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Antioxidant and Hypo-Ammonemic Activities of Alpha-Lactalbumin and Vitamin C in Thioacetamide-Induced Liver and Brain Damage in Rats

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ABSTRACT

Hepatic encephalopathy (HE) is a syndrome resulting from acute or chronic liver failure. The main hypothesis suggests a state of hyperammonemia which is responsible for both direct and indirect alterations in cerebral metabolism with increased production of reactive oxygen and nitrogen species. The effect of milk-derived alpha-lactalbumin (α-LAC) and vitamin C (vit. C) was evaluated in thioacetamide (TAA)-induced HEmodel in the current study. Animals were treated with TAA (100 mg/kg, i.p.) or saline thrice weekly for six weeks to induce HE then treatment groups received orally α-LAC (100 or150 mg/kg) and /or vit. C (500 mg/kg)daily for two weeks. Twenty-four hours after last treatment sera, liver and brain samples were collected to assess serum ammonia level, activities of alanine transaminase (ALT), and aspartate transaminase (AST), brain and liver oxidative stress parameters as well as histopathological investigations.TAA rats experienced increases in serum activities of ALT and AST as well as serum levels of ammonia. Furthermore, TAA induced hepatic and brain oxidative damage as indicated by increase in lipid peroxidation (LP), decrease in reduced glutathione (GSH) and decrease in superoxide dismutase (SOD) activity as well as increased nitric oxide (NO) levels. TAA caused distortion of hepatic and brain architecture as shown by histopathological examination. Treatment with α-LAC either alone or combined with vit. C resulted in improved liver functions by decline in serum AST and ALT activities and reduction in serum ammonia level. Alpha-LAC and vit. C reduced LP and NO levels while increased GSH concentration and SOD activity in hepatic and brain tissues. Finally, α-LAC-vit. C combination improved the hepatic and brain histological picture. Alpha-LAC-vit. C combination may be a promising pharmacological tool in providing a natural source of branched-chain amino acids and powerful antioxidants to combat hepatic encephalopathy-associated hyperammonemia and its consequential oxidative damage in liver and brain.

INTRODUCTION

Hepatic encephalopathy (HE) is a syndrome of reversible impairment of brain function in patients with acute or chronic liver failure. This leads to a spectrum of neurological impairments ranging from subclinical brain dysfunction to coma. The mechanisms underlying brain dysfunction are still largely unclear (McPhail *et al.*, 2010; Seyan *et al.*, 2010).But ammonia and the downstream consequences of ammonia uptake by astrocytes remain fundamental to the process. Ammonia not only leads to astrocyte swelling, but also alters neurotransmission, mitochondrial function, and induces oxidative and nitrosative stress (Norenberg *et al.*, 2004a; Norenberg *et al.*, 2007; Seyan *et al.*, 2010).

Moreover, as compared to the acute and end-stage HE, low mild-grade HE is prevalent in the patients with chronic liver failure (CLF)(Albrecht, 2007).

Viral hepatitis, alcoholism, drug intoxication, and long-term drug abuses are the main inducers of liver diseases in any population, and all of them cause CLF. About 60% to 80% of liver cirrhotic patients have been reported to show minimal overt HE symptoms with serious consequences in their daily life (Poordad, 2007; Bajaj, 2008). Therefore, it is important to elucidate neuro- and hepato-biochemical alterations associated with the pathogenesis of mild-grade HE (MHE) in a suitable CLF model.

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Among the various toxicity models, thioacetamide (TAA) is frequently used to produce liver injury in animals and further to evaluate the therapeutic potential of drugs.

TAA administration has been shown to cause fibrosis and cirrhosis in liver eventually leading to hepatocarcinoma (Tsukamoto *et al.,* 1990). One of the mechanisms of damage is the generation of ROS and instigation of oxidative stress (Wang *et al.,* 2000). Moreover, TAA causes a wide spectrum of pathophysiological changes which parallel to those observed in patients with hepatic encephalopathy (Zimmermann *et al.,* 1989). Malnutrition tends to be more common in patients with advanced liver disease and HE.

Nutritional deficits such as decreased lean body mass (muscle is important in ammonia uptake) and hypoalbuminemia (which increases free tryptophan levels) could promote HE (Sourkes, 1978). Hyperammonemia may lead to increased uptake of tryptophan by the brain which may lead to increased synthesis and release of serotonin and anorexia. This symptom may render the patient prone to chronic catabolism and malnutrition, and in turn to increased ammonia load, resulting in a vicious cycle (Bachmann *et al.,* 2004; Cohn and Roth, 2004).

Inadequate dietary protein intake or low levels of branched-chain amino acids (BCAAs) may have a deleterious effect on HE (Cordoba *et al.,* 2004). Since hyperammonemia results in increased utilization of (BCAAs), which are largely metabolized by the muscle, it would be anticipated that providing BCAAs could facilitate ammonia detoxification by supporting muscle glutamine synthesis (Tomiya *et al.,* 2002). BCAAs (leucine, isoleucine, and valine) cannot be synthesized *de novo* but must be obtained from dietary sources and have a unique role in amino acid metabolism, regulating the intra- and interorgan exchange of nitrogen and amino acids by different tissues (Felig and Wahren, 1974).

Being rich in BCAAs (22.3%) (Morifuji *et al.,* 2009), whey proteins can be introduced in HE to compensate excessive loss of BCAAs. Being an evolving concept in the pathogenesis of HE, oxidative stress can be attenuated by free radical scavengers. Ascorbic acid or "vitamin C" is a monosaccharide antioxidant found in both animals and plants. As it cannot be synthesized in humans and must be obtained from the diet, it is a vitamin (Smirnoff, 2001).

In cells, it is maintained in its reduced form by reaction with glutathione, which can be catalyzed by protein disulfide isomerase and glutaredoxins (Meister, 1994). Ascorbic acid is a reducing agent and can reduce and thereby neutralize ROS such as hydrogen peroxide (Padayatty *et al.,* 2003; Lobo *et al.,* 2010). Vitamin C has been reported by researchers to have hepatoprotective effect due to its antioxidant property (Netke *et al.,* 1997; El-Gendy *et al.,* 2010; Abhilash *et al.,* 2012).

Thus, the current study is designed to investigate the hypoammonemic and antioxidant ability of milk-derived alpha-lactalbumin (α-LAC) and vitamin C (vit. C) in modulating TAA-induced hepatic encephalopathy in rats.

MATERIAL AND METHODS

Animals

Female Sprague-Dawley rats, weighing 180-200 g, were used throughout the experiment. Animals were housed under standard environmental conditions (23 ± 1ºC, 55 ± 5 % humidity and a 12-h light: 12-h dark cycle) and maintained with free access to water and a standard laboratory diet *ad libitum*. Animal care and the experimental protocols were approved by the National Research Centre, Animal Care and Use Committee and are in accordance with the guidelines of the International Association for the Study of Pain Committee for Research and Ethical Issues (Zimmermann, 1983, 1986).

Treatment protocol

Thioacetamide (TAA, Sigma Chemical Co., St. Louis, MO, USA) was administered intraperitoneally (i.p.) at 100 mg/ kg (in saline solution) thrice weekly for six weeks to induce HE as reported earlier(Singh and Trigun, 2010; Singh *et al.*, 2014) with modification, whereas alpha-lactalbumin (Lacprodan® alpha -10 kindly supplied from Arla food amba, Denmark) was administered orally at 100 and 150 mg/kg (in distilled water prior to use) for two weeks after induction of HE by TAA, dose selection was according to previous studies of our team (Nada, 2009; Mansour *et al.,* 2013; Eliwa *et al.,* 2014; Mansour *et al.,* 2015; Nada *et al.,* 2015). Vitamin C (L (+)-ascorbic acid, Merck Co., Darmstadt, Germany) was administered orally at 500 mg/kg for two weeks after induction of HEby TAA.

Experimental procedures

Fifty-six female Sprague Dawley rats, weighing 180-200 g, were divided into 7 groups (8 rat /group). Normal control group received vehicle throughout the experiment. Thioacetamide (TAA) group received TAA (100 mg/kg, i.p) 3 times/week for 6 weeks to induce hepatic encephalopathy. To minimize weight loss, hypoglycemia, and renal failure in the experimental rats, 5% dextrose containing 0.45% NaCl and 20 meq/L KCl will be administered to all the rats through the drinking water as supportive therapy (Sathyasaikumar *et al.,* 2007). Alpha lactalbumin (α-LAC) groups received TAA as previously described followed by oral α-lactalbumin at 100 or 150 mg/kg daily for two weeks. Vitamin C (vit. C) group received TAA as previously described followed by oral vit. C at 500 mg/kg daily for two weeks. Alpha-LAC and vitamin C groups received TAA as (previously described) followed by α-LAC, at 100 or 150 mg/kg, in combination with vitamin C (500mg/kg), respectively, orally for two weeks.

Methods

Collection of blood and tissue samples

Twenty-four hours after last treatment, blood samples were withdrawn from the retro-orbital vein of each animal, under light anesthesia by diethyl ether(Cocchetto and Bjornsson, 1983).

Blood was allowed to coagulate and then centrifuged at 3000 rpm for 15 min. The obtained serum was used to determine ammonia level and the activities of ALT and AST enzymes. Immediately after blood sampling, animals were sacrificed by cervical dislocation; liver and brain tissues were rapidly removed, washed in ice-cold saline, plotted dry and weighed. The left lobe of each liver and part of brain tissues were dissected and placed in 10% formalin in phosphate buffered saline (PBS) to be used for histopathological examination. A weighed part of each liver and brain was homogenized with ice-cooled PBS to prepare 20% w/v homogenate.

The homogenate was then centrifuged at 4000 rpm for 5 min. at 4 °C using a cooling centrifuge to remove cell debris. The aliquots were kept at -80°C till the day of analysis.

Measurement of reduced glutathione (GSH)

The reduced glutathione (GSH) content was measured in homogenates of liver and brain tissues (Ellman, 1959). Although this method measures all acid-soluble thiols, GSH represents more than 90% of the reactive thiol groups. This assay is based on the reduction of 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) by SH groups of glutathione to form 1 mol of 2-nitro-S-mercaptobenzoic acid per mole of glutathione. The product was measured spectrophotometrically at 412 nm, with the extinction coefficient 13.7 mM/cm.

Measurement of lipid peroxidation (LP)

The formation of malondialdehyde (MDA) as a lipid-peroxidation product in tissue homogenates of liver and brain was determined spectrophotometrically according to the method described previously (Ohkawa *et al.,* 1979); the determination of MDA depends on the reaction between one molecule of malondialdehyde (MDA) with two molecules of 2-thiobarbituric acid under acidic conditions (pH 3.5). The pink-coloured product can be detected spectrophotometrically at 532 nm, with an extinction coefficient of 156 mM/cm.

Measurement of Nitric Oxide (NO)

Nitric oxide was determined in rat liver and brain homogenates using a test reagent kit (Montgomery and Dymock, 1961).

Measurement of superoxide dismutase (SOD) activity

The superoxide dismutase (SOD) activity in rat liver and brainhomogenates was measured as the degree of inhibition of auto-oxidation of pyrogallol at an alkaline pH (Marklund and Marklund, 1974)**.**

Measurement of serum Ammonia level

Determination of serum ammonia level was assessed colorimetrically using a test reagent kit according to the method described previously (Konitzer and Voigt, 1963).

Measurement of serum liver function enzyme activities

Determination of serum ALT and AST activities were assessed colorimetrically using test reagent kits (Reitman and Frankel, 1957).

Histopathological examination

Specimen from liver and brain of all examined groups were washed, dehydrated in ascending grades of ethyl alcohol, cleared in xylene and embedded in paraffin wax. Sections of 5-6μm in thickness were cut out, deparaffinized and stained with Hematoxylin and Eosin (H&E) for examination under light microscope (Bancroft *et al.,* 1994).

Statistical Analysis

Results are expressed as mean of eight experiments ± SE of the mean. Data were analyzed by one-way analysis of variance (ANOVA). When the variation among groups was proved significant, Tukey's multiple comparison tests was performed to compare significance between groups. The data were analyzed with GraphPad Prism v. 5.0 (GraphPad Software, Inc., CA, USA). Difference was considered significant when p value is ≤ 0.05.

RESULTS AND DISCUSSION

Results of the current study revealed significant decrease in GSH of liver and brain tissues in animals treated with TAA, (100 mg/kg, i.p) thrice weekly for six weeks, compared to normal control. Treatment with α-LACalone (100 or 150 mg/kg, p.o), significantly elevated GSH levels in liver and brain of TAA-treated rats. Animals treated with vit. C (500mg/kg), alone or with α-LAC, significantly increased GSH in liver and brain of TAA-treated rats. However, α-LAC alone exerted significant increase in hepatic GSH than vit. C alone or with α-LAC. On the other hand, vit. C alone elevated GSH significantly compared to α-LAC, alone or with vit. C, in brain tissue of TAA-treated animals **(Table 1)**.Lipid peroxidation (LP) in liver and brain of TAA-treated animals was significantly elevated compared to normal control. Alpha-LACor vit. C alone showed significant decrement in hepatic and brain LP compared to TAA-treated animals. While vit. C with α-LAC (100 mg/kg) decreased significantly liver and brain LP than in animals treated with vit. C alone **(Table 1)**.

It is clear that TAA administration resulted in significant elevation of NO content in liver and brain tissues compared to normal control. Treatment with α-LAC or vit. C, alone or in combination, decreased NO content in liver and brain significantly compared to TAA-treated animals. It is note-worthy that vit. C with α-LAC exerted significant decrement in hepatic NO compared to vit. C alone; this effect was not observed on brain NO content, in which vit. C effect was insignificant compared to vit. C with α-LAC. On the other hand, TAA showed significant inhibition of SOD activity in liver and brain compared to normal control. Animals treated with α-LAC or vit. C, alone or in combination, reported significant increment in SOD activity of both liver and brain compared to TAA-treated animals.

Table 1: Effects of α-LAC and vit. C on glutathione and lipid peroxidation in liver and brain of rats treated with thioacetamide (TAA).

Treatment	Glutathione (mg/g)		Lipid peroxidation (nmol/g)	
	Liver	Brain	Liver	Brain
Control	54.5 ± 1.1 [a]	24.6 ±1.2 [ac]	147 ± 3.2 [ac]	91.6 ± 1.7 [ac]
TAA	22.9 ±2.4 [b]	16.1 ± 0.74 [b]	198 ±.2.6 [b]	117.2 ± 2.9 [b]
TAA+ α-LAC 100	58.3 ±1. 9 [a]	23.5 ±0.92 [a]	155.1 ±.2.8 [a]	88.3 ±2.09 [a]
TAA+ α-LAC 150	66.4 ±1.8 [c]	22.8 ±0.6 [a]	149.5 ±.2.7[ac]	100.4 ±2.1 [c]
TAA+ vit. C	41.6 ±1.6 [d]	28.3 ±0.9 [c]	157.1 ±2.2 [a]	106.5 ±1.6 [c]
TAA+α-LAC100+ vit. C	35.3 ±2.5 [de]	23.3 ±1.47 [a]	142.6 ±3.1 [c]	87.9 ±1.3 [a]
TAA+α-LAC150+ vit. C	33.1 ±1.3 [e]	24.8 ±0.85 [a]	153.5 ±2.3 [ac]	104.3 ± 1.8 [c]

Within each column, means superscript with different letters are significantly different (P≤0.05)

Table 2: Effects of α-LAC and vit. C on nitric oxide content and superoxide dismutase (SOD) activity in liver and brain of rats treated with thioacetamide (TAA)

Treatment	Nitric oxide (nmol/g)		SOD (U/mg tissue)	
	Liver	Brain	Liver	Brain
Control	76.1 ± 1.2 [a]	52.7±1.7 [a]	1.85± 0.005 [a]	1.74± 0.013 [a]
TAA	98.4 ±2.1 [b]	60.5±1.8 [b]	1.57 ±0.031 [b]	1.47±0.011 [b]
TAA+ α-LAC 100	89.5 ±1.7 [c]	40.1±0.9 [c]	1.83±0.008 [ac]	1.57±0.016 [c]
TAA+ α-LAC 150	82.4 ±1.2 [a]	47.9±1.7[a]	1.75 ±0.021[cd]	1.62±0.021 [c]
TAA+ vit. C	91.2 ±1.7 [c]	53.5±2.1[a]	1.73±0.033 [d]	1.65±0.005 [ac]
TAA+α-LAC100+ Vit. C	82.5 ±1.6 [a]	54.7±0.6[a]	1.85 ±0.003 [a]	1.61± 0.01 [c]
TAA+α-LAC150+ Vit. C	81.1 ±1.4 [a]	51.4±1.8[a]	1.78 ±0.029[ad]	1.63±0.047 [c]

Within each column, means superscript with different letters are significantly different (P≤0.05)

Table 3: Effects of α-LAC and vit. C on serum ammonia level and serum aminotransferases of rats treated with thioacetamide (TAA).

Treatment	Ammonia (µmol/L)	AST (IU/L)	ALT (IU/L)
Control	93.4± 0.38 [ac]	251.2 ± 1.83 [a]	98.85±0.39 [a]
TAA	118 ± 2.9 [b]	303.2 ±3.3 [b]	118±1.2 [b]
TAA+ α-LAC 100	95.05 ± 1.7 [ac]	264.8±0.89 [cd]	101.6±0.43 [ac]
TAA+ α-LAC 150	98.7± 1.04 [a]	261.6±1.3 [c]	103.7±1.07 [c]
TAA+ vit. C	97.7± 1.5 [a]	271.5±1.5 [d]	102.4±0.69 [ac]
TAA+α-LAC100+ vit. C	90.3± 0.93 [c]	267.6±1.5 [cd]	101.8±0.71 [ac]
TAA+α-LAC150+ vit. C	99.2 ± 1.7 [a]	282.3±0.76 [e]	104.4±0.78 [c]

Within each column, means superscript with different letters are significantly different (P≤0.05).

Vit. C with α-LAC (100 mg/kg) significantly increased hepatic SOD activity compared to vit. C alone. While in brain tissues, SOD activity of combined treatments showed insignificant increase compared to individual treatments (**Table 2)**.Serum ammonia level was assessed in TAA-treated animals which showed remarkable significant increase in serum ammonia compared to normal control (**Table 3)**. Treatment with α-LAC or vit. C, alone or in combination, reduced serum ammonia significantly compared to TAA-treated animals. Vit. C with α-LAC (100 mg/kg) reported significant reduction in ammonia compared to α-LAC (150 mg/kg), vit. C alone or combined with α-LAC (150 mg/kg). Regarding aminotransferases activity, TAA significantly elevated serum AST and ALT activities compared to normal control. Animals treated with α-LAC or vit. C, alone or in combination, significantly reduced both AST and ALT activities compared to TAA-treated group **(Table 3)**.

Regarding histopathological examination of rat liver tissues, there were apparently normal hepatic parenchyma in normal control group **(Fig.1A)**. While in liver treated with TAA (100 mg/kg,i.p), thrice weekly for six weeks, hepatocytes were infiltrated and replaced by mononuclear inflammatory cells **(Fig.1B)**. This picture was improved to healthy hepatic tissue when liver treated with α-LAC (100 mg/kg) daily for two weeks after TAA**(Fig.1C)**.In α-LAC (150 mg/kg)-treated group, daily for two weeks after TAA, liver showed dilated central vein with congested hepatic blood sinusoids **(Fig.1D).** By administration of vit. C (500mg/kg), daily for two weeks after TAA, focal areas of leucocytic cell infiltration were recorded **(Fig.1E)**. While combined treatment with vit. C (500mg/kg) and α-LAC (100 mg/kg)normal histological findings were noticed **(Fig.1F)**, whereas slightly congested central vein was reported when vit. C (500mg/kg)was combined with α-LAC (150 mg/kg)as shown in **Fig.1G**.

Concerning histopathological examination of rat brain tissues, normal control group showed apparently normal neuronal histology **(Fig.2A)**. Perineuronal edema was observed in brain of rats treated with TAA (100 mg/kg, i.p) **(Fig.2B).** Normal neuronal cells were observed in the treatment group with α-LAC (100 mg/kg); while α-LAC (150 mg/kg) administration showed degeneration in neuronal cells **(Fig.2D).** Moreover, brain of animals treated with vit. C (500 mg/kg) alone showed focal areas of leucocytic cell infiltration **(Fig.2E).** The combined treatment with vit. C (500 mg/kg) and α-LAC (100 mg/kg) normalized the neuronal cells structure **(Fig.2F).** However, brain of rats treated with vit. C (500 mg/kg) andα-LAC (150 mg/kg) exhibited focal perineuronal edema as shown in **Fig.2G.**

Our previous reports about the antioxidant and hepatoprotective activities of milk-derived alpha-lactalbumin (Nada, 2009; Mansour *et al.,* 2013; Eliwa *et al.,* 2014; Mansour *et al.,* 2015; Nada *et al.,* 2015), inspired us to investigate the

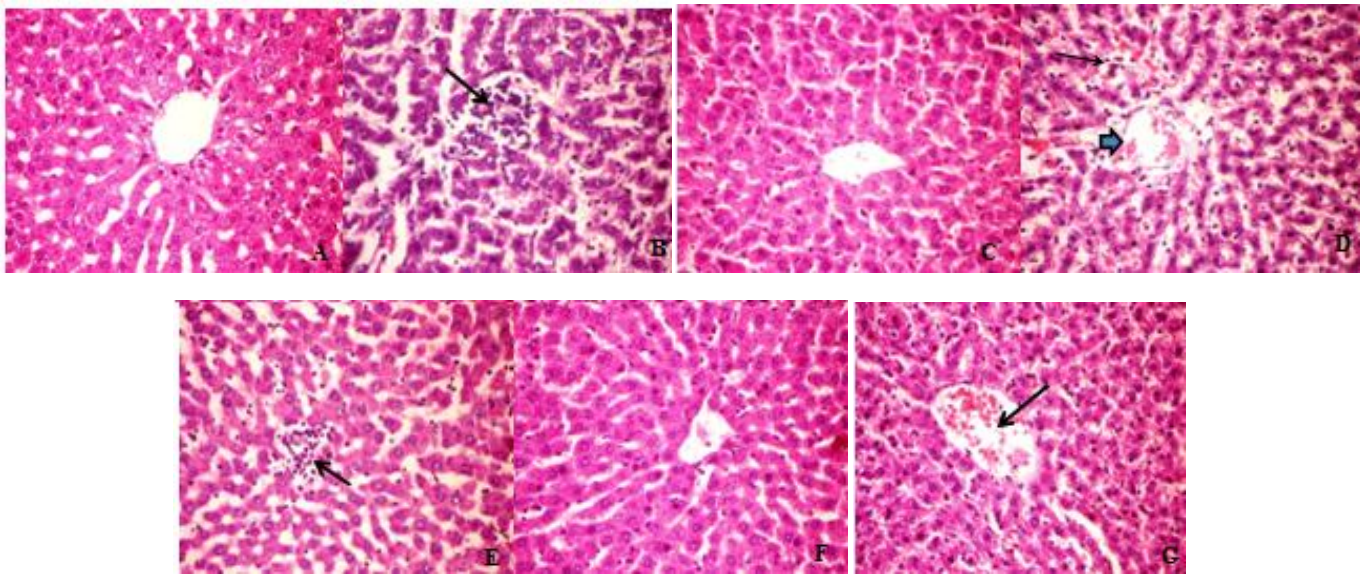

Fig. 1: Haematoxylin and eosin-stained liver sections (H&E, 400X)**. (A) Normal control group,** showing apparently normal hepatic parenchyma. **(B) TAA-intoxicated group,** showing focal area of necrosed hepatocytes infiltrated and replaced by mononuclear inflammatory cells (arrow). **(C) Alpha-lactalbumin (α-LAC 100 mg/kg)-treated group after TAA**, showing apparently healthy hepatic tissue. **(D) Alpha-lactalbumin (α-LAC 150 mg/kg)-treated group after TAA**, showing dilated central vein (arrow head) with congested hepatic blood sinusoids (arrow). **(E) Vit. C (500 mg/kg)-treated group after TAA,** showing focal area of leucocytic cell infiltration (arrow). **(F)α-LAC (100 mg/kg) and Vit. C-treated group after TAA,** showing normal histological findings. **(G) α-LAC (150 mg/kg) and Vit. C-treated group after TAA**, showing slightly congested central vein (arrow).

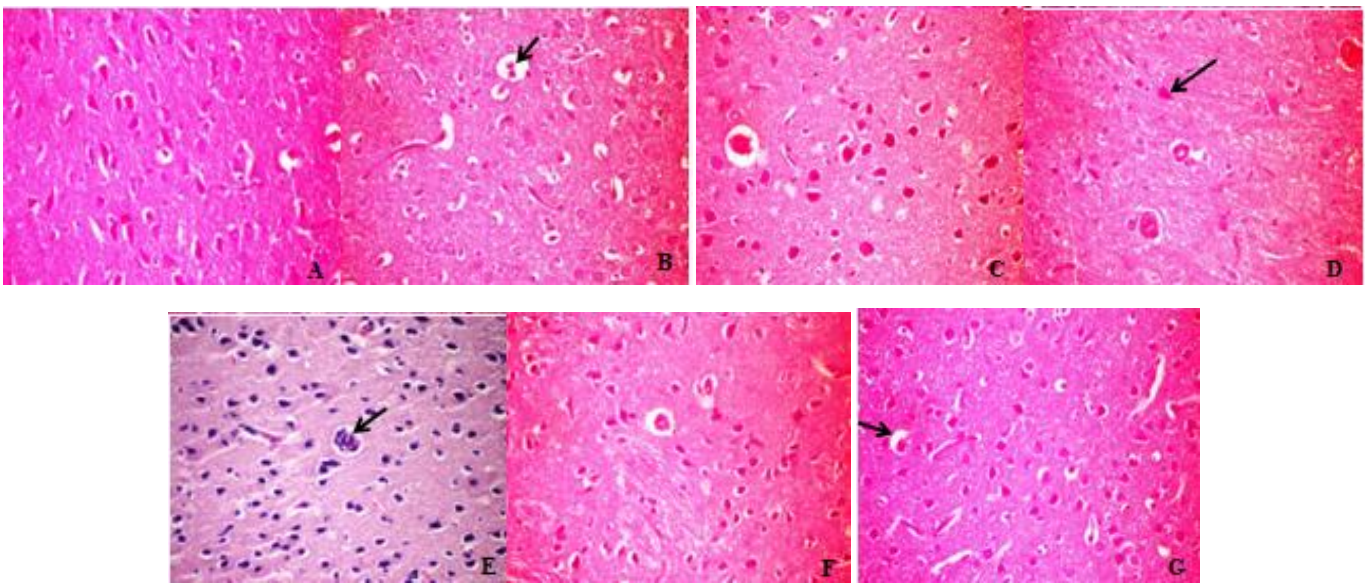

Fig. 2: Haematoxylin and eosin-stained brain sections (H&E, 400X)**. (A) Normal control group,** showing apparently normal neuronal histology. **(B) TAA-intoxicated group,** showing perineuronal edema (arrow). **(C) Alpha-lactalbumin (α-LAC 100 mg/kg)-treated group after TAA**, showing apparently normal neuronal cells. **(D) Alpha-lactalbumin (α-LAC 150 mg/kg)-treated group after TAA**, showing degenerated neuronal cells (arrow). **(E) Vit. C (500 mg/kg)-treated group after TAA**, brain tissue showing focal area of leucocytic cell infiltration (arrow). **(F)α-LAC (100 mg/kg) and Vit. C-treated group after TAA**, showing normal neuronal cells. **(G) α-LAC (150 mg/kg) and Vit. C-treated group after TAA**, showing focal perineuronal edema (arrow).

antioxidant and the possible hypoammonemic effects of alpha-lactalbumin (α-LAC) with or without vitamin C (vit. C) in thioacetamide (TAA)-model of minimal hepatic encephalopathy (MHE). Challenging of experimental animals with TAA causes a wide spectrum of pathophysiological changes which parallel to those observed in patients with hepatic encephalopathy (Zimmermann *et al.*, 1989). In this respect, TAA-induced liver failure model sounds a better alternative as TAA is a liver-specific toxicant whose metabolic products (sulfoxide and sulfone) cause hepatocytes damage and thereby derange overall liver biochemistry (Saran *et al.*, 2004) resulting into induction of different grades of liver dysfunction in a concentration-dependent manner in the rodent models (Schmandra *et al.*, 2001; Hernandez *et al.*, 2004; Sathyasaikumar *et al.*, 2007). To verify the role of oxidative stress (OS) in HE; GSH, MDA, SOD and NO were assessed in liver and brain tissues of TAA-treated rats. OS has been strongly associated with astrocyte swelling in acute liver failure (ALF) as well as the induction of mitochondrial transition

pore in chronic liver failure (CLF)(Norenberg *et al.,* 2004a). Thioacetamide (TAA), intraperitoneal administration at 100 mg/kg thrice weekly for six weeks, was reported to induce chronic liver damage (Schmandra *et al.,* 2001). According to the recommended classification of HE models, depending on the severity of liver damage accompanied with neurological complications developed, the minimal or low grade HE has been recommended as a subdivision of the subcategory of persistent HE under type C HE models (Ferenci *et al.,* 2002; Singh and Trigun, 2010). Hence, the present model of TAA-induced HE can be referred as minimal HE (MHE). GSH is a tripeptide that plays important roles in maintaining reducing equivalents in the cells facing oxidative challenges (Dickinson and Forman, 2002). In the present study, GSH was remarkably decreased by TAA both in liver (Al-Attar, 2012; Amin *et al.,* 2012) and brain (Singh *et al.,* 2014) of MHE rats. The increased production of reactive oxygen and nitrogen oxide species (ROS/RNOS) in the brain cells, in turn, is likely to amplify the neuronal derangements associated with HE pathogenesis (Haussinger and Gorg, 2010). Moreover, results demonstrated significant elevation in LP during TAA-induced HE in liver and brain. Most of the hepatotoxic agents including TAA damaged liver mainly by inducing lipid peroxidation (LPO) directly or indirectly (Sanz *et al.,* 2002; Hessien *et al.,* 2010; Chi *et al.,* 2011; Anbarasu *et al.,* 2012). As brain is structurally rich in polyunsaturated fatty acids and has high oxygen demand, making it the most vulnerable organ to undergo oxidative damage during un-physiological challenges. Therefore, MDA level, the first stable product of lipid peroxidation; serves as immediate indicators for the oxidative damage produced by ROS in the brain cells(Halliwell, 2006). Concurrently, TAA-induced elevation in nitric oxide (NO) level in liver and brain that confirms the contribution of nitrosative stress in the pathogenesis of HE. **Singh and coworkers** reported significant increase in the level of neuronal nitric oxide synthase (nNOS) protein coinciding with the similar increment in the level of NO in the cerebellar extracts from CLF rats(Singh and Trigun, 2010). Elevation in the levels of both MDA and NO as oxidative markers in liver and brain of the CLF rats may result in declined antioxidant mechanisms to counterbalance deleterious effects of ROS in CLF rats. On light of our results significant and concordant decline in the activity of the antioxidant enzyme superoxide dismutase (SOD) in liver and brain of CLF rats was reported. SOD is generally considered one of the first lines of antioxidant defense (Noor *et al.,* 2002). This enzyme converts superoxide anion into hydrogen peroxide, which is then removed by either catalase (CAT) or GSH-peroxidase (GPx) (Fakhrzadeh *et al.,* 2004). **Nada and coworkers** reported severe decline in the activity of SOD and level of GSH while increase in level of MDA and NO in TAA model of liver fibrosis (Nada *et al.,* 2015).

The pathogenesis of hepatic encephalopathy (HE) is associated with hyperammonemia (HA) and subsequent exposure of the brain to excess of ammonia (Hilgier *et al.,* 2004). OS participates as a critical factor in the mechanism of HE production, especially in hyperammonemic conditions (Kosenko *et al.,* 1997; Norenberg, 2003). Results from the present study revealed marked increase in serum ammonia levels of TAA-treated rats after 6 weeks, compared to normal control that was consistent with other investigations(Chen *et al.,* 2008; Farjam *et al.,* 2012; Huang *et al.,* 2012). Ammonia also reduced intracellular levels of glutathione (GSH) (Murthy *et al.,* 2001). Additionally, ammonia inhibits cystine uptake into cells (Bender *et al.,* 2000). As the cellular uptake of cystine is critical for GSH synthesis, the reduction in GSH levels would place astrocytes at risk for oxidative damage. Also, the elevated levels of circulatory liver markers and lipid peroxidation products in ammonium chloride (AC) rats might be due to the liver damage caused by ammonia-induced free radical generation (Essa and Subramanian, 2006). Ammonia-induced increase of NO and subsequently extracellular cGMP is a good indicator of the over-stimulation of NMDA receptors in rat brain, a process that contributes to increased reactive oxygen and nitrogen species (ROS/RNS) production (Hermenegildo *et al.,* 2000; Kosenko *et al.,* 2003; Hilgier *et al.,* 2004). Superoxide and NO have the ability to generate hydroxyl radicals (Hensley *et al.,* 1997). This leads to oxidative stress (Lena and Subramanian, 2004; Norenberg *et al.,* 2004b), which eventually results in increased levels of lipid peroxidation products (MDA) and decreased levels of antioxidants (GSH and SOD) in TAA-treated rats. Consequently, CLF following TAA administration is well established by the elevated activities of liver transaminases (ALT and AST) in the present study, indicating cellular leakage and loss of functional integrity of hepatic membrane(Chen *et al.,* 2008; Al-Attar, 2012; Anbarasu *et al.,* 2012; Farjam *et al.,* 2012). The extensive liver injury induced by TAA through its free radical generation mechanism, which in turn has the ability to cause hepatic damage resulting in increased leakage of cellular enzymes. Thioacetamide is metabolized to acetamide and thioacetamide–s–oxide. The latter binds to tissue macromolecules and is responsible for the change in cell permeability, increased intracellular concentration of Ca^{++}, increase in nuclear volume and enlargement of nucleoli and inhibits mitochondria activity eventually leading to hepatic necrosis (Ansil *et al.,* 2011). Histopathological studies of liver and brain tissue of TAA rats confirmed hepatic necrosis and mononuclear inflammatory cells infiltration. Further perineuronal edema in brain tissues was evident; which might be a consequence of chronic liver damage, oxidative stress and hyperammonemia. The findings in the present investigation derives its importance from the evidence that oxidative stress has an important role in development and progression of both acute and chronic liver disease (Lucena *et al.,* 2002) and in the pathogenesis of hepatic encephalopathy (Norenberg *et al.,* 2004a). Therefore, alpha-lactalbumin (α-LAC) and vit. C can be introduced in alleviating HE-associated oxidative stress and hyperammonemia. Results from TAA rats treated with α-LAC (100 and 150 mg/kg) and/or vit. C (500mg/kg) demonstrated significant increase in GSH level while decreased MDA of liver and brain was reported as well. Combined treatment of vit. C with α-LAC (100mg/kg) showed stronger antioxidant effect on the level of MDA both in liver and brain of TAA rats

than in rats treated with either compounds. This result further confirms the potent antioxidant activity of both α-LAC(Brown *et al.,* 2004; Kume *et al.,* 2006; Mansour *et al.,* 2013; Mansour *et al.,* 2015; Nada *et al.,* 2015) and vit. C (Patra *et al.,* 2001; El-Gendy *et al.,* 2010; Mustafa *et al.,* 2013).

Regarding nitric oxide (NO), treatment with α-LAC (100 and 150 mg/kg) and / or vit. C decreased hepatic and brain nitric oxide (NO). Alpha-LAC (100 mg/kg) and vit. C combination exerted significant effect on liver NO content compared to individual treatments. Generation of RNS could also explain the loss of GSH and the appearance of markers of oxidative stress (Crespo *et al.,* 2001); the mechanism that may be interrupted by α-LAC(Nada *et al.,* 2015) and vit. C (Mustafa *et al.,* 2013) treatment. Moreover, it has been shown that down-regulation of NF-κB with antioxidants resulted in decreases in the amount of NO released in the circulation, a treatment effectattributed to the reduction in iNOS expression (Hagar, 2009). ROS production mainly superoxide anion and hydroxyl radical, may be a major cause of TAA-induced oxidative stress (Al-Attar, 2012; Nada *et al.,* 2015). Current study reported decline in SOD activity during TAA-induced HE, it may be due to increased usage in scavenging free radicals induced by the TAA thus causing irreversible inhibition of the activity (Sanz *et al.,* 2002). Two-week treatment with α-LAC (100 and 150 mg/kg) or vit. C (500 mg/kg) exerted significant improvement in liver and brain SOD activity in TAA-treated rats, while their combination maintained normal SOD activity in liver tissues. Current data is in accordance with other investigators who reported that whey proteins, including α-LAC, suppressed hepatic lipid peroxidation and stimulated the antioxidant defense system by increasing the level of glutathione and the activity of glutathione-S-transferase (GST), glutathione peroxidase (GPx) and superoxide dismutase (SOD) in wounded diabetic rats (Ebaid *et al.,* 2013)**,**α-LACattenuated the decrease in hepatic SOD activity in TAA-induced fibrosis (Nada *et al.,* 2015) and improved hepatic
SOD activity in LPS-treated rats (Mansour et al., 2015). In addition, vit. C increased the activities of GST, SOD and CAT, and the levels of SH-group and protein in stannous chloride-induced oxidative damage (Yousef *et al.,* 2007).

As oxidative stress participates as a critical factor in the mechanism of HE production, especially in hyperammonemic conditions (Kosenko *et al.,* 1997; Norenberg, 2003). **Bai et al.(2001)** have proposed that ammonia induces mitochondrial permeability transition in cultured astrocytes, which may be a factor in mitochondrial dysfunction associated with HE and other hyperammonemic states (Bai *et al.,* 2001). Accordingly, the results of the current study reported elevated levels of serum ammonia consistent with oxidative and nitrosative stress in both liver and brain of TAA-treated rats. It must be recalled that the presence of oxidative stress in brain without adequate defenses aggravates the CNS condition during acute or chronic liver damage (Lemberg and Fernandez, 2009). Since hyperammonemia results in increased utilization of (BCAAs) (Tomiya *et al.,* 2002),treatment with α-LAC, a rich source of BCAAs (Morifuji *et al.,* 2009), could facilitate ammonia detoxification by supporting muscle glutamine synthesis (Tomiya *et al.,* 2002) while vit. C could counteract oxidative stress (Patra *et al.,* 2001; Yousef *et al.,* 2007; Mustafa *et al.,* 2013). Groups treated with either α-LAC, vit. C or their combination reported normal serum ammonia levels, the current results support the substantial evidence of contribution between hyperammonemia and oxidative stress in aggravation of HE.

On the level of liver function test, the present study demonstrates that TAA-induced HE resulted in impairment in liver functions that was manifested by increased serum ALT and AST activities. Treatment with α-LAC (100 or 150 mg/kg) for two weeks improved liver function enzymes. Moreover, vit. C (500 mg/kg) alone exerted significant reduction in AST and ALT activities. While combined treatment showed no significance from individual treatments. Noteworthy, α-LAC (100mg/kg), vit. C and their combination maintained normal serum ALT activity. Results, herein, are in accordance with previous literature that reported the ability of whey proteins, including α-LAC, in reducing or maintaining normal liver function in several models of hepatotoxicity (Kume *et al.,* 2006; Gad *et al.,* 2011; Mansour *et al.,* 2013; Eliwa *et al.,* 2014; Mansour *et al.,* 2015; Nada *et al.,* 2015).

Further, vit. C exerted hepatoprotective activity by lowering liver function enzymes in TAA-induced acute liver injury (Mustafa *et al.,* 2013), choline-deficient rat model of non-alcoholic fatty liver disease (Oliveira *et al.,* 2003) and CCl4-induced liver damage (Ismail *et al.,* 2009).

Histopathological examination revealed that TAA showed various histological changes in both liver and brain of rats. These changes include necrosed hepatocytes, infiltrated and replaced by mononuclear inflammatory cells and perineuronal edema in brain(Kucera *et al.,* 2011; Farjam *et al.,* 2012).The hepatic architecture of TAA-treated rats resulted in necrotic changes and inflammatory cell infiltration, which basically supported the alterations observed in biochemical analysis. It might be due to TAA-intermediates and the reactive oxygen species that can covalently bind to biologically important molecules and increase cellular oxidative stress (Pallottini *et al.,* 2006; Chilakapati *et al.,* 2007), and lipid peroxidation, and glutathione depletion (Sanz *et al.,* 2002; Kucera *et al.,* 2011).TAA interferes with the movement of RNA from the nucleus to the cytoplasm which may cause membrane injury(Anbarasu *et al.,* 2012), also astrogliosis was evaluated at the area of cerebellum and hippocampus in TAA-treated animals (Avraham *et al.,* 2009), which may explain changes observed in brain histology. Alpha-LAC markedly counteracted the histological alterations induced by TAA in liver and brain tissues. This can be attributed to its antioxidant and chelating activities, which significantly reduced the oxidative threat leading to reduction of pathological changes and restoration of normal physiological functions (Gad *et al.,* 2011).

Moreover, Administration of BCAAs has been shown to stimulate hepatic protein synthesis; indeed, leucine stimulates the synthesis of hepatocytes growth factor by stellate cells (Tomiya *et*

al., 2002). Alpha-LAC (100mg/kg) showed improvement in liver and brain histology either alone or combined with vit. C compared to α-LAC (150mg/kg), vit. C or their combination.

CONCLUSION

It can be concluded that administration of α-LAC (at lower dose) in combination with vitamin C can provide new intervention for liverand brain damage in minimal hepatic encephalopathy (MHE) due to their antioxidant and hypoammonemic effects.

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