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Original Article

Lutein prevents alcohol-induced liver disease in rats by modulating oxidative stress and inflammation

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Abstract: Objective: Oxidative stress and inflammation play an important role in pathogenesis of alcohol-induced liver injury. The present study was designed to investigate the protective role of Lutein against alcohol-induced liver injury. Treatment: Wistar rats weighing 150-200 g were divided into 3 groups, control, EtOH treatment, Lutein followed by EtOH treatment. Ethanol-treated rats received EtOH [5 g/kg body weight] by gavage every 12 hours for a total of 3 doses. For Lutein pre-treatment, Lutein at a dose of 40 mg/kg was dissolved in the EtOH and gavaged 30 mins before EtOH treatment. Methods: Oxidative stress markers-(reactive oxygen species, lipid peroxidation, protein carbonyls and sulfhydryls content), liver markers (ALT, AST, ALP and LDH) were determined. Antioxidant enzyme activities and its master regulator Nrf-2 expression were analyzed. Further, inflammatory proteins NF-κB, COX-2, iNOS and inflammatory cytokines (TNF-α, MCP-1, IL-1β, IL-6) were analyzed. Results: The results showed significant decrease in oxidative stress markers and liver markers in the lutein pre-treatment. Lutein treatment down regulated inflammatory proteins and cytokines with concomitant up regulation in Nrf-2 levels and antioxidant enzymic activities. Conclusion: The present study showed that Lutein treatment exerted potent antioxidant and anti-inflammatory property and offered significant cytoprotection against alcohol-induced liver injury.

Keywords: Alcohol, liver injury, lutein, oxidative stress, inflammation, cytokines

Introduction

Alcoholic liver disease (ALD) is one of the major causes of morbidity and mortality worldwide. ALD leads to spectrum of liver diseases, including steatosis, steatohepatitis, cirrhosis and hepatocellular carcinoma. Alcohol once entered into the system is metabolized in the liver, which is the target organ. Metabolism and removal of alcohol from the system results in production of toxic metabolites and which in turn activates redox signaling [1]. Redox imbalance is one of the well known mechanisms in alcohol induced liver injury [2]. Further, oxidative stress mediates inflammatory responses and the innate immune cells of hepatic cells, kupffer cells are one of the early mediators of inflammation in alcohol-induced liver injury; however, the progressive events are led by recruiting inflammatory cells like parenchymal cells, macrophages etc [3]. Major signaling cascades activated during alcohol induced liver injury (ALI) include, deregulation in calcium homeostasis, activation of MAPK's, and redox sensitive transcription factors and finally cell death mechanism by apoptosis [4-7]. Thus, effective amelioration of alcohol-induced liver disease can be achieved by targeting oxidative stress, since it is the central mediator in the AIL. The purpose of the present study was to analyze the protective role of lutein against alcohol-induced liver injury in Wister rats.

Lutein (C40H5602), also known as tetraterpenoids is rich in eggs, dark green leafy vegetables, such as kale and spinach [8, 9]. Lutein shows antioxidant property and involved in cytoprotection against a range of diseases mainly involving oxidative stress. In its protective role, lutein has been involved in inhibiting hydrogen peroxide-induced apoptosis leading in promotion of survival and differentiation of photoreceptors [10]. Lutein prevents early atherosclerosis by preventing MDA levels and inflammatory mechanisms [11]. Further, inhibition of NF-κB pathway and promotion of anti-inflammatory effect of lutein was observed in LPS-induced inflammation in macrophages

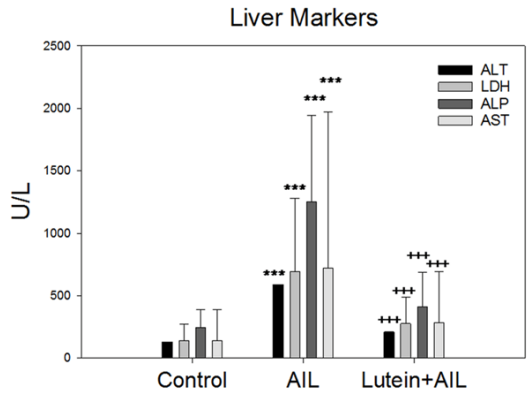

Figure 1. Lutein reduces serum liver marker enzymes. The results were expressed as U/mg of protein. Group I (control); Group II (AIL-rats); Group III (Lutein + AIL-rats). ***P<0.001, when compared to control group. +++P<0.001, when compared to AIL rats. Results are given as the mean ± SEM for 6 rats in each group (One way ANOVA followed by Tukey's multiple comparison).

[12]. Skin inflammation mediated through ultra violet-irradiation in keratinocytes was reduced by lutein, by down regulating COX-2, inflammatory cytokines and MMP levels [13]. However, the protective role of lutein against liver damage induced by paracetamol, carbon tetrachloride and ethanol have been studied earlier; however the results are limited to its antioxidant status [14]. The present study was designed to analyze the exact mechanism of lutein's cytoprotection and anti-inflammatory effect in alcohol induced liver injury. In the current study, we tested the ability of lutein against liver markers, oxidative stress markers, antioxidant activities, Nrf-2 level. Further, inflammatory proteins such as NF-κB, COX-2 and iNOS and pro-inflammatory cytokines (IL-6, IL-1β, TNF-α and MCP-I) were analyzed to determine its anti-inflammatory property.

Materials and methods

Animal and experimental treatment

The study was approved by the institutional animal care and use committee in China-Japan Friendship Hospital, China. Wistar rats weighing 150-200 g were divided into 3 groups, control, EtOH treatment, Lutein followed by EtOH treatment (6 rats/group). Because control and Lutein values were virtually identical, only control treatment is shown for ease of viewing. Ethanol-treated rats received EtOH [5 g/kg body weight] by gavage every 12 hours for a total of 3 doses. Control rats received an isocaloric maltose solution. For Lutein pre-treatment, Lutein at a dose of 40 mg/kg was dissolved in the EtOH and gavaged 30 min before EtOH treatment. After the treatment period, the rats were anesthetized and serum was collected for determining liver markers and interleukin levels. The tissue samples were homogenized and used for various biochemical and protein expression studies.

Measurement of serum enzymes

The serum samples were analyzed for alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) in a biochemical analyzer (7180, HITACHI, Japan).

Lipid peroxidation

The tissue levels of MDA were determined by estimating the amount of TBA reactants (Lipid peroxidation products) as described previously by Devasagayam [15]. The tissue samples were boiled for 1 hour in the presence of TBA reagent and the formed pink color chromogen was measured spectrophotometrically at 532 nm. The results were expressed as TBARS formed/mg of protein.

Reactive oxygen species

The tissue homogenate was incubated with 2', 7'-dichlorofluorescein diacetate DCF-DA at 37°C for 15 min. After 15 minutes, the reaction mixture was centrifuged for 15 minutes at 10,000 rpm. The supernatant was discarded and the resultant pellet was re-suspended in phosphate buffered saline and incubated for 60 min at 37°C. Followed by which the ROS levels were measured spectrofluorimetrically at excitation (485 nm) and emission (528 nm) wavelength. The results were expressed as percentage of ROS generation by comparing the levels to that of control (100%) Hashimoto et al. [16].

Protein carbonyls and sulphydryl content

Protein carbonyls: The Protein carbonyls formed were measured as described by Dalle-Donne et al., [17]. Briefly, the reaction between carbonyl groups in the sample and 2, 4-dinitrophenylhydrazine (DNPH) results in the formation of 2, 4-dinitrophenylhydrazone which is

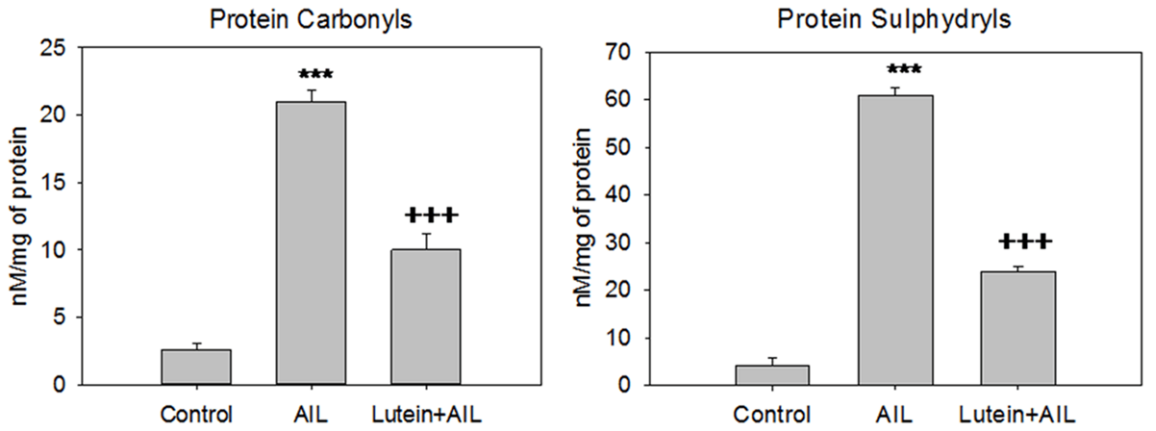

Figure 2. Lutein reduces oxidative stress in AIL-rats. Lipid peroxidation: The results are expressed as nano moles of TBARS formed/mg of protein. ROS generation: The results are expressed as ROS generated (%) when compared to control rats. ***P<0.001, when compared to control group. +++P<0.001, when compared to AIL rats. Group I (control); Group II (AIL-rats); Group III (Lutein + AIL-rats). Results are given as the mean ± SEM for 6 rats in each group (One way ANOVA followed by Tukey's multiple comparison).

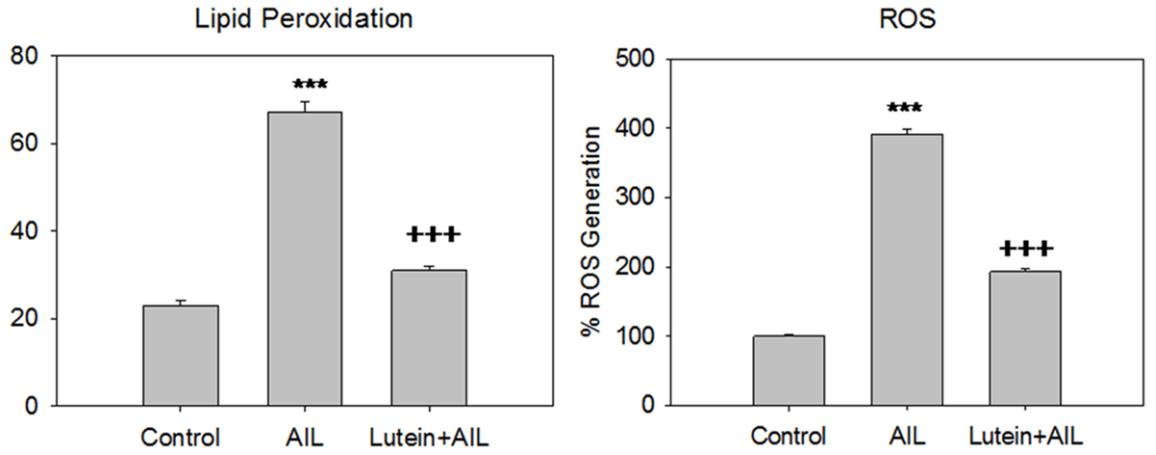

Figure 3. Lutein inhibits protein damage. Protein carbonylation: Results are expressed as nmol/mg of protein. Protein sulfhydryls: Results are expressed as nm/mg of protein. ***P< 0.001, when compared to control group. +++P<0.001, when compared to AIL rats. Group I (control); Group II (AIL-rats); Group III (Lutein + AIL- rats). Results are given as the mean ± SEM for 6 rats in each group (One way ANOVA followed by Tukey's multiple comparison).

quantified spectrophotometrically at 365 nm. The results were expressed as nmoles of protein carbonyls formed per milligram of protein.

Protein sulphydryls: The tissue protein sulphydryls were determined using Ellman's reagent (5, 5-dithiobis-2-nitrobenzoic acid) as described previously by Ellmann, [18]. The results were expressed as nmoles protein sulphydryls per mg protein.

Glutathione (GSH) content

The GSH content was determined as described by Beutler, [19], with some modification. The tissue sample was added with 0.3 M of Na2HP04·2H20 and 0.2 ml of dithiobisnitrobenzoic (0.4 mg/ml in 1% sodium citrate). After incubation, the absorbance was measured at 412 nm. The results were expressed as nmoles of GSH/mg of protein.

Antioxidant enzyme activities

SOD-Superoxide dismutase activity: The SOD activity was determined as described by Sun et al., [20]. The assay is based on reduction of nitroblue tetrazolium (NBT). 1 U of SOD activity = amount required for 50% inhibition of NBT

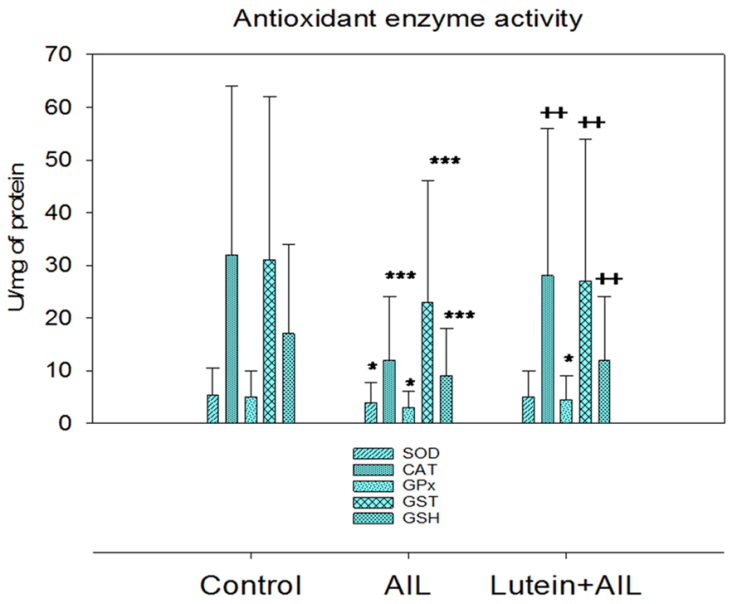

Figure 4. Lutein enhances antioxidant status. GSH-expressed as nmol of GSH/mg of protein. SOD, CAT, GST and GPX expressed in Units/mg protein. Results are given as the mean ± SEM for 6 rats in each group. $^{*}P<0.05$; $^{***}P<0.001$ when compared to control. $^{+}P<0.05$; $^{++}P<0.01$ when compared to AIL-rats. Group I (control); Group II (AIL-rats); Group III (Lutein + AIL-rats) (One way ANOVA followed by Tukey's multiple comparison).

reduction. The SOD activity is expressed as U/mg of protein. Catalase (CAT) activity: The activity was determined according to the method described by Aebi, [21]. The reaction mixture contained tissue homogenate and 30 mM H_2O_2 in a 50 mM phosphate buffer pH 7.0. The activity was estimated by decreased in absorbance of H_2O_2 at 240 nm. Glutathione-S-Transferase (GST) activity: The reaction between 1-chloro-2, 4-dinitro benzene (CDNB) and reduced glutathione results in formation of dinitrophenyl thioether which is measured at 340 nm (Habig et al., 1974) [22]. 1 U = Amount of enzyme producing 1 mmol of CDNB-GSH conjugate/min. Glutathione Peroxidase (GPx) activity: The GPx was performed as described by Pagia and Valentine, [23]. The oxidized glutathione (GSSG) is reduced by glutathione reductase and NADPH. The oxidation of NADPH to $NADP^+$ is measured by a decrease in absorbance at 340 nm. GPx activity is expressed as U/mg of protein.

Inflammatory cytokines by ELISA: IL-6, TNF- α, MCP-1, IL-1β levels

The serum interleukin levels were detected using ELISA kits (Sigma). The levels of interleukins were expressed as pg/ml. The absorbance was measured using an ELISA reader (MTP-800 Microplate reader; Corona Electric, Tokyo, Japan).

Immunoblot

Whole cell lysate was used for iNOS, COX-2 expression while nuclear extract was used for NF-κB p65 and Nrf-2 expression. The sample aliquots containing 50 µg of protein were separated on 8~12% SDS-polyacrylamide gels and transferred onto polyvinylidene fluoride (PVDF) membranes using glycine transfer buffer (192 mM glycine, 25 mM Tris-HCl (pH 8.8), 20% MeOH (v/v)). After proteins are transferred to membranes, the nonspecific sites were blocked with 5% non-fat dried milk for 1 hour at RT. The membrane was washed with TBST thrice and incubated with specific primary mouse monoclonal antibodies against iNOS, Nrf-2, COX-2, NF-κB p65 (1:1000) at 4°C overnight. Each membrane was further incubated for 30 min with secondary peroxidase-conjugated goat anti-mouse or -rabbit IgG (1:5000). The bands were visualized with an enhanced chemiluminescence (ECL) system according to the manufacturer's instructions. Densitometric analyses of the western blot bands were performed using optical density scanning and Image J software (GE Healthcare Life Sciences).

Statistical analysis

Data are expressed as mean ± standard deviation. All the experimental data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. All biochemical experiments were performed thrice in triplicates to ensure reproducibility.

Results

Lutein decreases serum liver markers

Rats treated with ethanol showed significant elevation in liver markers ALT, AST, LDH and

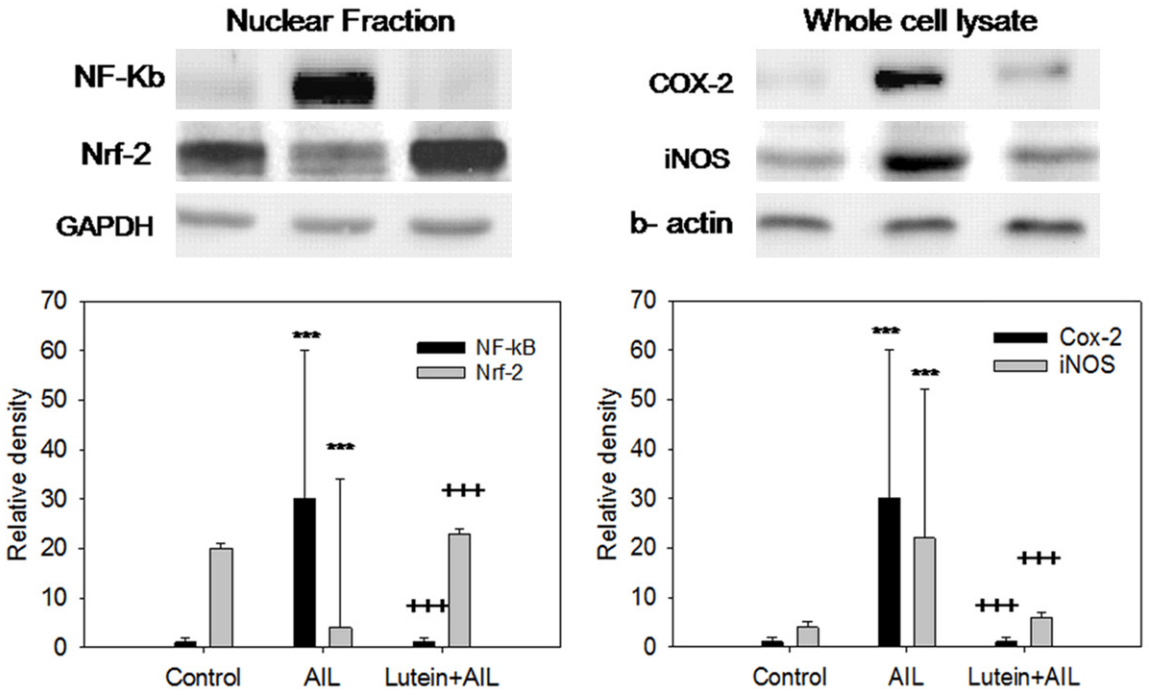

Figure 5. Lutein up regulates Nrf-2 and down regulates inflammatory proteins. Inflammatory proteins (NF-κB, COX-2, iNOS) were significantly up regulated. ***P<0.001 and Nrf-2 levels were down regulated $^{+++}$P<0.001 in AIL rats compared to control. Results are given as the mean ± SEM for 3 rats in each group (One way ANOVA followed by Tukey's multiple comparison).

ALP in serum when compared to control rats. Hepatoprotection was achieved by lutein pre-treatment followed by ethanol administration through significant decrease in the liver markers compared to AIL rats (**Figure 1**).

Lutein inhibits oxidative stress through reducing ROS, lipid peroxidation, protein carbonyl and sulfhydryls

The present study showed a significant increase (*P*<0.001) in oxidative stress markers along with ROS levels in ethanol-treated rats compared to control. Significant inhibition (*P*<0.001) in the oxidative stress status (LPO, protein carbonyls and sulfhydryls) was observed in rats pre-treated with lutein followed by ethanol administration when compared to ethanol treated rats alone (**Figures 2**-**3**).

Lutein enhances antioxidant status through Nrf-2 up regulation

Rats treated with ethanol showed significant decrease in antioxidant status compared to control rats. The protective effect of lutein on antioxidant enzymes was reflected with significant increase in antioxidant status compared to AIL rats (**Figure 4**). In addition, the master regulator of cytoprotective enzyme Nrf-2 was significantly down regulated (P<0.001) in ethanol treated rats; however the Nrf-2 protein expression was significantly enhanced (P< 0.001) in lutein pre-treatment followed by ethanol administration (**Figure 4**).

Lutein exerts anti-inflammation in alcohol-induced liver injury

Figure 5 shows a significant increase (*P*<0.001) in redox sensitive transcription factor NF-κB and its downstream target gene COX-2 and iNOS during ethanol treatment. Further, the expression of inflammatory proteins was significantly down regulated (*P*<0.001) during lutein pre-treatment. **Table 1** shows significant decrease (*P*<0.001) in pro-inflammatory cytokines during lutein pre-treatment compared to ethanol treated rats.

Discussion

Oxidative stress and its associated inflammatory activation play a major role in alcohol-induced liver injury. Oxidative stress during

Table 1. Lutein down regulates pro-inflammatory cytokines

Interleukins	Group I	Group II	Group III
TNF-α	53±2.08	47±2.13***	25±1.90+++
MCP-1	34±2.27	267±2.17***	75±2.12+++
IL-6	24±1.09	62±1.05***	36±1.91+++
IL-1β	26±1.56	66±2.01***	54±2.1+++

Interleukin levels (IL-1 β, IL-6, TNF- α, MCP-1) were expressed as pg/mg of protein. ***P<0.001, when compared to control group. +++P<0.001, when compared to AIL rats. Group I (control); Group II (AIL- rats); Group III (Lutein+ AIL- rats). (One way ANOVA followed by Tukey's multiple comparison).

alcohol-induced liver injury ensues as a result of imbalance between ROS generation and antioxidant scavenging mechanisms [24]. Enhanced ROS generation during alcohol exposure activates various signaling cascade and ultimately results in tissue damage [25]. Thus, for an efficient protection against alcohol-induced liver injury, there must be an effective barrier in combating the initially generated reactive species. The present study shows the protective role of lutein on alcohol-induced liver injury by virtue of its anti-oxidative and anti-inflammatory properties in its protective role in preventing alcohol-induced liver injury.

Alcohol-induced liver damage is associated with an increase in serum liver markers [26, 27]. The present study showed a significant increase in the liver markers such as ALT, AST, LDH and ALP in ALD rats; however, these levels were significantly down regulated during lutein treatment when compared to ethanol-treated rats. The results suggest a protective effect of lutein against alcohol-induced liver injury. Similar protective effect of lutein in reducing lung injury is consistent with the present study (Ammar et al., 2013). Evidence shows that ROS and downstream events which get activated play an important mediator in alcohol-induced liver injury [3, 26]. The present study shows alcohol-induced liver injury resulted in a significant increase in ROS generation and various oxidative stress mediators such as lipid peroxides, protein carbonyls and sulfhydryls. However, treatment with lutein offered protection against alcohol-induced liver injury by reducing oxidative stress and protection against lipid and protein on free radical mediated attack. The protection offered by lutein on free radical mediated oxidative stress has been demonstrated earlier by Li et al. [29]. As a defense against oxidative stress, cells have certain endogenous antioxidants as adaptive mechanisms to counteract stress. It is well known tenant that Glutathione, a non-enzymic antioxidant present in the cells which readily scavenge ROS. However, when the oxidative stress exceeds normal defense mechanisms, cells activate various enzymic antioxidants such as superoxide dismutase, Catalase, Glutathione-S-Transferase, Glutathione peroxidase etc. [30]. Superoxide dismutase functions to combat oxidative stress by scavenging superoxide anion radical to hydrogen peroxide. Further the formed H_2O_2 is effectively degraded into H_2O and O_2 by the enzyme Catalase, an iron containing hemoprotein [31, 32]. GPx functions with selenium ion as a cofactor and in conjugation with GSH protect the cells from toxic free radicals [33]. The present study showed that alcohol-induced liver injury resulted in an increased ROS generation with subsequent decline in antioxidant status. The present study results are in line with Lu et al. [34] clearly showing the imbalance in redox homeostasis in alcohol-induced liver injury. Lutein treatment completely restored the redox imbalance and increased the antioxidant status during alcohol-induced liver injury. The antioxidant effects of lutein have been largely studied, one such recent study which is consistent with the present results shows that, significant decrease in oxidative stress in retinal pigment epithelium; indicating its major role in age related macular degeneration [35]. Further, the current study shows significant up regulation in Nrf-2 levels during lutein treatment. This enhanced oxidative stress in alcohol-induced liver disease might be associated with a decline in master regulator of antioxidant enzymes, Nrf-2. Similar effect of lutein by enhancing Nrf-2 and inhibiting oxidative stress has been reported recently by Miyake et al. [36] in PC12D cells.

The protective role of lutein has been attributed to its antioxidant and other interrelated pathways. Recent studies have shown that one of the mechanisms by which lutein offers cytoprotection has been attributed to anti-inflammatory effects [37, 38]. The present study has shown that alcohol-induced liver injury resulted in significant up regulation of inflammatory pro-

teins NF-κB, COX-2, iNOS and pro-inflammatory cytokines (TNF-alpha, IL-6, MCP-1and IL-1β) when compared to control rats. In recent times, alcohol-induced liver injury and its associated inflammatory responses have been well studied [3, 39]. Overproduction of pro-inflammatory cytokines during liver injury has been reported in various studies [40-42]. However, rats treated with lutein followed by ethanol treatment showed protection by down regulating the inflammatory mediators. Hadad and Levy, [43] demonstrated the synergistic effect of lutein with other antioxidants in exerting anti-inflammatory effect against LPS induced inflammation. Further, Li et al. [44] showed that anti-inflammatory role of lutein in retinal ischemic/hypoxic injury by NF-κB pathway.

The present investigation highlights the protective role of lutein in alcohol-induced liver injury in Wister rats. The protection shown by lutein was associated with inhibition of oxidative stress and enhanced antioxidant status. Further, lutein ameliorated alcohol-induced liver injury induced inflammatory proteins and pro-inflammatory cytokines. Thus the study shows some novel insights on lutein mediated cytoprotection against alcohol-induced liver injury, which might be attributed to its antioxidant and its associated anti-inflammatory mechanisms.

Acknowledgements

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Disclosure of conflict of interest

None.

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