lawhorn, 1978; Mukherjee, 2003). GalN administration in rats disrupts the membrane permeability of the plasma membrane causing leakage of the enzymes from the cell, which leads to elevation in levels of marker enzymes (Han, *et al*., 2006). In our study, the rise in AST, ALT, ALP, and LDH levels induced by GalN administration was significantly reduced by ALA pretreatment suggesting that its hepatoprotective activity might be due its effect against cellular leakage and loss of functional integrity of the cell membrane in hepatocytes.

LPO distorted the levels of some free radical scavengers, which indirectly reveals the oxidative stress in liver (El-Khatib, *et al*., 2001). LPO involves the direct reaction of oxygen and lipid to form radical intermediates and to produce semistable peroxides, which in turn damage the enzymes, nucleic acids, membranes, and proteins (Sivalokanathan, *et al*., 2005). LPO is thought to be an important mechanism of liver injury, and MDA is one of its end products, and measurement of MDA can be used to assess LPO levels (Kurata, *et al*., 1993). It has been well documented that GalN administration produces the MDA-protein adducts which is reflective of LPO (Wong, *et al*., 2007). Previous reports confirmed that ALA scavenges hydroxyl radicals, H_2O_2, singlet oxygen, superoxide radicals, and peroxyl radicals thereby preventing the free radicals-mediated LPO (Packer, *et al*., 1995). In our study, ALA pretreatment diminished the MDA levels and protected the membrane damage caused by GalN. Earlier reports have shown that LPO induced by GalN is concomitant with GSH depletion (Seckin, *et al*., 1993).

GSH is the intracellular non-protein sulfhydryl compound that act as both nucleophile and an effective reductant by interacting with various electrophilic and oxidized compounds, and its depletion results in increased defenselessness of cell to oxidative stress (Yuan, *et al*., 1991; Atmaca, 2004). The consequence of decrease in cellular GSH levels is an increase of free radical intermediates leading to oxidative stress and potent cellular damage. The reduced form of GSH becomes readily oxidized to oxidized glutathione (GSSG) with the GPx on interacting with free radicals (Bose and Agarwal, 2007; Butzer, *et al*., 1999). In our present study, pretreatment of the rats with ALA significantly increased the concentration of GSH. It has been known that the ALA plays an important role in improving the GSH status through the mechanism of increasing the cystine availability because cystine is the rate-limiting factor in GSH biosynthesis. ALA induces the cystine uptake and thereby increases GSH synthesis. In our present investigation, pretreatment with ALA augments the hepatic GSH level which is in accordance with the previous studies (Han, *et al*., 1997).

A major defense mechanism in the human system involves the antioxidant enzymes including SOD, CAT, and GPx which convert active oxygen molecules into non-toxic compounds, which includes free radical scavengers and chain reaction terminators (Narasimhanaidu, *et al*., 2005; Proctor and McGinness, 1986). CAT is the key component of the antioxidant defense system; inhibition of this protective mechanism leads to free radicals-induced cellular damage. The observed decrease in the

activity of CAT in oxidative stress is consistent with earlier studies (Al-Qirim, *et al*., 2002, Yargicoglu, *et al*., 2003; Zaidi and Banu, 2004). ALA is able to increase glucose uptake *in vitro* (Haugaard and Haugaard, 1970). Enhanced glucose uptake by cells serve as a fuel for both the pentose phosphate shunt and oxidative phosphorylation, thus bringing up the cellular levels of nicotinamide adenine dinucleotide phosphate (NADPH) and nicotinamide adenine dinucleotide (NADH) and thereby enhances the activity of CAT in stress condition (Arivazhagan, *et al*., 2000). SOD is an enzyme extensively used as a biochemical indicator of pathological states associated with oxidative stress. At the first step of the defense system against oxidative stress, it catalyzes dismutation of the super oxide anion into hydrogen peroxide. H_2O_2 is one of the most active oxygen species; at the second step of the antioxidant defense system, GPx and CAT independently degrade H_2O_2 to water. Several lines of evidence indicated that SOD activity should therefore produce an excess H_2O_2 that must be efficiently neutralized by either GPx or CAT (Chance, *et al*., 1979; Hebbel, 1986). Our data indicated that GalN-induced oxidative stress causes a significant inhibition of SOD and CAT. The possible reason for this finding could be the decrease of this enzyme caused by enhanced LPO in the stress condition (Chaudiere and Ferrari-iliou, 1999).However, diminished SOD and CAT activity in liver tissues of GalN-treated rats was brought back to a normal status with pretreatment of ALA. The GPx which is coupled with GSH, together with catalase functions as a major cellular reducer of hydrogen peroxide (Arteel and Sies, 2001). The stress-induced decrease in the activity of GPx documented in our study is corroborated by earlier investigations (Al-Qirim, *et al*., 2002; Yargicoglu, *et al*., 2003). GPx activity in the liver of stressed rats was also maintained at a normal level with administration of ALA. GR is responsible for the transformation of the GSSG into its reduced form, GSH; the diminution in the concentration of GSH leads to fast accumulation of lipid peroxides in cells (Quinlan, *et al*., 1988). A decrease in the activity of these antioxidants can lead to an excess availability of superoxide anion and hydrogen peroxide in biological systems, which in turn generate hydroxyl radicals, resulting in initiation and propagation of LPO (Murugan and Pari, 2007). ALA indirectly influences the activities of SOD, and apart from this, it increases intercellular GSH content that might also activate the GSH-depended enzymes, GPx thereby preventing the accumulation of H_2O_2, and also increases the activity of CAT (Balachandar, *et al*., 2003). The previous report shows that the decline in levels of antioxidant enzymes such as SOD, CAT, GR, and GPx in GalN induced oxidative stress (Korda, 1996; Hwa-Kyung, *et al*., 2000). It has been showed that treating with ALA increases the enzyme levels by directly reacting with various reactive oxygen species and also nullify the oxidation process in lipids and intercellular components (Kokilavani, *et al*., 2005). In our study, pretreatment with ALA prevented the leakage of liver marker enzymes and increased the levels of antioxidant enzymes, and by this way it might have protected the liver against oxidative stress induced by GalN.

In conclusion, the results of this study show that the administration of GalN induced extensive oxidative stress in liver, which is associated with membrane damage and hepatocyte dysfunction. ALA supplementation may reduce oxidative stress in liver of GalN-treated rats by decreasing the LPO and ROS propagation by scavenging free radicals, or by improving the actions of antioxidant enzymes. The function of ALA in the regulation of GSH and other intracellular antioxidants may be significant in the restitution of redox status. Further comprehensive studies to elucidate the mechanism action of ALA against GalN are in progress.

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