Ind J Clin Biochem (Oct-Dec 2010) 25(4):349–356
DOI 10.1007/s12291-010-0073-3

ORIGINAL ARTICLE

Protective Role of Catechin on D-Galactosamine Induced Hepatotoxicity Through a p53 Dependent Pathway

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Received: 16 February 2010 / Accepted: 7 June 2010 / Published online: 14 September 2010

Abstract Objective of this study was to obtain a better understanding of the mechanism responsible for the D-galactosamine (D-GalN) induced hepatotoxicity and to study the effect of catechin against D-GalN induced hepatotoxicity. Catechin 50 and 100 mg/kg b.wt was administered for 1 week by oral route. Liver damage was induced by intra-peritoneal administration of 400 mg/kg b.wt D-galactosamine on the last day of catechin treatment. At the end of treatment all animals were killed and liver enzyme levels were estimated. Dissected hepatic samples were used for histopathology, RNA isolation, expression studies of Bax, Bcl-2 and p53 mRNA levels and mitochondrial membrane potential studies. We found that increases in the liver enzyme activity and decrease in antioxidant enzyme activity by D-GalN were significantly restricted by oral pretreatment with catechin. Disruption of mitochondrial membrane potential, up regulation of p53, Bax and down regulation of Bcl-2 mRNA levels in the liver of D-GalN intoxicated rats were effectively prevented by pretreatment with catechin.

Keywords Mitochondria · Catechin · D-Galactosamine · p53 · Bax · Bcl-2 · Hepatoprotective

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Introduction

Herbal medicines derived from plant extracts are being used to treat a wide variety of clinical disease [1]. More attention has been paid to the protective effects of natural antioxidants against drug-induced toxicity studies especially whenever free radical generation is involved [2]. Flavonoids have been found to play a very important role in protection against oxidative stress [3, 4]. Flavonoids are group of polyphenolic compounds that occur widely in fruit, vegetables, tea, cocoas and red wine [5, 6]. Fresh tea leaves are rich in flavanol monomers known as catechins [7]. Studies have been carried out exploring different biological activities of catechins.

Scavenging and protective activities of green tea polyphenols [8], preventive effect of green tea catechins on experimental tumor metastasis [9], protective effect of green tea on ethanol-induced lipid peroxidation [10] and tamoxifen-induced liver injury [11] have been reported. Catechins have beneficial effects in prevention of cardiovascular diseases including LDL oxidative susceptibility [12]. There have been reports supporting the hepatoprotective effects of green tea against ethanol intoxication [13–15]. Effect of green tea and epigallo catechin gallate on ethanol-induced toxicity in HepG2 Cells was also reported [16]. However, the effect of catechin on D-galactosamine induced toxicity and the molecular mechanism involved are not established. Further the effect of catechin on the expression pattern of important genes involved in liver is not clear. Therefore the aim of this study was to investigate catechin for its protective effects against D-galactosamine induced hepatotoxicity, using expression of Bax, Bcl-2 and P53 as markers genes.

Materials and Methods

Materials

All routine chemicals were obtained from SD-fine chemicals, Mumbai, India. Mitochondrial isolation and staining kit, Primers for Bax, Bcl-2, and GAPDH, standard enzymes SOD, CAT, D-galactosamine and catechin pure sample were purchased from Sigma–Aldrich Co., St. Louis, MO, USA. Primers for p53 was synthesized and purified by Bioserve, India. Cobas kits for AST, ALT, ALP and LDH were purchased from Roche diagnostics, USA. Trizol Reagent, dNTPs and 100 bp DNA ladder was purchased from Invitrogen Life Technologies Co, USA. RT PCR enzymes, RT PCR buffers, oligo dT primers and *Thermus thermophilus* DNA polymerase enzyme was procured from Merck, Germany.

Animals

Colony bred Wister strain adult albino rats (150–200 g) 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).

Catechin Administration and D-Galactosamine Challenge

Twenty-four Wistar strain albino rats (180–200 g) of either sex were used. The animals were divided into four groups of six animals in each group. Liver damage was induced by intra-peritoneal administration of 400 mg/kg b.wt D-galactosamine (D-GalN). Group I received only the vehicle (sodium CMC 0.3%) and served as normal control. Group II served as toxicant D-GalN (400 mg/kg b.wt) treated toxicant control. Group III received catechin (50 mg/kg b.wt). Group IV received catechin (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-GalN intra-peritoneal. In the entire treatment period, the rats were weighed once daily, and the consumption of food and water was monitored every day.

Tissue Homogenate Preparation and Serum Collection

After 24 h of intoxication, on the eighth day all animals were killed and serum was collected by centrifugation of blood at 3,500×*g* (Eppendorf 5415R, Germany) for 10 min at 4°C and stored at −80°C (Sanyo, Japan). Rinsed with ice cold isotonic saline, dissected hepatic samples were quickly dried by blotting between two pieces of filter paper; one lobe is immersed into liquid nitrogen for over 10 min and then stored at −80°C used for Reverse transcriptase PCR. Other fresh liver sample is processed for histopathology and mitochondrial isolation. An accurately weighed piece of hepatic sample was homogenized with ice cold 0.1 M potassium phosphate buffer (pH 7.4) containing 5 mM EDTA to yield a 10% (w/v) tissue homogenate. The homogenate was centrifuged at 3,500×*g* for 10 min at 4°C to remove cell debris and nuclei and the resulting supernatant was employed for various biochemical assays.

Determination of Serum AST, ALT, ALP and LDH Level

Serum AST, ALT, ALP and LDH levels were measured with enzymatic kinetic method by automatic biochemical analyzer (Cobas, USA) using kits.

Determination of Hepatic Superoxide Dismutase (SOD) Activity

SOD activity was estimated according to available standard protocol [17] with minor modification. Briefly, the reaction was initiated by mixing appropriate water dilution of homogenate supernatant with 0.5 mM hypoxanthine, 0.5 mM hydroxylamine, and 0.01 U xanthine oxidase in the buffer consisting of 104 mM potassium phosphate, 78 mM sodium borate and 0.025 mM EDTA (pH 7.0) at 37°C for 30 min with 1 ml of reaction system. The reaction was terminated by adding 2.0 ml of 16% (v/v) acetic acid solution containing 2.6 mM sulfanilic acid and 38.6 μM naphthyl ethylene diamine, and the absorbance at 550 nm was recorded to calculate the SOD activity. Under these conditions, one nitroso unit (Nu) of enzyme activity was expressed as the inhibiting 50% of the oxidation of hydroxylamine without sample as enzyme source.

Determination of Hepatic Catalase (CAT) Activity

CAT activity was determined by mixing 10 μl samples with 1 ml substrate (0.1 mmol H_2O_2 in phosphate buffer) for 60 s followed by adding 1 ml of 32.4 mM ammonium molybdate to terminate the reaction. The absorbance against blank at 405 nm was measured and CAT activity was calculated by standard curve. One unit of the enzyme was defined as mmol H_2O_2 disproportionated per min per mg tissue [18].

Histopathology

The fresh liver samples were processed according to the classical procedure using hematoxylin–eosin staining [19].

Briefly, liver tissues were cut into about 3-mm-thick slices and fixed with 10% phosphate-buffered formalin (pH 7.4). The tissue slices were dehydrated and embedded in paraffin. Tissue sections of 5–8 μm were stained by hematoxylin and eosin, and were observed with Olympus phase contrast microscope with Motic imaging system, China.

Isolation of Total RNA and Reverse Transcription Polymerase Chain Reaction (RT PCR)

Total RNA from hepatic tissues was extracted using trizol reagent following the manufacturer's instructions and quantified by DNA protein enzyme analyser (Shimadzu, Japan). cDNA synthesis and amplification was performed by PCR apparatus (Eppendorf, Germany) in a volume of 50 μl comprising of 2 μl total RNA, 2 μl oligo(dT), 25 μl of RT PCR master mix, 3 μl of 50 mMMn$(0Ac)_2$, 2 μl of respective forward and reverse primers and 16 μl of RNAse free water. Polymerase activation was done at 90°C for 30 s and reverse transcription was performed at 60°C for 30 min. *Thermus thermophilus* DNA polymerase enzyme was used for cDNA synthesis step and PCR amplification step. The sequences of the PCR primers for Bax (NM_017059) were 5′-CCA AGA AGC TGA GCG AGT GTC TC-3′ (forward) and 5′-AGT TGC CAT CAG CAA ACA TGT CA-3′ (reverse), Bcl-2 (NM_016993) were 5′-GGA GCG TCA ACA GGG AGA TG-3′ (forward) and 5′-GAT GCC GGT TCA GGT ACT CAG-3′ (reverse), p53 (NM_030989) were5′-CAG CTT TGA GGT TCG TGT TTG T-3′ (forward) and 5′- ATG CTC TTC TTT TTT GCG GAA A-3′ (reverse) and the sequence for GAPDH (NM_017008) were 5′-CCA AGA AGC TGA GCG AGT GTC TC-3′ (forward) and 5′-CCT GCT TCA CCA CCT TCT TG-3′ (reverse). The cycle condition of PCR amplification process consisted of 40 cycles, including denaturation at 94°C for 1 min, annealing at 60°C for GAPDH, 51°C for Bax and Bcl-2 and 46°C for p53 for 30 s, and extension at 72°C for 1 min with 1 cycle of final extension at 60°C for 7 min. The predicted sizes of the amplified products of Bax, Bcl-2, p53 and GAPDH were 487, 127, 82 and 349 bp, respectively. Equal amounts of corresponding products of Bax, Bcl-2, p53 and GAPDH were separated by 1.5% agarose gel electrophoresis (Bangalore Genei, India) and optical densities of ethidium bromide-stained DNA bands were quantified by Alpha Innotech software, USA.

Mitochondrial Isolation

Mitochondria were isolated from rat liver as previously described [20]. Briefly, the tissue was manually homogenized by four strokes with a Teflon pestle in solution I containing 230 mM mannitol, 70 mM sucrose, 1 mM ethylene glycol tetra acetic acid (EGTA) and 5 mM HEPES (pH 7.4) on ice. After centrifugation ($100\times g$ for 80 s at 4°C), the supernatant was layered in solution II [460 mM mannitol, 14 mM sucrose, 1 mM EGTA and 10 mM HEPES (pH 7.4)] and centrifuged at $2{,}000\times g$ for 5 min at 4°C. The mitochondrial pellet was resuspended in 215 mM mannitol, 71 mM sucrose, 10 mM succinate and 10 mM HEPES (pH 7.4), and kept on ice until mitochondrial staining procedure.

Isolated Mitochondrial Staining

Isolated mitochondrial preparation was stained with help of JC-1 (5,5,6,6′-tetrachloro-1,1′-3,3′-tetra ethyl benzimidazolocarbocyanine iodide) dye. The concentration of mitochondrial preparation was diluted to 40 μg/ml and used for staining. Final concentration of JC-1 staining solution was 0.2 μg/ml. 90 μl of JC-1 staining solution was added to 10 μl of isolated mitochondrial sample and excitation wave length of 490 nm and emission wave length of 590 nm was used to visualize the samples with help of Olympus inverted microscope with fluorescence attachment (Olympus, USA).

Statistical Analysis

The statistical analysis was carried out by one way analysis of variance (ANOVA). The values are represented as mean ± S.E.M. Probability value of $P < 0.05$ was determined to be statistically significant.

Results

Determination of Serum AST, ALT, ALP and LDH Level

All rats survived the experimental period until sacrifice. Intoxication of rats with D-GalN 400 mg/kg b.wt significantly altered the biochemical parameters when compared with the normal control rats ($P < 0.001$, Table 1). A significant increase in the levels of AST, ALT, ALP and LDH levels were observed in toxicant group. Treatment with catechin at 50 and 100 mg/kg b.wt significantly decreased the level of AST, ALT, ALP and LDH levels towards normal. All these significant changes in the levels of biochemical parameters refer to the effect of catechin in protecting the liver by restoring the altered levels in rats.

Determination of Hepatic SOD and CAT Level

A significant decrease in the levels of antioxidant enzymes SOD and CAT was observed in animals which received a dose of D-GalN 400 mg/kg b.wt when compared with that

Table 1 Effect of catechin on the biochemical parameters of D-galactosamine intoxicated rats

Treatment group	Concentration (mg/kg)	ASAT U/L	ALAT U/L	ALP U/L	LDH U/L
Normal	–	66.60 ± 2.207	27.51 ± 1.15	325.30 ± 4.874	333.04 ± 6.399
D-Galactosamine (D-GalN)	400	149.30 ± 1.623[a]	68.76 ± 0.848[a]	538 ± 1.37[a]	657.5 ± 1.346[a]
Catechin	50	88.11 ± 0.985[b]	44.40 ± 0.25[b]	379.8 ± 0.653[b]	390.7 ± 1.017[b]
	100	81.52 ± 1.371[b]	41.73 ± 0.746[b]	360.3 ± 1.971[b]	381.6 ± 1.338[b]

[a] $P < 0.001$, when compared to normal group

[b] $P < 0.001$ when compared to D-galactosamine treated group

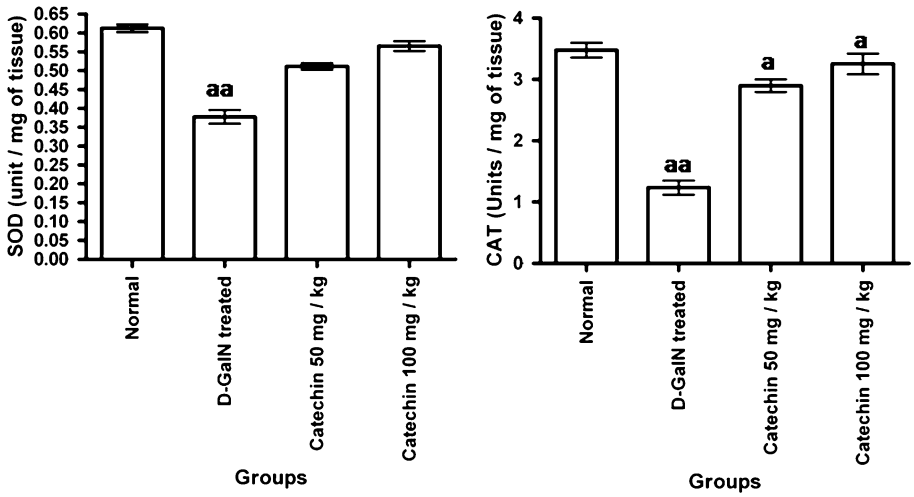

Fig. 1 Determination of hepatic SOD and CAT level: A significant decrease in the levels of antioxidant enzymes SOD and CAT was observed in animals which received a dose of D-GalN 400 mg/kg b.wt when compared with that of the normal untreated group. Groups which received catechin at a dose of 50 and 100 mg/kg b.wt, showed significant increase in the levels of both the enzymes. [aa] Significant decrease over untreated group, $P < 0.001$. [a] Significant increase over toxicant group, $P < 0.001$

of the normal untreated group. Groups which received catechin at a dose of 50 and 100 mg/kg b.wt, showed significant increase in the levels of both the enzymes ($P < 0.001$, Fig. 1) even after D-GalN challenge. These significant changes in the levels of antioxidant parameters refer to the effect of catechin in protecting the liver by restoring the altered levels in rats.

Histopathology

Normal histological structures of hepatic lobules were observed in normal liver (Fig. 2). Group treated with D-GalN showed complete damage to hepatocytes with hepatocellular vacuolization, focal hepatic necrosis and congestion of hepatic sinusoids (Fig. 3). Catechin at a dose of 50 and 100 mg/kg b.wt showed good improvement in hepatocytes as compared to that of D-GalN treated animals (Figs. 4, 5). There was slight congestion and mild vacuolization observed in catechin treated groups.

Reverse Transcriptase PCR Analysis

Reverse transcriptase PCR was used to analyze the levels of Bax, Bcl-2 and p53 mRNA levels in livers of rats treated with D-GalN and their control group in comparison with catechin (50 and 100 mg/kg b.wt) treated groups. Amplification of Bax, Bcl-2 and p53 was done with help of specific primers using cDNA as template strand prepared from livers, and the specificity was confirmed using agarose gel electrophoresis.

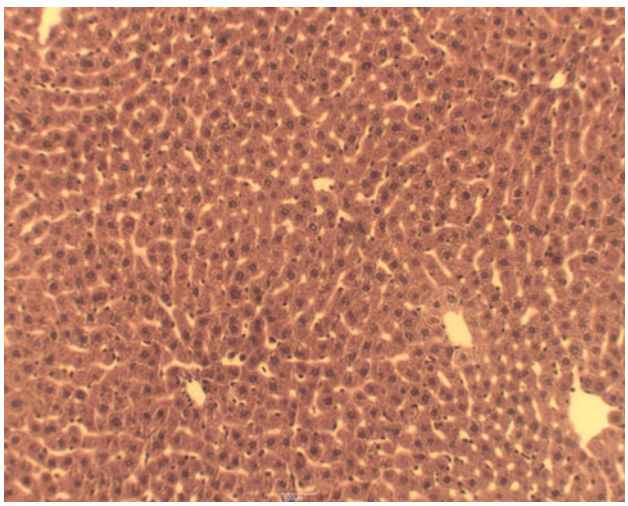

Fig. 2 Normal liver having histological structures of normal hepatic lobules

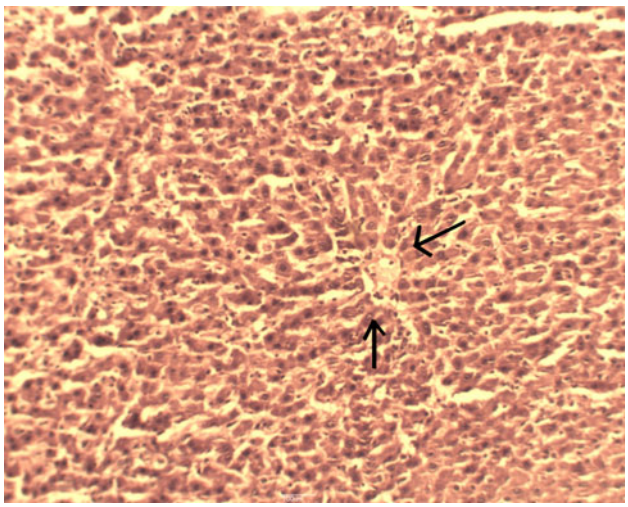

Fig. 3 D-GalN treated liver, arrows showing damage to hepatocytes with hepatocellular vacuolization, focal hepatic necrosis and congestion of hepatic sinusoids

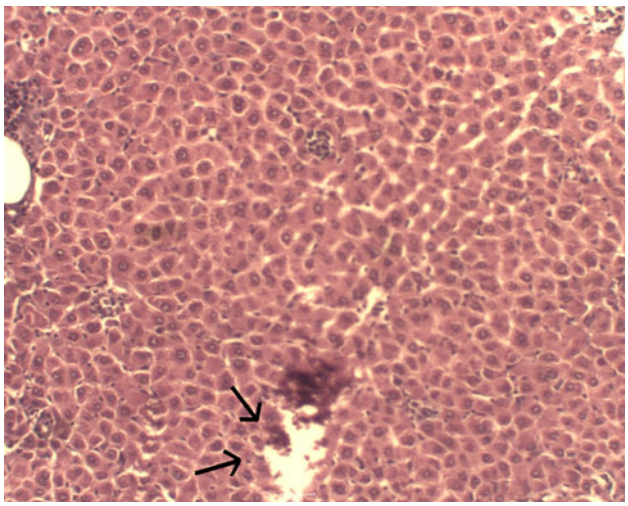

Fig. 4 Catechin (50 mg/kg b.wt) treated group. Arrows showing mild vacuolization

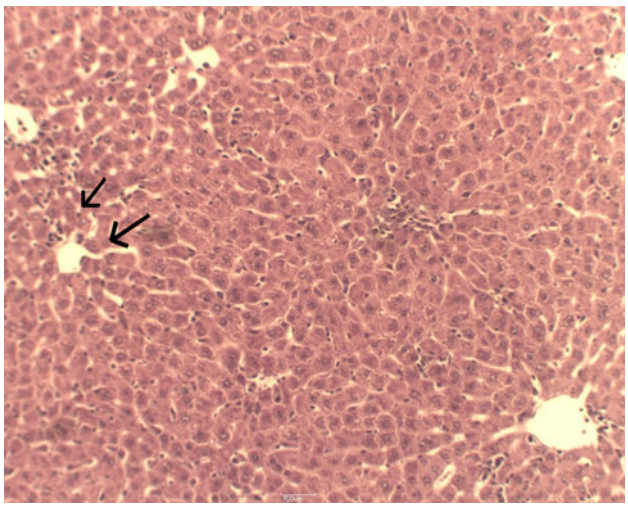

Fig. 5 Catechin (100 mg/kg b.wt) treated group showing apparently normal hepatocytes. Arrows indicate mild vacuolization

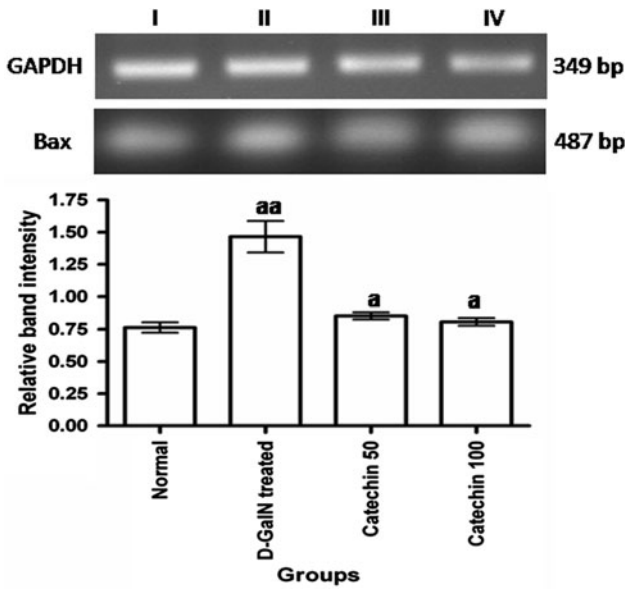

Fig. 6 The mRNA expression of Bax by RT PCR. Gr I: untreated, Gr II: D-GalN treated, Gr III: Catechin 50 mg/kg b.wt + D-GalN treated, Gr IV: Catechin 100 mg/kg b.wt + D-GalN treated. The data is represented as the mean of ± S.E.M. of five independent experiments. [aa] Significant increase over untreated Gr I, $P < 0.001$. [a] Significant decrease over Gr II, $P < 0.001$

Bax mRNA Expression

The mRNA levels of Bax in livers are shown in Fig. 6. The levels of Bax in livers of D-GalN treated rats increased significantly compared with control group. The levels of Bax were maintained almost normal in catechin treated groups administered prior to D-GalN challenge. The values were found to be statistically significant ($P < 0.001$) when compared with the toxicant.

Bcl-2 mRNA Expression

Figure 7 presents Bcl-2 mRNA levels in livers of rats exposed to D-GalN with and without catechin treatment in comparison with control group. D-GalN at a dose of 400 mg/kg b.wt caused significant decrease of Bcl-2 mRNA levels when compared with that of the normal group. Whereas no alteration in the expression of Bcl-2 levels was observed in livers from rats treated with catechin (50 and 100 mg/kg b.wt).

p53 mRNA Expression

D-GalN caused a significant increase in p53 levels in the livers when compared with the normal control livers. The

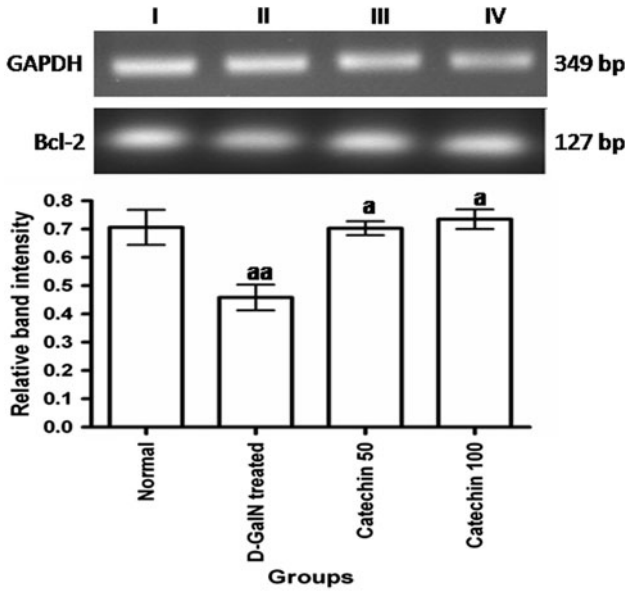

Fig. 7 The mRNA expression of Bcl-2 by RT PCR. Gr I: untreated, Gr II: D-GalN treated, Gr III: Catechin 50 mg/kg b.wt + D-GalN treated, Gr IV: Catechin 100 mg/kg b.wt + D-GalN treated. The data is represented as the mean of ± S.E.M. of five independent experiments. [aa] Significant decrease over untreated Gr I, $P < 0.001$. [a] Significant increase over Gr II, $P < 0.001$

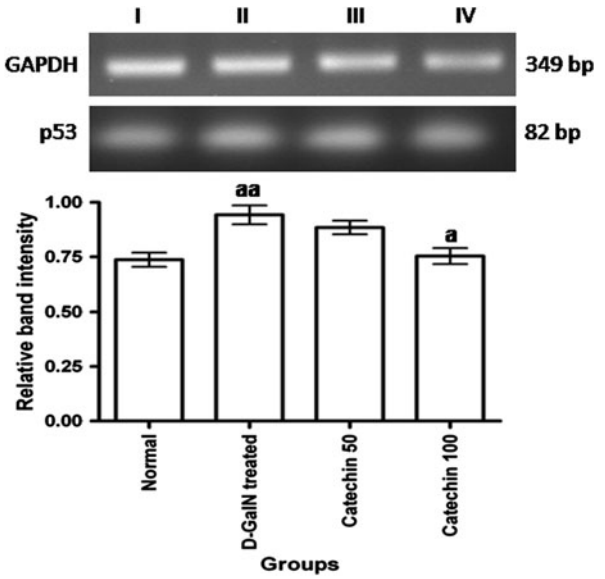

Fig. 8 The mRNA expression of p53 by RT PCR. Gr I: untreated, Gr II: D-GalN treated, Gr III: Catechin 50 mg/kg b.wt + D-GalN treated, Gr IV: Catechin 100 mg/kg b.wt + D-GalN treated. The data is represented as the mean of ± S.E.M. of five independent experiments. [aa] Significant increase over untreated Gr I, $P < 0.001$. [a] Significant decrease over Gr II, $P < 0.001$

treatment with catechin (100 mg/kg b.wt) resulted in significant decrease, whereas catechin (50 mg/kg b.wt) showed marginal decrease in the level of p53 as compared with that of the D-GalN treated group (Fig. 8).

Isolated Mitochondrial Staining to Study Inner Membrane Potential

Mitochondrial fraction was prepared from the livers of rats from normal group, D-GalN treated groups and catechin treated group (Fig. 9). Mitochondrial inner membrane potential was studied from the uptake of the cationic carbocyanine dye JC-1 into the mitochondrial matrix. In normal group, this dye concentrates in the matrix and bright red fluorescence was observed. In D-GalN treated group a shift from red to green fluorescence was observed which indicates damage to the inner membrane potential, hence prevents the accumulation of the JC-1 dye in the mitochondrial matrix. In catechin (50 and 100 mg/kg b.wt) treated groups showed red and mild green fluorescence which indicates mitochondrial inner membrane integrity was maintained.

Discussion

The main objective of this study was to obtain a better understanding of the mechanism responsible for the D-GalN induced hepatotoxicity and to study the effect of catechin against D-GalN induced hepatotoxicity. The changes associated with D-GalN induced liver damage are similar to that of acute viral hepatitis [21, 22]. Hence, D-GalN mediated hepatotoxicity was chosen as the experimental model. D-GalN dose of 400 mg/kg b.wt was finalized after acute toxicity studies and following standard protocol [23]. 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 hepatic enzymes [24]. 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 [24]. D-GalN has been found to induce extensive liver damage within a period of 24 h following intra-peritoneal administration. Hence we decided to study the levels of expression of important genes such as p53, Bax and Bcl-2 involved in regulation of apoptosis before and after D-GalN intoxication.

Apoptosis is programmed cell death characterized by cytoplasmic condensation and nuclear fragmentation [25]. Apoptosis signaling pathways fall under two categories: receptor-mediated apoptosis and mitochondrial-dependent apoptosis. Mitochondrial- dependent apoptosis occurs in response to certain liver insults and to drug administration [26]. Bcl-2, an onco protein, functions as a suppressor of apoptosis, and its down regulation causes tumor regression [27]. On the other hand, predominance of Bax, a pro apoptotic protein over Bcl-2 promotes apoptosis [28].

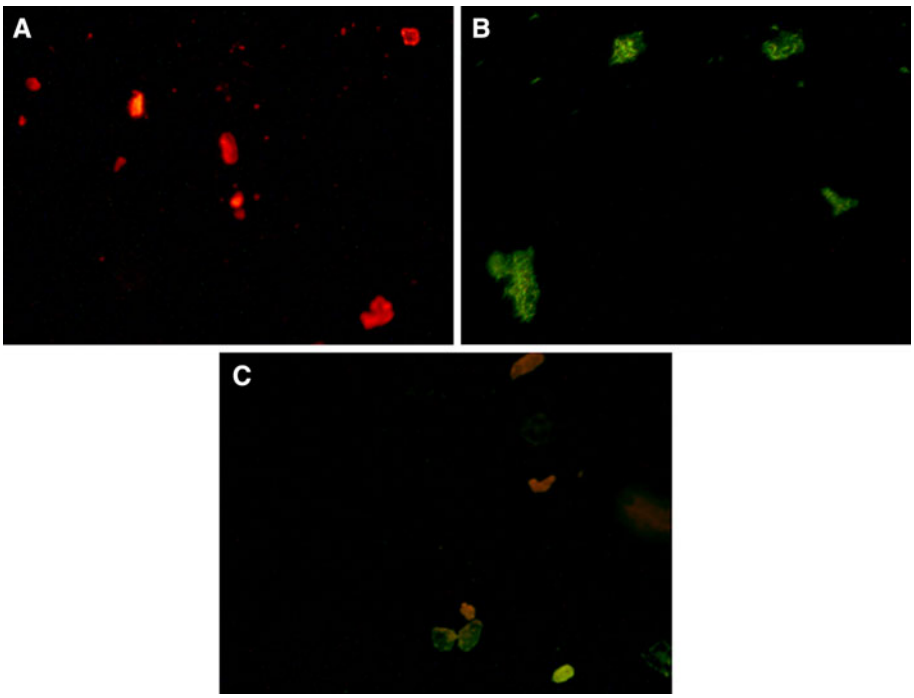

Fig. 9 Isolated mitochondrial staining. **a** Normal liver, the dye JC-1 concentrates in the matrix and bright red fluorescence was observed. **b** D-GalN treated liver. Shift from *red* to *green* fluorescence was observed up on incubation with JC-1 dye, which indicates damage to the inner membrane potential. **c** Catechin (100 mg/kg b.wt) + D-GalN treated liver showed *red* and *mild green* fluorescence up on incubation with JC-1 dye, which indicate mitochondrial inner membrane integrity was maintained

Studies have also shown that the ratio of Bax to Bcl-2 proteins increases during apoptosis [29]. The process is initiated by the translocation or activation of pro apoptotic genes to the mitochondrial membrane. In our study catechin prevented the up regulation of Bax after D-GalN treatment, thus prevented the liver cell death. Also Bcl-2 levels after catechin treatment were found to be similar to that of the normal group; hence the ratio of Bax and Bcl-2 was maintained normal, thus preventing apoptosis. Hence, the protective role of catechin could be related to its ability to maintain the normal levels of Bax and Bcl-2 even after D-GalN challenge.

Further, p53 plays a key role in the process of apoptosis. p53 is a nuclear phosphoprotein induced in response to cellular stress, functioning as a transcriptional transactivator in DNA repair, apoptosis and tumor suppression pathways [30]. p53 mediated apoptosis involves multiple mechanisms including modulation of the expression of Bcl-2, Bax and other BH3 only proteins, amplification of death signals and activation of caspases [31]. D-GalN intoxication up regulated the P53 levels whereas catechin prevented the up regulation of P53 levels after D-GalN treatment hence protected the cell death.

The results of the present study validate our conviction that D-GalN causes hepatic damage via up regulation of p53, Bax levels and down regulation of Bcl-2 levels and inducing mitochondrial damage. Further catechin at a dose of 50 and 100 mg/kg b.wt prevented the up regulation of Bax and maintained mitochondrial integrity hence, protected the liver from D-GalN induced toxicity in rats. Thus, these observations hold promise for further molecular target oriented studies.

Acknowledgment We thank All India Council of Technical Education (AICTE) for funding (200-62/FIN/04/05/1784/268).

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