ORIGINAL ARTICLE

EFFECT OF LECITHIN AND SILYMARIN ON D-GALACTOSAMINE INDUCED TOXICITY IN ISOLATED HEPATOCYTES AND RATS

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ABSTRACT

To investigate Lecithin for its hepatoprotective activity against D-galactosamine (D-GalN) induced toxicity in freshly isolated rat hepatocytes and animal models. Freshly isolated rat hepatocytes were exposed to D-galactosamine (30 mM) along with/without lecithin (100 µg/ml) and the levels of selected liver enzymes were measured. Thirty six Wistar strain albino rats were used for the in vivo investigations. Lecithin 50 and 100 mg/kg.b.wt were administered for one week by oral route. Liver damage was induced by intra peritoneal administration of 400 mg/kg b.wt D-galactosamine. The antihepatotoxic effect of lecithin was observed in freshly isolated rat hepatocytes at concentration 100 µg/ml and was found to be similar to that of the standard silymarin used. Its in vivo hepatoprotective effect at 100 mg/kg b.wt was comparable with that of the standard silymarin at 100 mg/kg body weight. Lecithin was able to normalise the biochemical levels which were altered due to D-galactosamine intoxication in freshly isolated rat hepatocytes and also in animal models.

KEY WORDS

Lecithin, Silymarin, D-galactosamine, Hepatoprotective activity, Hepatocytes, Animal models.

Lecithin, an important phospholipid is found in the major organs in our body such as the heart, the liver, and the kidneys (1-2). Lecithin, a component of most cells, will help in transport and responsible for overall health of the body. Though it is produced within our own bodies, we do not always consume enough of the nutrition needed to produce it in adequate amounts (1-2). As a result, lecithin supplementation is necessary for overall health and prevention of many conditions and diseases.

Lecithin is composed of phosphoric acid, choline, fatty acids, glycerol, glycolipids, triglycerides and phospholipids (e.g., phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol)(1-2). However, lecithin is sometimes used

as a synonym for pure phosphatidylcholine, a phospholipid that is the major component of its phosphatide fraction. It may be isolated either from egg yolk or soya beans.

Few studies have been carried out exploring different biological activities of lecithin. Soya-derived lecithin have significant effects on lowering cholesterol and triglyceride, while increasing HDL levels in the blood (1-2). Effect of lecithin on ethanol induced oxidative stress was reported (3). Phosphatidylcholine synthesis in hepatocytes (4), lipoprotein secretion from hepatocytes (5-6), role of phosphatidylcholine biosynthesis (7), The role of phosphatidylethanolamine methylation in hepatocytes (8) and role of lecithin and favorable changes in lipoprotein composition in hypercholesterolemic rats were studied (9-10). Also hepatic uptake and metabolism of phosphatidylcholine associated with high density lipoprotein was reported (11). However, no investigation on *in vitro* and *in vivo* hepatoprotective activity of lecithin against has been reported. Since antioxidant activity of lecithin is well known, it will be interesting to know its hepatoprotective nature. Hence, in this study we report the activity of lecithin in D-galactosamine induced toxicity in freshly isolated rat hepatocytes and in rat models.

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MATERIALS AND METHODS

Materials: All routine chemicals were obtained from SD-fine chemicals, Mumbai, India. Collagenase, insulin, dexamethasone, F-12 Coon's medium, Ham's F12 medium, minimum essential medium (MEM), new born calf serum, D-galactosamine, silymarin (standard drug) and antibiotics were purchased from Sigma chemicals Co., St. Louis, MO, USA. Cobas kits were purchased from Roche diagnostics, USA. Soy lecithin pure sample was a gift from Perfect Biotech, Nagpur, India.

Preparation of drug sample : 10 mg of lecithin were dissolved separately in 1 ml of dimethyl sulphoxide (DMSO) and volume was made up to 10 ml with F12 Coon's/MEM to obtain a stock solution of 1 mg/ml concentration and stored at -20°C prior to use. Further dilutions were made to obtain different concentrations ranging from 1000 µg/ml to 100 µg/ml with the respective media and used for *in vitro* investigations. Solutions of standard silymarin powder was also prepared (1 mg/ml) in a similar manner. Both lecithin and standard silymarin were suspended in sodium CMC (0.3%) in distilled water and used for *in vivo* investigations.

Hepatoprotective effect of lecithin in freshly isolated rat hepatocytes : Liver cells were isolated by a slight modification of procedure described earlier (12). The calcium free HEPES buffer and collagenase solutions were warmed in a water bath (37°C). The abdomen of the rat was opened under phenobarbital sodium (35 mg/kg.b.wt) anesthesia. A midline incision was made and a loosely tied ligature was placed around the portal vein approximately 5 mm from the liver and the cannula was inserted up to the liver and then the ligature was tightened and heparin was injected into the femoral vein (1000 IU). The inferior vena cava was cut below the renal vein. Perfusion was performed for 20 min (37°C) with calcium free HEPES buffer, containing 1% bovine serum albumin fraction V at a flow rate of 30 ml/min. The liver swells during this time, slowly changing its color from dark red to grayish white. The swollen liver was then perfused with TPVG (trypsin, Phosphate buffered saline, versine, glucose) solution (50 ml) followed by perfusion with calcium free HEPES buffer, which contained additional collagenase (0.075%) and calcium chloride (4 mM) at a flow rate of 15 ml/min for 20 min. After the perfusion, the lobes were removed and transferred into a sterile Petri dish containing calcium free HEPES buffer and dispersed gently. It was transferred into a sterile conical flask and the crude cell suspension was stirred with the help of a magnetic stirrer for 5 min to release hepatocytes into the solution. The cell suspension was filtered through a nylon mesh (250 µ) and

the cell suspension was centrifuged at 1000 rpm for 15 min. The supernatant was aspirated off and the loosely packed cell pellet was resuspended in calcium free HEPES buffer. This washing procedure was repeated three times. Cell viability was determined by the trypan blue dye exclusion method (13). These isolated hepatocytes were cultured in Ham's F12 medium, supplemented with 10% newborn calf serum, antibiotics, 10⁻⁶M dexamethasone and 10⁻⁸ M bovine insulin. The cell suspension was incubated at 37°C for 30min in a humidified incubator with 5% CO₂.

In-vitro hepatoprotective activity of lecithin : Isolated hepatocytes after incubation for 24h were exposed to the fresh medium containing D-galactosamine (30 mM) along with/without lecithin (100 µg/ml) or the medium alone (as normal). After 60 min of D-galactosamine challenge, concentrations of aspartate amino transferase (ASAT), alanine amino transferase (ALAT), alkaline phosphatase (ALP), triglycerides, total proteins, albumin, total bilirubin and lactate dehydrogenase (LDH) in the medium were measured as an indication of hepatocytes necrosis using kits.

In vivo hepatoprotective effect of lecithin : Colony bred Wistar stain adult albino rats (150-200g) of either sex were used for the investigations. All the animals were maintained under standard husbandry conditions with food and water *ad libitum*. The experimental procedures were approved by the Institutional Animal Ethics Committee (IAEC), KMC, Manipal, India (No.IAEC/KMC/06/2006-2007). The animals were divided into six groups of six animals in each group. Liver damage was induced by intra peritoneal administration of 400 mg/kg.b.wt D-galactosamine. Group I received only the vehicle (sodium CMC 0.3%) and served as control. Group II served as toxicant D-galactosamine (400 mg/kg.b.wt) treated control. Group III received lecithin (50 mg/kg.b.wt). Group IV received lecithin (100 mg/kg.b.wt). Group V received standard silymarin (50 mg/kg.b.wt). Group VI received standard silymarin (100 mg/kg.b.wt). The animals received these treatments by the oral route for a period of 7 days. On the seventh day except group I, all other groups received 400 mg/kg body weight D-galactosamine intra-peritoneal. After 24 h of intoxication, on the 8th day, blood was withdrawn and collected in sterile centrifuge tubes and allowed to clot. Serum was separated and used for the estimation of ASAT, ALAT, ALP, triglycerides, total proteins, albumin, total bilirubin and LDH using kits.

Histopathology : Liver was removed, part of the liver sample is fixed overnight in 10% buffered formalin and paraffin-embedded. The sections were stained with hematoxylin and eosin (H&E) for histological evaluation and examined under

light microscope. In brief, 4 μ m thick sections of paraffin-embedded rat liver were dewaxed in xylene, rehydrated in graded alcohol series, and washed with distilled water for 2 min. Subsequently, the sections were stained with hematoxylin for 5 min at room temperature. After 15 min, the sections were counter-stained with eosin for 2 min, dehydrated in graded alcohol series, washed with xylene, and blocked by rosin. H&E stained slides were observed under microscope at 40 $\times$ magnifications (Fig 1, 2, 3 & 4).

DNA extraction and electrophoresis : DNA was extracted from excised liver of toxicant treated rat group, normal control, lecithin and standard silymarin treated groups. The liver tissues were lysed by exposing them at -70°C and subsequent trituration with liquid nitrogen before extraction. The tissues were lysed with digestion buffer (pH 7.5) containing 0.5% SDS, 25 mM tris-HCl, 0.5% mg/ml proteinase K and 5 mM EDTA at 55°C overnight. After extracting the cell lysates with

Fig 1: Normal liver having normal histological structures of hepatic lobules

Fig 2: Toxicant treated liver showing damage to hepatocytes with hepatocellular vacuolization, focal hepatic necrosis and congestion of hepatic sinusoids

Fig 3: Lecithin drug treated group showing mild vacuolization

Fig 4: Silymarin drug treated group showing apparently normal hepatocytes

phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1), DNA was precipitated with 3M sodium acetate (pH 5.2) and absolute ethanol, and was washed, dried and resuspended with tris-EDTA buffer containing RNase A (100 $\mu\text{g/ml}$) at 37°C for 30 min. Approximately 15 μg DNA was electrophoresed on a 1% agarose gel and was visualized with ethidium bromide staining under UV light (Fig 5).

Statistical analysis : The statistical analysis was carried out by one way analysis of variance (ANOVA). The values are represented as mean $\pm$ SEM. Comparison of mean values of different groups treated with different dose levels of the drug and positive control with normal were estimated by Turkey's multiple comparison test. $P < 0.05$ was considered as significant.

Fig 5: DNA Fragmentation lane 1 marker DNA ladder, lane 2, 3 & 4 showing single intact band of normal group. Lane 5 toxicant treated group revealed ladder of DNA fragmentation in agarose gel electrophoresis. Lane 6 and 7 showing lecithin and silymarin treated samples were able to protect the toxic effect of D-galactosamine and were able to prevent the DNA damage. The figure is the representative of three separate independent experiments

RESULTS

Hepatoprotective effect of lecithin in freshly isolated rat hepatocytes : The effects of lecithin on freshly isolated rat hepatocytes intoxicated with D-galactosamine are recorded Table 1. A significant increase in the levels of ASAT, ALAT,

ALP, total bilirubin and a significant reduction in levels of triglycerides, total proteins, albumin and LDH were observed in hepatocytes exposed to D-galactosamine (30 mM) in comparison to untreated cells. Lecithin (100 µg/ml) treatment resulted in significant dose depended restoration of the biochemical parameters towards the normal. Similar results were observed with the treatment of standard silymarin. The restoration of biochemical parameters by lecithin was comparable to that of standard silymarin used.

In-vivo hepatoprotective activity : The *in vitro* hepatoprotective activity of lecithin against D-GalN induced toxicity was potent and comparable with that of standard silymarin used at the same test dose levels. Hence in *in vivo* hepatoprotective activity using D-GalN intoxicated rat model, two similar dose levels of lecithin and standard silymarin (50 and 100 mg/kg.b.wt.) were studied.

Intoxication of rats with D-galactosamine (400 mg/kg) significantly altered the biochemical parameters when compared with the normal control rats ($P < 0.001$, Table 2). A significant increase in the levels of ASAT, ALAT, ALP, total bilirubin and LDH and significant decrease in triglycerides, total proteins, and albumin levels were observed in toxicant group. Treatment with lecithin at both 50 and 100 mg/kg.b.wt. dose levels significantly decrease the level of ASAT, ALAT, ALP, total bilirubin and LDH levels towards normal. The triglyceride, albumin and total protein levels were restored towards normal values. All these significant changes in the levels of biochemical parameters refer to the effect of lecithin in protecting the liver by restoring the altered levels in rats.

Histopathology : Normal histological structures of hepatic lobules were observed in normal liver (Fig 1). Group treated with D-galactosamine showed complete damage to hepatocytes with hepatocellular vacuolization, focal hepatic

Table 1: Effect of lecithin treatment on the biochemical parameters of D-galactosamine intoxicated freshly isolated rat hepatocytes

Treatment groups	ASAT U/L	ALAT U/L	ALP U/L	Triglyceride mg/dl	Total Protein g/dl	Albumin g/L	Total Bilirubin mg/dl	LDH U/L
Control	12.67 ± 0.61	13.83 ± 0.47	27.50 ± 0.50	159.8 ± 1.13	3.048 ± 0.019	0.692 ± 0.003	0.922 ± 0.002	112 ± 2.36
D-galactosamine (30 mM)	78.83 ± 0.79 ^a	95.83 ± 0.60 ^a	96.17 ± 0.87 ^a	72.5 ± 0.67 ^a	1.615 ± 0.006	0.308 ± 0.002	1.965 ± 0.005	289.2 ± 2.36 ^a
lecithin (100 µg/ml)	15.33 ± 0.55 ^b	26.83 ± 1.07 ^b	39 ± 0.25 ^b	155.2 ± 1.49 ^b	2.758 ± 0.0091	0.5173 ± 0.0023	0.9957 ± 0.0023	136.3 ± 1.11 ^b
Standard Silymarin (100 µg/ml)	15.50 ± 0.76 ^b	24.33 ± 0.55 ^b	33.67 ± 0.42 ^b	164.2 ± 1.70 ^b	2.937 ± 0.019	0.5757 ± 0.0017	0.9440 ± 0.0050	129.3 ± 0.33 ^b

Number of independent experiments = 3, 5 replicates, mean ± SEM a = $P < 0.001$, when compared to normal group, b = $P < 0.001$, when compared to D-galactosamine treated cells

Table 2: Effect of treatment with lecithin on the biochemical parameters of D-galactosamine intoxicated rats

Treatment group	Concentration	ASAT U/L	ALAT U/L	ALP U/L	Triglyceride mg/dl	Total Protein g/dl	Albumin g/L	Total Bilirubin mg/dl	LDH U/L
Control	-	66.60 ± 2.21	27.51 ± 1.15	325.3 ± 4.87	72.47 ± 1.09	6.80 ± 0.31	4.56 ± 0.25	0.41 ± 0.02	333.04 ± 6.40
D-galactosamine	400 mg/kg	103.2 ± 2.53 ^a	61.43 ± 0.56 ^a	503.1 ± 1.88 ^a	27.52 ± 0.23 ^a	4.08 ± 0.03	2.07 ± 0.01	0.99 ± 0.01	655.8 ± 3.84 ^a
lecithin	50 mg/kg	85.83 ± 1.25 ^{a,y}	43.87 ± 1.03 ^{b,y}	391.7 ± 1.41 ^{a,x}	54.99 ± 1.16 ^{b,x}	5.14 ± 0.04	3.08 ± 0.02	0.49 ± 0.01	393 ± 2.1 ^{a,x}
	100 mg/kg	78.79 ± 1.87 ^x	36.23 ± 1.51 ^x	350.7 ± 7.92 ^{a,x}	66.05 ± 1.03 ^x	5.71 ± 0.12	3.72 ± 0.14	0.44 ± 0.01	378.8 ± 9.22 ^{a,x}
Standard silymarin	50 mg/kg	81.11 ± 1.08 ^x	42.51 ± 0.75 ^{c,x}	384.5 ± 1.79 ^{a,x}	55.65 ± 0.60 ^{b,x}	5.18 ± 0.02	3.15 ± 0.03	0.49 ± 0.01	387.4 ± 3.82 ^{a,x}
	100 mg/kg	74.89 ± 1.20 ^x	33.17 ± 0.45 ^x	344.8 ± 1.42 ^{a,x}	68.82 ± 0.49 ^x	6.03 ± 0.05	3.99 ± 0.03	0.42 ± 0.01	373.7 ± 3.29 ^{a,x}

Values are ± SEM of six animals. Superscripts a, b and c denote statistical significance in comparison to normal group $P < 0.001$, $P < 0.01$ and $P < 0.05$ respectively. Superscripts x and y denote statistical significance in comparison to D-Galactosamine (D-GalN) group at $P < 0.001$ and $P < 0.01$ respectively.

necrosis and congestion of hepatic sinusoids (Fig 2). Lecithin and silymarin treated group showed good improvement in hepatocytes as compared to that of D-galactosamine treated animals (Fig 3 and 4).

There was slight congestion and mild vacuolization observed in lecithin and silymarin treated groups.

DNA fragmentation studies : The results of DNA fragmentation studies were given in figure 5. Single intact band was observed in case of normal liver and D-galactosamine treated group revealed ladder of DNA fragmentation in agarose gel electrophoresis. Lecithin and silymarin treated samples was able to protect the toxic effect of D-galactosamine and was able to prevent the DNA damage.

DISCUSSION

Liver injuries induced by D-GalN are the best characterized system of xenobiotic-induced hepatotoxicity and commonly used models for the screening of anti-hepatotoxic and/or hepatoprotective activities of drugs. The changes associated with D-GalN induced liver damage are similar to that of acute viral hepatitis (14-15). Hence, D-GalN mediated hepatotoxicity was chosen as the experimental model. D-GalN acts directly or indirectly and alters antioxidant status that makes certain organs, more susceptible to oxidative stress. Several studies have shown that D-GalN causes alteration of liver marker enzymes (16). D-GalN is known to selectively block the transcription and indirectly hepatic protein synthesis and as a consequence of endotoxin toxicity, it causes fulminant hepatitis (15-16). The toxicity of D-GalN results from inhibition of RNA and protein synthesis in the liver. The metabolism of D-GalN may deplete several uracil nucleotides including UDP-glucose, UDP-galactose and UTP, which trapped in the formation of uridine- diphosphogalactosamine. Accumulation of UDP-sugar

nucleotide may contribute to the change in the rough endoplasmic reticulum and to the disturbance of protein metabolism (14-15). Intense galactosamine of the membrane structures is thought to be responsible for loss in the activity of ionic pumps. The impairment in the calcium pumps, with consequent increase in the intracellular calcium is considered to be responsible for cell death (15-16). D-GalN intoxication is known to cause marked elevation in liver enzyme levels (14). Silymarin is used as standard hepatoprotective compound since it is reported to have a protective effect on the plasma membrane of hepatocytes (17).

To our knowledge, this is the first study which reveals the hepatoprotective effect of lecithin against D-GalN induced toxicity in isolated rat hepatocytes and in animals. D-GalN has been found to induce extensive liver damage within a period of 24 h following intra-peritoneal administration. As a result of this, accumulation of fat in the liver and necrosis in the centrilobular region of the liver occurs. As a consequence, the microsomal enzyme activities are found to decrease and due to lipid peroxidation, the water-soluble enzymes leak into plasma from the liver. It is shown by the significant decrease in triglycerides and proteins in D-GalN intoxicated rat hepatocytes or animals in the present studies. Treatment with lecithin exhibited significant restoration of the altered biochemical parameters towards normal in D-GalN intoxicated rat hepatocytes and in rats. The protective activity of lecithin was comparable with that of standard silymarin use in both the test concentration. The result of histopathology and DNA fragmentation studies also supported the in vivo animal study.

CONCLUSION

The hepatoprotective effect observed in the present study may be mainly due to the presence of Phosphatidylcholine, which occurs in all cellular organisms being one of the components

of the phospholipid portion of the cell membrane. The results from the present study indicate a good correlation between the *in vivo* and *in vitro* studies. In conclusion, lecithin merits further investigation in identifying the mechanism of action for this activity.

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