International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 8, Issue 3, 2016

Original Article

UMBELLIFERONE EXTENUATES THE ABNORMALITIES IN LIPID METABOLISM DURING D-GALACTOSAMINE AND LIPOPOLYSACCHARIDE-INDUCED FULMINANT HEPATIC FAILURE IN RATS

TAMILARASI SASIVARNAM[1], PRAMILA CHENGALVARAYAN SUBRAMANI[1], INDUMATHI SELVANATHAN[1], DEVAKI THIRUVENGADAM[1]

[1]Department of Biochemistry, University of Madras, Guindy Campus, Chennai 600025, TN, India
Email: devakit@yahoo.co.uk

Received: 03 Oct 2015 Revised and Accepted: 13 Jan 2016

ABSTRACT

Objective: Coumarins and coumarin-related compounds are a plentiful source of potential drugs candidate in relation to its safety and efficacy. Hence, the present study was designed to evaluate the protective nature of Umbelliferone (UMB), a coumarin derivative on alterations in lipid metabolism during D-Galactosamine (D-GalN) and Lipopolysaccharides (LPS) induced Fulminant hepatic failure (FHF).

Methods: Male wistar rats were challenged with a single intraperitoneal injection of D-GalN/LPS, and the protective nature of UMB was examined with reference to Total Cholesterol, Phospholipids, Triglycerides, free fatty acids(plasma, liver, and kidney), lipid metabolizing enzymes and lipoprotein fraction.

Results: Anomalies in lipid status, lipid metabolizing enzymes and lipoprotein fractions induced by D-GalN/LPS was restored to near normal levels upon pretreatment with UMB which was further confirmed by Transmission electron microscopic observations.

Conclusion: The outcome of the above findings evidenced the protective nature of UMB on alterations in lipid metabolism during D-GalN/LPS induced FHF in rats.

Keywords: Umbelliferone, D-Galactosamine, Lipopolysaccharides (LPS), Lipid metabolism, Liver failure, Lipid metabolizing enzymes, Lipoproteins, Transmission electron microscope.

INTRODUCTION

Liver diseases pose a major threat to mankind worldwide today with limited options of prevention and treatment [1]. FHF synonymous with acute liver failure is an end-stage liver disease with a sudden onset of necrosis and massive degeneration of hepatocytes in the context of previously normal liver function [2]. FHF leads to multiorgan failure causing high mortality rates (80%-90%) despite the advent of liver transplantation, which is also limited by the chronic shortage of liver donors [3]. Several experimental models, contribute to develop therapeutic strategies by investigating the underlying mechanisms of liver dysfunction [4]. Among which, the combination of D-GalN and LPS is a widely established experimental model as it mimics the clinical FHF [5]. D-GalN, a well known hepatotoxicant renders its hepatotoxic effect via depletion of UTP and inhibition of mRNA synthesis and also has been suggested to react by activating hepatic macrophages to produce ROS. LPS, a toxic component of gram negative bacteria when co-administered with D-GalN elicits immune response by stimulating inflammatory and hepatic kupfer cells to produce various proinflammatory cytokines thus implicated in the pathology of liver failure [6].

Coumarin, a benzopyrone compound is a plant-derived natural product widely distributed in numerous species belonging to different botanical families with the richest source being the Umbelliferae and Rutaceae families [7]. Despite the extensive distribution of coumarins in entire parts of the plant, the highest levels are consistently found in fruits followed by roots, stems and leaves [8]. They are also available in some essential oils such as cassia oil [9], cinnamon bark oil [10] and lavender oil [11]. The search for useful pharmaceutical has led to a resurgence of interest in coumarins because these substances display potent and relevant pharmacological activities that are structure-dependent, while at the same time appearing to lack toxicity in mammalian systems [12].

Umbelliferone (UMB) or 7-hydroxy coumarin, the major biotransformation product (75%) of coumarin is a widespread natural antioxidant with a short half-life [13] found predominantly in the edible fruits of golden apple (Aegle marmelos Correa) [14] and bitter orange (Citrus aurantium)[15]. It is a yellowish-white crystalline solid which has a slight solubility in hot water, but high solubility in ethanol [16]. UMB exhibits a wide spectrum of pharmaceutical effects including antioxidant [17], anti-lipidemic [18], anti-diabetic, anti-hyperglycemic [19], radioprotective [20] and anti-inflammatory properties [21]. Further, it has been used in the synthesis of anticancer drugs and in the treatment of asthma and allergic disorders. UMB can be invoked as a fluorescence indicator for metal ions such as copper and calcium and also serves as a pH indicator in the 6.5-8.9 range. [22].

Fascinated by its diverse pharmacological properties, researchers trying to explore the pure coumarin and their derivatives for their applicability as drugs. Hence, the present study was aimed to evaluate the protective nature of UMB on lipid metabolism during D-GalN/LPS induced Fulminant hepatic failure in rats.

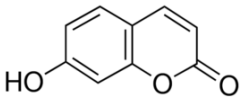

Fig. 1: Structure of Umbelliferone ($C_9H_6O_3$)

MATERIALS AND METHODS

Chemicals

LPS (Escherichia coli, 0111: B4), D-GalN and Umbelliferone were purchased from Sigma Chemicals (St. Louis, MO, USA). All other chemicals used were of analytical grade obtained from Sisco Research Laboratories Pvt. Ltd, Mumbai, India.

Animals

Adult male albino rats of the Wistar strain weighing about 150-180g were utilized in this study. They were maintained in clean, sterile,

polypropylene cages and fed with commercial pelleted rat chow (M/s. Hindustan Lever Ltd., Bangalore, India), water ad libitum and kept in a well-ventilated room with 12 h light/dark cycles throughout the experimental period. All the experiments were designed and conducted according to the ethical norms approved by the institutional animal ethical committee guidelines. (IAEC. No.36/ 03/2014).

Experimental design

The experimental animals were divided into four groups with each groups comprising of six animals.

Group 1 (Control)

Normal control rats received 10% DMSO which served as a vehicle throughout the experimental period along with standard diet and drinking water.

Group 2 (D-GalN/LPS)

Rats received the standard diet and drinking water throughout the experimental period and were given single intraperitoneal injections of D-GalN (500 mg/kg body weight) and LPS (50 µg/kg body weight) [23] 24 h before the end of the experimental period.

Group 3 (UMB+D-GalN/LPS

Rats were pretreated with UMB (30 mg/kg body weight dissolved in10%DMSO) for 10 d prior to the induction of D-GalN/LPS

Group 4 (UMB)

Rats were treated with UMB (dissolved in10%DMSO) at a dosage of 30 mg/kg body weight via intragastric intubation on all the days of the experimental period (10 d).

At the end of the experimental period, i.e., 24h after the intraperitoneal injection of D-GalN/LPS the animals were fasted overnight and anesthetized with Ketamine (90 mg/kg) and Xylazine (10 mg/kg) and sacrificed by cervical decapitation. Blood was obtained from sinus orbital with and without anticoagulant for the separation of plasma and serum respectively. The liver and kidney tissue was excised immediately and washed with ice-cold saline. Accurately weighed liver and kidney were homogenized in 0.1M Tris HCl buffer and the homogenates was used for biochemical estimation.

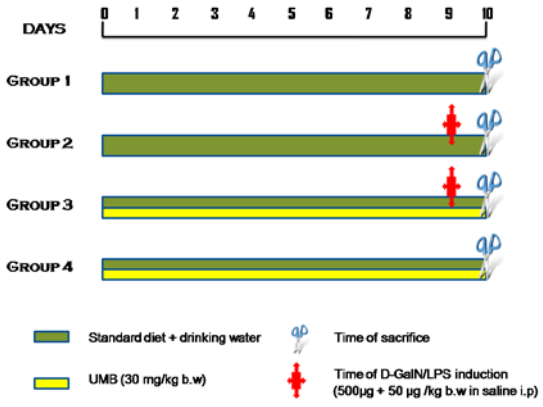

Fig. 2: Schematic representation of the experimental design

Biochemical parameters

Lipid analysis

Total cholesterol [24], Phospholipids [25], Triglycerides [26] and free fatty acid [27] contents were estimated in plasma, liver and kidney. Free and ester cholesterol contents were assayed in plasma [24].

Estimation of lipid metabolizing enzymes

The activity of lipoprotein lipase [28], Lecithin cholesterol acyltransferase [29] and hepatic triglyceride lipase [30] was estimated.

Estimation of lipoprotein fractions

Lipoproteins such as LDL, HDL and VLDL were fractionated from serum by dual precipitation technique [31].

Transmission electron microscopy of liver tissue

A portion of the liver tissue was instantaneously immersed in 25 g/l of glutaraldehyde solution, buffered with 0.1 mol/l sodium cacodylate (pH 7.4). The specimen was subsequently placed in the buffer fixative medium, followed by washing with sodium cacodylate and fixation in 20 g/l osmium tetroxide buffered with 0.1 mol/l sodium cacodylate. After dehydration in a graded series of alcohol and propylene oxide, the tissues were transferred to propylene oxide: ethanol mixture (1:1) and embedded in resin. The specimens were placed on epoxy resin blocks and left in the oven at 65^{o}C for 72 h. Thin sections were cut with an ultramicrotome, stained with uranyl acetate and lead citrate, and then examined on a TEM.

Statistical analysis

All the grouped data were evaluated with SPSS/22 software. Hypothesis testing methods included one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. *P* values of less than 0.05 were considered to indicate statistical significance. All these results were expressed as mean±SD for six animals in each group.

RESULTS

Effect of UMB on lipid status

The levels of cholesterol (total, free and ester), phospholipids, triglycerides and free fatty acids in the plasma respectively are presented in table 1. Significant elevation of cholesterol (total, free and ester), triglycerides and free fatty acids with a reduction in the levels of phospholipids were observed in Group 2 rats injected with D-GalN/LPS. These altered levels were brought back to near normal in Group 3 (UMB+D-GalN/LPS) animals. Group 4 UMB alone treated animals exhibited similar activity with that of Group 1 control animals.

Fig. 3 and fig. 4 represents the effects of UMB on total cholesterol, phospholipids, triglycerides and free fatty acids in the liver and kidney of a normal and experimental group of rats. The levels of total cholesterol, triglycerides and free fatty acids were significantly elevated ($p<0.05$) while the levels of phospholipids were reduced in Group 2 D-GalN/LPS intoxicated rats when compared with Group 1 control rats. UMB exhibited a characteristic property of significantly reducing the total cholesterol, triglycerides and free fatty acids levels and elevated the levels of phospholipids in Group 3 (UMB+D-GalN/LPS) animals. Thus, UMB pretreatment significantly maintained the tissue lipids status at near normal levels as compared to the Group 2 rats.

Table 1: Levels of cholesterol (Total, free and ester), phospholipids, triglycerides and free fatty acids in the plasma of control and experimental group of rats

Plasma	Total cholesterol	Free cholesterol	Ester cholesterol	Phospholipids	Triglycerides	Free fatty acids
Group 1 (Control)	51.25±3.63	11.55±1.00	44.76±4.41	123.59±11.28	52.23±5.08	12.06±1.17
Group 2 (D-GalN/LPS)	83.95±7.66^{a}	54.51±5.24^{a}	22.76±2.19^{a}	91.74±7.37^{a}	151.19±13.95^{a}	25.16±1.59^{a}
Group 3 (UMB+D-GalN/LPS)	62.39±5.88^{a,b}	24.39±1.61^{a,b}	35.54±2.70^{a,b}	104.54±9.91^{a,b}	72.43±6.00^{a,b}	15.61±1.20^{a,b}
Group 4 (UMB)	51.37±3.83^{b,c}	11.66±0.68^{b,c}	44.05±4.34^{b,c}	123.04±11.06^{b,c}	53.79±5.27^{b,c}	12.04±1.18^{b,c}

Results are expressed as mean±SD for six rats in each group. Statistical significance at $p<0.05$ compared with ^{a}group 1, ^{b}group 2 and ^{c}group 3 based on Duncan's multiple range tests. Units: mg/dl

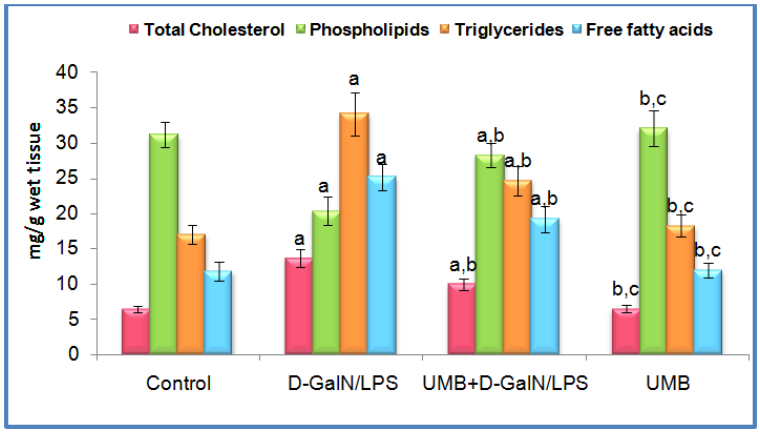

Fig. 3: Levels of total cholesterol, phospholipids, triglycerides and free fatty acids in the liver of control and experimental group of animals

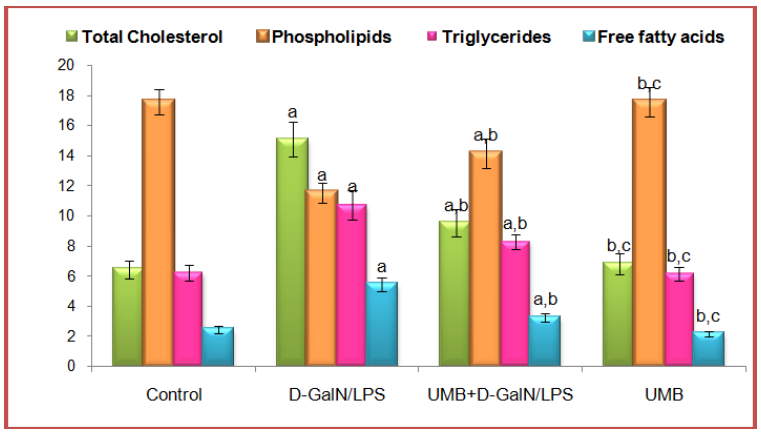

Fig. 4: Levels of total cholesterol, phospholipids, triglycerides and free fatty acids in the kidney of control and experimental group of animals

Results are expressed as mean±SD for six rats in each group. Statistical significance at $p<0.05$ compared with [a]group 1, [b]group 2 and [c]group 3 based on Duncan's multiple range tests.

Units: mg/g wet tissue

Effect of UMB on lipid metabolizing enzymes

Altered lipid composition modifies the activity of lipid metabolizing enzymes which leads to striking changes in the pattern of integrated metabolism. Table 2 indicates the activity of the lipid metabolizing enzymes such as lecithin cholesterol acyl transferase, lipoprotein lipase in the plasma and hepatic triglyceride lipase in the liver of control and an experimental group of rats. A significant decrease($p<0.05$) in the activities of these enzymes was noted in D-GalN/LPS induced Group 2 rats while prior oral administration of UMB to Group 3 animals produces a significant reversal of the enzyme activity towards near normalcy. Group 4 animals treated with UMB alone exhibited similar activity with that of Group 1 control animals.

Table 2: Activities of lipid metabolizing enzymes in the control and experimental group of animals

Particulars	Lipoprotein lipase	Lecithin cholesterol acyltransferase	Hepatic triglyceride lipase
Group 1 (Control)	5.17±0.13	99.40±0.87	17.68±1.22
Group 2 (D-GalN/LPS)	2.32±0.16[a]	56.35±0.47[a]	10.02±0.73[a]
Group 3 (UMB+D-GalN/LPS)	3.68±0.21[a,b]	83.15±0.53[a,b]	14.59±1.18[a,b]
Group 4 (UMB)	5.41±0.40[b,c]	99.85±0.92[b,c]	17.41±1.21[b,c]

Results are expressed as mean±SD for six rats in each group. Statistical significance at $p<0.05$ compared with [a]group 1, [b]group 2 and [c]group 3 based on Duncan's multiple range tests, Units: LPL-mmol of glycerol liberated/hr/ml plasma; LCAT-µg of cholesterol esterified/min/l of plasma; HTGL-µg of free fatty acid released/min/mg protein.

Lipoprotein fraction

As shown in table 3, significantly elevated levels ($p<0.05$) of LDL and VLDL with a concomitant reduction in HDL levels were obvious in Group 2 animals intoxicated with D-GalN/LPS. However, Group 3 animals treated with UMB+D-GalN/LPS showed a substantial improvement in the levels of HDL with a parallel inhibitory action on the elevation of LDL and VLDL levels as compared to Group 2 D-GalN/LPS animals. There was no marked difference between UMB alone Group 4 rats and Group 1 control rats.

Table 3: Levels of HDL, LDL and VLDL in the serum of control and experimental group of animals

Particulars	HDL	LDL	VLDL
Group 1 (Control)	36.00±3.08	42.82±4.23	9.44±0.32
Group 2 (D-GalN/LPS)	24.00±1.78[a]	82.15±7.72[a]	14.83±1.13[a]
Group 3 (UMB+D-GalN/LPS)	30.18±1.94[a,b]	53.79±5.31[a,b]	12.42±1.04[a,b]
Group 4 (UMB)	36.04±2.83[b,c]	43.36±3.79[b,c]	9.32±0.23[b,c]

Results are expressed as mean±SD for six rats in each group. Statistical significance at $p<0.05$ compared with [a]group 1, [b]group 2 and [c]group 3 based on Duncan's multiple range tests. Units: mg/dl

Findings on transmission electron microscopic images

Intraperitoneal injection of D-GalN/LPS in Group 2 rats resulted in significant changes in liver morphology as evidenced from fig.5A, showing accumulation of assorted lipid droplets with swollen mitochondria. It is very clear from Fig.5B, that UMB+D-GalN/LPS treated Group 3 rat livers showed meager lipid droplet with serrated edged nucleus and scanty swollen mitochondria.

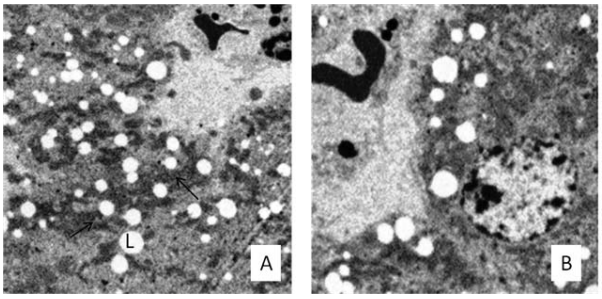

Fig. 5: Transmission electron microscopic observations of D-GalN/LPS intoxicated rat liver (A), and UMB+D-GalN/LPS treated rat liver (B)

A-Group 2 D-GalN/LPS intoxicated rat livers with arrows indicating swollen mitochondria and (L) assorted lipid droplets. B-Group 3 UMB+D-GalN/LPS treated rat liver with meager lipid droplets and scanty mitochondria.

DISCUSSION

The liver is the major site for the synthesis and metabolism of cholesterol, bile acids and phospholipids [32]. Marked alterations in lipid metabolism have been declared in D-GalN induced hepatic injury in rats [33] which corroborates well with the present investigation. Any liver disease will show an increased blood cholesterol level [34]. The sizable increase of cholesterol in the plasma, liver and kidney sample might have been due to the inability of the diseased liver to remove cholesterol from circulation. This finding could be related to the results of the previous studies [35].

The administration of D-GalN/LPS causes the imbalance between the rate of synthesis and the rate of release of triglycerides by the parenchymal cells into the circulation [36] and also elevates the levels of intracellular calcium. These changes in lipid metabolism led to the accumulation of triglycerides and free fatty acids. The elevated triglyceride and free fatty acids levels in the plasma, liver and kidney sample of rats found in the present study are in accordance with the previous report that showed a parallel increase in the lipid levels in D-GalN/LPS intoxicated rats [37]. Phospholipids are vital components of bio membranes rich in polyunsaturated fatty acids, which are a susceptible substrate for free radicals and also important for the maintenance of cellular integrity, microviscosity, and survival [38]. The significant decrease in the levels of phospholipids in the plasma, liver and kidney of D-GalN/LPS intoxicated rats may be explained by the accelerated degradation of membrane phospholipids probably by the activation of phospholipase A2 activity as a consequence of intracellular calcium overload [39]. Rats pretreated with UMB prior to the induction of hepatic damage showed a restoration of the altered lipid levels induced by D-GalN/LPS towards near normalcy thereby showing the modulating effect of UMB against D-GalN/LPS induced changes in the lipid levels in rats.

The lipid metabolizing enzymes such as lipoprotein lipase, lecithin cholesterol acyltransferase and hepatic triglyceride lipase are the key players involved in lipoprotein metabolism. LPL activity varies within tissues depending upon the nutritional and stress state of an animal. LPL is associated with the initial catabolism of triglyceride-rich lipoproteins, VLDL and chylomicron by the extrahepatic tissues [40]. Thus, decreased activity of LPL indicates decreased uptake of these lipoproteins by the tissues resulting in hypertriglyceridemia in D-GalN/LPS induced rats. Among the activities most drastically decreased after D-GalN/LPS challenge is that of plasma LCAT. This deficiency leads to low levels of plasma cholesterol esters and in severe damage, the fall in cholesterol ester content in the plasma was attributed to the lack of LCAT[35]. Earlier reports are also proposed that D-GalN injection results in severe LCAT deficiency in rats [41]. HTGL is allegedly a role in the catabolism of VLDL and chylomicron remnants. The efficient clearance of VLDL and chylomicron from circulation by the liver requires the action of HTGL. Hence reduced activities of hepatic lipase results in increased accumulation of lipoproteins (LDL, VLDL) and triglycerides in the liver [42]. The activities of above lipid metabolizing enzymes were reverted to near normal upon pretreatment with UMB. This assures the hypolipidemic effect of UMB.

HDL, beneficial lipoprotein helps in the scavenging of cholesterol from the extrahepatic tissues in the presence of LCAT and brings it to the liver. The lowered HDL levels can be ascribed to decreased serum LPL and LCAT activity. In this context, it has been demonstrated that elevated activity of plasma LPL leads to an increase in HDL production and reduction in LDL constituents [43]. It has clearly been shown that a relationship exists between increased concentration of HDL-C and decreased morbidity and mortality rate in cardiovascular patients [44]. Similarly, recent research has revealed that a 4-5% decrease in LDL-cholesterol results in a 5-10% decrease in the occurrence of coronary heart disease (CHD) [45]. Numerous studies have already reported the increased levels of LDL,VLDL and a concomitant reduction in the HDL values during D-GalN/LPS induced liver failure [38, 46]. Our present findings clearly coincide with the above findings. The ability of UMB to normalize the abnormal levels of lipoproteins during D-GalN/LPS liver failure in our investigation justifies the foregoing findings and confirms its anti dyslipidemic effect.

Intraperitoneal injection of D-GalN/LPS in rats resulted in significant changes in liver morphology. As shown in Fig.5A assorted lipid droplets with swollen mitochondria and polymorphic mitochondria were evident. These alterations in liver cell morphology and appearance of lipid droplets were consistent with the severe alterations in the lipid profile. The appearance of severe degeneration of mitochondria could be caused by severe depletion of glutathione and other non-enzymatic antioxidants.

CONCLUSION

The results of the present investigation clearly suggests that UMB is able to influence lipid metabolism by restoring the altered levels of lipids status and lipid metabolizing enzymes and stabilizing the derangement of lipoprotein levels during experimentally induced hepatic failure. The anti dyslipidemic effect of UMB during D-GalN/LPS induced fulminant hepatic failure may be attributed due to its ability to combat the oxidative stress and or the involvement of

some cellular regulatory mechanism that preserves the cellular integrity against chemically induced toxicity. However, further dose dependent and mechanistic studies could eventually reinforce its use in modern medicine as a remedy for lipid-associated disorders.

ACKNOWLEDGEMENT

Principal author S. Tamilarasi, gratefully acknowledges the University Grant Commission (UGC), New Delhi, India, for the financial assistance in the form of UGC-BSR Research Fellowship.

CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interest.

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