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Research Paper

Carotenoid lutein protects rats from paracetamol-, carbon tetrachloride- and ethanol-induced hepatic damage

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Abstract

Objectives Carotenoids are a class of natural fat-soluble pigments that are found in many fruits and vegetables. Consumption of a diet rich in carotenoids has been epidemiologically correlated with a lower risk for several diseases. In the present study the carotenoid lutein (3,3′-dihydroxy-β,ε-carotene) was evaluated for its hepatoprotective activity in rats.

Methods Paracetamol, 20% ethanol and carbon tetrachloride were used to induce liver toxicity.

Key findings Levels of serum glutamate oxaloacetate transaminase, serum glutamate pyruvate transaminase and alkaline phosphatases, which were increased in the serum, were found to be significantly reduced by the treatment of lutein in a dose-dependent manner, indicating that lutein may reduce the hepatotoxicity induced by these agents. Serum bilirubin was also significantly lower in lutein-treated groups compared with control. Increased lipid peroxidation, conjugated diene and hydroperoxides in the liver tissue produced by the administration of paracetamol were found to be reduced in the lutein-treated groups. Levels of antioxidant enzymes, like superoxide dismutase, catalase, glutathione peroxidase and glutathione, were found to be increased in lutein-treated groups compared with control group during alcohol- and CCl_4-induced liver toxicity. Hydroxyproline, which is an indicator of fibrosis in liver tissue, was high in the ethanol-treated control group. Hydroxyproline levels were decreased by simultaneous lutein administration.

Conclusions Histopathological evidence confirmed the protection offered by lutein from the tissue damage caused by hepatotoxins. The hepatoprotective action may be due to lutein's ability to scavenge reactive oxygen radicals.

Keywords antioxidants; carotenoids; hepatoprotection; lutein; oxidative stress

Introduction

Free radical-induced damage to mammalian tissues is believed to contribute to the ageing process and to the development of several degenerative diseases. Reactive free radicals react with polyunsaturated fatty acids (PUFA) of the membrane lipids and initiate lipid peroxidation.[1] The excessive lipid peroxidation caused by the free radicals leads to a condition of oxidative stress, which causes a variety of diseases, including liver diseases, which are a serious health problem. Several chemicals used in day-to-day life are well known for the induction of free radicals and cause liver damage through mechanisms of covalent binding and lipid peroxidation. Experimentally, hepatic injury can be produced by the administration of carbon tetrachloride, paracetamol, ethanol and thioacetamide, which can generate free radicals in the body. Antioxidants which scavenge free radicals could therefore reduce liver injury.[2]

Carotenoids are naturally occurring plant pigments that are involved in light-harvesting reactions and protect plant organelles from singlet-oxygen-induced damage. Many fruits and vegetables are rich sources of carotenoids.[3] Other sources for high lutein content are *Perillia frutescens*, *Spinacia oleraceae* and microalgae. Dietary carotenoids serve as antioxidants in the tissues and protect the body from oxidative damage.[4] Numerous epidemiological studies support a strong inverse relationship between consumption of carotenoid-rich fruits and vegetables and incidence of degenerative diseases.[5]

The carotenoid lutein (3,3′-dihydroxy-β,ε-carotene) is widely present in many green leafy vegetables, fruits and flowers,[6] and is commercially prepared from the marigold flower (*Tagetus erecta* L) in which it occurs at 1.5–1.8%. Lutein, zeaxathin and mesozeaxanthin are

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the only macular pigments and due to their extended conjugated structure have been shown to have significant antioxidant potential and a protective effect against the oxidative damage to macula induced by singlet oxygen produced by ultraviolet light. Lutein and zeaxathin are important carotenoid components in the human diet and several investigators have suggested that elevated intake of food rich in lutein is related to decreased macular degeneration and the risk of cataracts.[7] The recommended daily intake of lutein has been reported to be 6 mg, which has to be derived from either food or supplements.[8] Intake of foods rich in lutein and zeaxanthin is related to an increased level of carotenoids in the serum as well as in the macula.[9]

There are several multicentre studies currently underway aiming to establish more concrete data regarding the actual role of supplementation of lutein in preventing age-related macular degeneration (AMD).[10] Recent studies have suggested that lutein can reduce atherosclerosis and affords cardiac protection.[11] Lutein also reduces skin damage induced by ultraviolet rays.[12] However, there are no reports on the use of lutein in other degenerative conditions, such as liver damage, and in the present study we have looked into the use of lutein to reduce the hepatic toxicity produced by paracetamol, carbon tetrachloride (CCl_4) and ethanol administration in rats. This work will be strategic for determining effective means of resolving the clinical problems produced by these toxic agents.

Materials and Methods

Chemicals

Nitroblue tetrazolium (NBT), glutathione (GSH) and 5,5′dithiobis(2-niotrobenzoic acid) (DTNB) were purchased from Sisco Research Laboratories Pvt. Ltd, (Mumbai, India). Paracetamol was purchased from Variety Pharmaceuticals, Shornur, Kerala, India. Silymarin was purchased from Sigma-Aldrich (USA). Thiobarbituric acid was purchased from Himedia Laboratories (Mumbai, India). Span Diagnostics Ltd (Surat, India) supplied the biochemical kits for determining alkaline phosphatase, aspartate transaminase, alanine transaminase, total protein and bilirubin content. All other chemicals and reagents used were of analytical grade. Lutein isolated from marigold flowers (*Tagetes erecta* L.) (80%) was supplied by Omni Active Health Technologies Pvt. Ltd (Mumbai, India) as a 5% solution in sunflower oil.

Animals

Male Wistar rats (150–200 g) were purchased from the Small Animal Breeding Station, Kerala Agricultural University, Mannuthy, Kerala, India. The animals were maintained under standard environmental conditions and fed with standard rat feed (Sai Durga Food and Feeds, Bangalore, India) and water *ad libitum*. All the animal experiments were performed as per the instructions prescribed by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India, and implemented through the Institutional Animal Ethical Committee of the Research Centre.

Table 1 Experimental groups for paracetamol treatment

Group I	Without any treatment (normal)
Group II	Paracetamol + sunflower oil (control)
Group III	Paracetamol + lutein (50 mg/kg body weight)
Group IV	Paracetamol + lutein (100 mg/kg body weight)
Group V	Paracetamol + lutein (250 mg/kg body weight)
Group VI	Silymarin (100 mg/kg body weight)

Table 2 Experimental groups for carbon tetrachloride treatment

Group I	Normal without any treatment
Group II	CCl_4 + sunflower oil (control)
Group III	CCl_4 + lutein (50 mg/kg body weight)
Group IV	CCl_4 + lutein (100 mg/kg body weight)
Group V	CCl_4 + lutein (250 mg/kg body weight)

Table 3 Experimental groups for ethanol treatment

Group I	Normal without any treatment
Group II	Ethanol + sunflower oil (control)
Group III	Ethanol + lutein (100 mg/kg body weight)
Group IV	Ethanol + lutein (250 mg/kg body weight)

Dosing

Paracetamol treatment

Male Wistar rats were divided into six groups of six animals each as shown in Table 1. Groups II–VI received a single dose of paracetamol (3 g/kg body weight, 2 ml, oral) to produce acute hepatotoxicity. Lutein and silymarin treatment (oral) was started 7 days prior to paracetamol administration. All animals were sacrificed 24 h after paracetamol administration.[13]

Carbon tetrachloride treatment

Male Wistar rats were divided into five groups of six animals each as shown in Table 2. Groups II–V received a single dose (i.p.) of CCl_4 (2.5 ml/kg body weight, diluted with an equal volume of liquid paraffin) to produce acute hepatotoxicity. Lutein treatment (oral) was started 7 days prior to CCl_4 administration. All animals were sacrificed 24 h after CCl_4 administration.[14,15]

Ethanol treatment

Male Wistar rats were divided into four groups of eight animals each, as shown in Table 3. Groups II–IV received 20% ethanol, 95% ethanol diluted to 20% with distilled water (7.5 g/kg body weight, 5 ml in the forenoon and 5 ml in the afternoon, oral) and respective doses of lutein (1.5 ml, orally) dissolved in sunflower oil for 6 months.[16] At the end of 6 months all animals were sacrificed.

Analyses

Hepatic injury

Blood was collected by cardiac puncture. Serum was separated and used to determine the activities of serum glutamate pyruvate transaminase (SGPT), serum glutamate oxaloacetate transaminase (SGOT),[17] alkaline phosphatase (ALP)[18] and bilirubin[19] using commercial kits.

Table 4 Effect of lutein administration on serum parameters in carbon tetrachloride treated rats

Groups	ALP (KA units)	SGOT (IU/l)	SGPT (IU/l)	Bilirubin (mg/dl)
Normal	26.22 ± 6.56	97.60 ± 13.06	43.33 ± 4.13	0.596 ± 0.48
Vehicle control	125.64 ± 40.40***	541.91 ± 130.50***	626.4 ± 88.2***	2.00 ± 0.50***
Treated 50 mg/kg body weight	72.20 ± 12.19**	368.63 ± 217.87*	328 ± 51.92***	1.06 ± 0.52**
Treated 100 mg/kg body weight	45.98 ± 17.41***	264.81 ± 57.22**	218.20 ± 61.04***	0.60 ± 0.39***
Treated 250 mg/kg body weight	24.20 ± 2.29***	151.05 ± 27.43***	180 ± 74.83***	0.30 ± 0.27***

Lutein treatment for 7 days, followed by CCl_4 treatment, and sacrifice 24 h later. SGPT, serum glutamate pyruvate transaminase; SGOT, serum glutamate oxaloacetate transaminase; ALP, alkaline phosphatases. Values are mean ± SD, $n = 6$, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Table 5 Effect of lutein administration on serum parameters in ethanol treated rats

Groups	ALP (KA units)	SGOT (IU/l)	SGPT (IU/l)	Bilirubin (mg/dl)
Normal	25.01 ± 2.01	32.71 ± 2.01	35.00 ± 2.01	0.54 ± 0.20
Vehicle control	85.93 ± 12.93***	238.56 ± 21.22***	117.00 ± 21.96***	1.25 ± 0.32***
Treated 100 mg/kg body weight	31.55 ± 12.77***	126.29 ± 14.77***	54.37 ± 16.35***	0.73 ± 0.17***
Treated 250 mg/kg body weight	22.53 ± 3.16***	84.97 ± 5.16***	37.00 ± 10.59***	0.60 ± 0.23***

Duration of ethanol and lutein treatment = 6 months. SGPT, serum glutamate pyruvate transaminase; SGOT, serum glutamate oxaloacetate transaminase; ALP, alkaline phosphatases. Values are mean ± SD, $n = 6$, $^{***}P < 0.001$.

Table 6 Effect of lutein administration on serum parameters in paracetamol treated rats

Groups	ALP (KA units)	SGOT (IU/l)	SGPT (IU/l)	Bilirubin (mg/dl)
Normal	35.37 ± 0.70	93.66 ± 15.10	40.00 ± 11.50	0.75 ± 0.05
Vehicle control	127.15 ± 3.80***	385.33 ± 16***	564 ± 196.20***	2.80 ± 0.64***
Treated 50 mg/kg body weight	101.60 ± 21.70*	184 ± 68.40**	164.00 ± 63.80***	1.52 ±0.26***
Treated 100 mg/kg body weight	93.78 ± 6.70**	116.00 ± 49.29***	100 ± 48***	0.80 ±0.17***
Treated 250 mg/kg body weight	63.42 ± 19***	93.33 ± 18***	46.6 ± 10.30***	0.58 ± 0.09***
Treated silymarin (100 mg/kg body weight)	35.77 ± 0.81	94.00 ± 7.00	43.8 ± 12.70	0.75 ± 0.60

Lutein treatment for 7 days, paracetamol treatment 24 h before sacrifice. SGPT, serum glutamate pyruvate transaminase; SGOT, serum glutamate oxaloacetate transaminase; ALP, alkaline phosphatases. Values are mean ± SD, $n = 6$, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Oxidative stress

A 25% (1 volume of liver with 3 volumes of buffer) liver homogenate was prepared in 0.1 M Tris HCl. Lipid peroxidation in the liver homogenate was performed by the thiobarbituric acid method.[20] Tissue hydroperoxides and conjugated dienes were determined by the modified method of John and Steven.[21]

Antioxidant enzymes and glutathione

Liver homogenate was centrifuged at 10 000 rpm for 30 min at 4°C and the supernatant was used to estimate levels of antioxidant enzymes. Superoxide dismutase activity was measured by the NBT reduction method of McCord and Fridovich.[22] Catalase activity was estimated by the method of Aebi,[23] by measuring the rate of decomposition of hydrogen peroxide at 240 nm. Glutathione (GSH) levels were assayed by the method of Moron *et al*.,[24] based on the reaction with DTNB. The assay of glutathione peroxidase was performed by the method of Hafeman *et al*.,[25] based on the oxidation of GSH in the presence of hydrogen peroxide.

Liver homogenate was hydrolysed using 6 N HCl and the hydroxyproline level was estimated by the method of Newman and Logan.[26] A small piece of liver was excised, washed in ice-cold saline and a small portion was fixed in 10% formalin for histopathological analysis using haematoxylin and eosin stain.

Statistical analysis

The values were expressed as mean ± SD. Statistical evaluation of the data was performed by one-way ANOVA followed by Tukey's test (post-hoc) using the InStat 3 software package.

Results

Effect of lutein administration on hepatic serum markers

The levels of serum ALP, SGOT and SGPT were found to be significantly elevated in paracetamol-, CCl_4- and ethanol-treated animals, indicative of hepatic damage. Administration

Table 7 Effect of lutein administration on liver antioxidant enzymes and glutathione in carbon tetrachloride- and ethanol-treated rats

Groups	SOD (U/mg protein)	Catalase (U/mg protein)	GPx (U/mg protein)	GSH (nmol/mg protein)
Carbon tetrachloride				
Normal	1.05 ± 0.38	6.33 ± 0.48	8.60 ± 1.39	14.10 ± 1.43
Vehicle control	0.34 ± 0.20**	1.00 ± 0.20***	6.90 ± 0.58	2.90 ± 0.74***
Treated 50 mg/kg body weight	1.25 ± 0.20***	2.98 ± 0.82**	17.92 ± 2.02***	11.20 ± 2.16***
Treated 100 mg/kg body weight	2.11 ± 0.37***	6.32 ± 1***	25.18 ± 2.71***	16.20 ± 0.83***
Treated 250 mg/kg body weight	2.69 ± 0.42***	9.10 ± 1.90***	28.49 ± 2.52***	21.20 ± 3.96***
Ethanol				
Vehicle control	0.83 ± 0.01*	2.43 ± 0.95***	3.10 ± 0.75***	3.66 ± 0.50***
Treated 100 mg/kg body weight	1.22 ± 0.19***	9.50 ± 2.10***	10.59 ± 2.40***	9.85 ± 0.69***
Treated 250 mg/kg body weight	1.73 ± 0.23***	12.00 ± 2.33***	13.74 ± 3.36***	17.14 ± 1.21***

SOD, superoxide dismutase; GPx, glutathione peroxidase; GSH, glutathione. Values are mean ± SD, $n = 6$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

of lutein along with the hepatotoxins significantly decreased the serum levels of these markers in a dose-dependent manner (Tables 4–6).

Similarly serum bilirubin level, which was increased by treatment with hepatotoxins, was found to be significantly lowered by treatment with lutein in a dose-dependent manner (Tables 4–6). The levels of SGOT, SGPT and bilirubin in silymarin- and 250 mg/kg lutein-treated animals were found to be almost identical. On the other hand, serum ALP levels in the lutein treated (250 mg/kg) group were higher than those of the silymarin-treated group (Tables 4–6).

Effect of lutein administration on liver antioxidant enzymes and glutathione

The activities of liver antioxidant enzymes were significantly decreased in the CCl_4- and ethanol-treated control groups compared with normal animals, and the levels of antioxidant enzymes were significantly elevated in the lutein-treated groups (Table 7). Similarly, the levels of GSH, which were low in the CCl_4- and ethanol-treated groups and were significantly increased when also dosed with lutein (Table 7).

Inhibition of paracetamol-induced oxidative stress by lutein

There was a significant elevation of lipid peroxidation, formation of conjugated diene and hydroperoxides in the animals treated with paracetamol alone compared to normal rats, and the levels of these oxidative stress markers were significantly reduced to normal levels in the lutein-pretreated groups in a dose-dependent manner (Figure 1).

Lipid peroxidation in the control group was 4.06 ± 0.47 nmol/mg protein and in the 50, 100 and 250 mg/kg body weight lutein-treated groups they were 3.30 ± 0.73, 3.40 ± 0.59 and 2.53 ± 0.83 nmol/mg protein, respectively. The normal level was found to be 2.13 ± 0.46 nmol/mg protein. Conjugated diene formation in the control group was 1.50 ± 0.1 mmol/100 g tissue and in 50, 100 and 250 mg/kg body weight lutein-treated groups it was 0.95 ± 0.03, 0.88 ± 0.05 and 0.81 ± 0.10 mmol/100 g tissue, similar to that of untreated normal animals (0.79 ± 0.20 mmol/100 g tissue). Formation of tissue hydroperoxides in the control group was 8.60 ± 0.20 mmol/100 g tissue and there was a significant reduction with 50, 100 and 250 mg/kg body weight lutein pre-treatment (7.05 ± 0.47, 4.93 ± 0.52 and 4.18 ± 0.54 mmol/100 g, respectively).

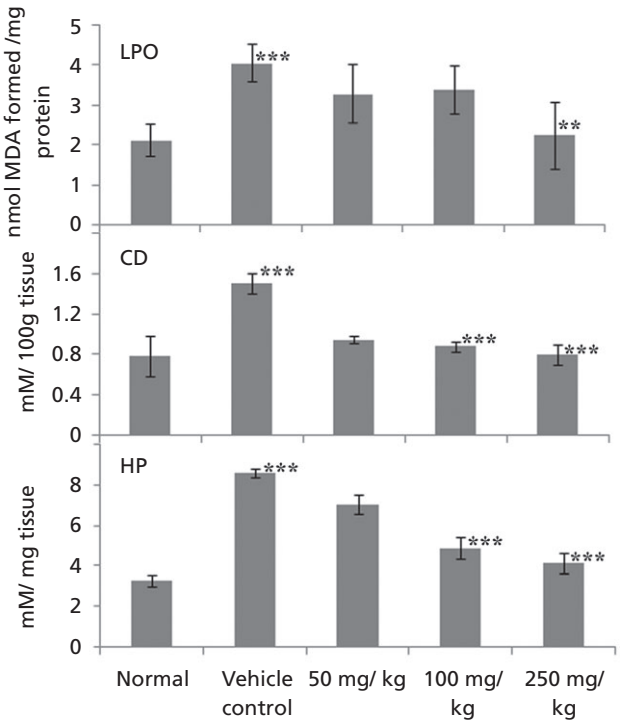

Figure 1 Effect of lutein on oxidative stress markers in paracetamol treated rats. LPO, lipid peroxidation; CD, conjugated diene; HP, hydroperoxides; MDA, malondialdehyde. **$P < 0.01$, ***$P < 0.001$.

The hydroxyproline level, which is an indicator of collagen content during fibrosis, was found to be increased to 5.57 ± 2.02 μg/mg protein in the ethanol-treated control group from the normal value of 2.54 ± 0.04 μg/mg protein. Treatment with lutein significantly reduced the hydroxyproline contents in the liver to 1.74 ± 0.41 μg/mg protein.

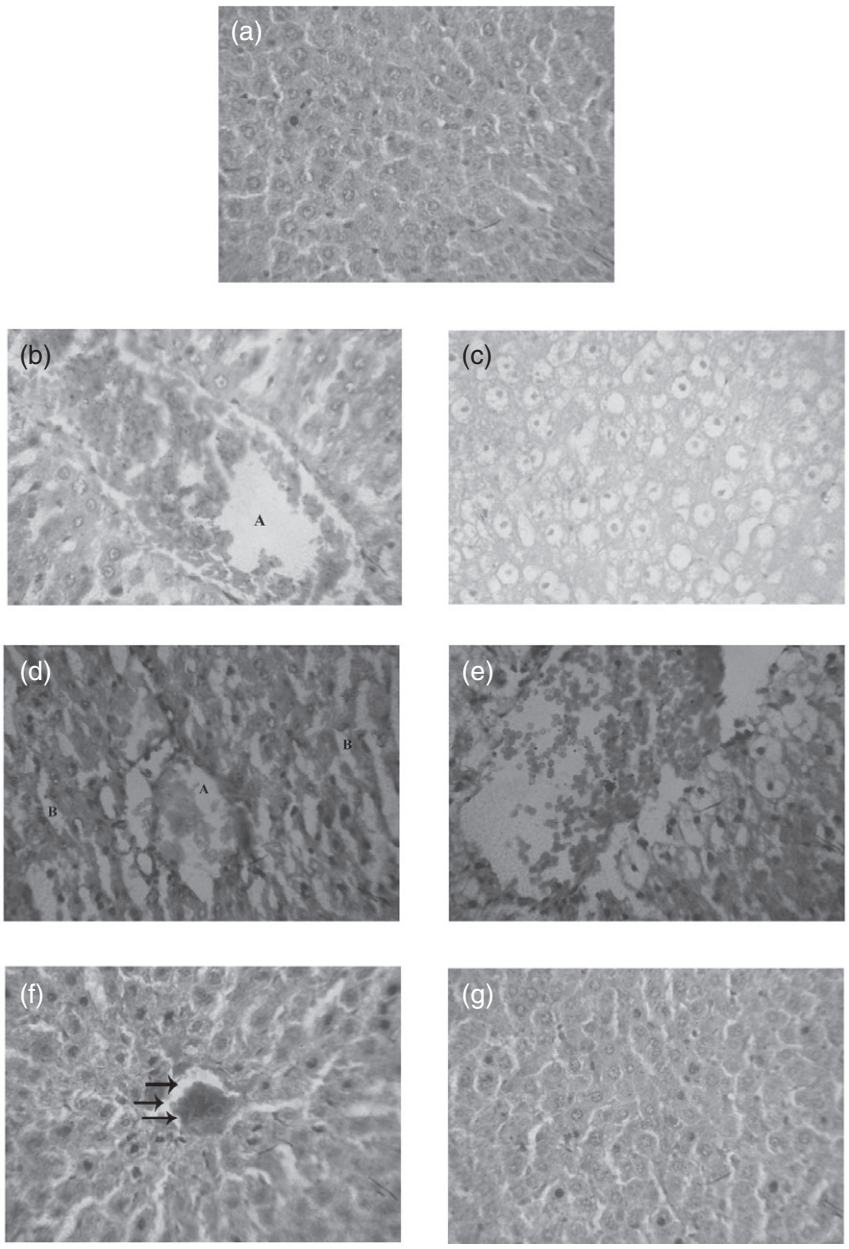

Figure 2 Histopathology of rat liver. Magnification, 400×. (a), Normal; (b) paracetamol + sunflower oil treated (A, dilated central vein); (c) paracetamol + 250 mg/kg body weight lutein treated; (d) CCl_4 + sunflower oil treated (A, portal areas showing hemorrhage and congestion; B, necrotic changes of hepatocytes); (e) CCl_4 + 250 mg/kg body weight lutein treated; (f) ethanol + sunflower oil treated (arrows indicate proliferative fibrosis); (g) ethanol + 250 mg/kg body weight lutein treated.

Effect of lutein administration on liver histopathology

Histopathological analysis confirmed the protective effect of lutein against the actions of the hepatotoxins used here. There was only a mild change in the paracetamol-treated control group when compared with normal liver tissue, which may be due to the short duration of exposure to paracetamol (Figure 2b and c). In the CCl_4-treated control groups the liver section showed haemorrhage and congestion in the portal area, hepatocytes showed necrotic changes, sinusoidal spaces were congested and there were areas of haemorrhage and vacuolation. The tissue damage was found to be less in the lutein-treated group (Figure 2d and e). In the ethanol-treated group, there was a proliferative fibrosis, which was absent in the section of the lutein-treated group indicating its protective role (Figure 2f and g).

Discussion

As liver is the most important organ that metabolises xenobiotics, it is highly affected by toxic agents. Paracetamol is a common antipyretic agent, which is safe in therapeutic doses

but at toxic doses can produce fatal hepatic necrosis in humand, rats and mice.[27] Paracetamol is mainly metabolised in the liver to excreatable glucuronide and sulphate conjugates.[28] The hepatotoxicity of paracetamol has been attributed to the formation of toxic metabolites when a part of the molecule is activated by hepatic cytochrome P-450[29] to a highly reactive metabolite, *N*-acetyl-*p*-benzo-quinoneimine.[30] This toxic metabolite covalently interacts with thiol groups in the proteins of liver and consequently stimulates lipid peroxidation.[31] The lipid peroxidative degradation of biomembrane is one of the principal causes of hepatotoxicity induced by toxins. Since lipids constitute nearly 60% of the compounds in biomembranes, major perturbation is bound to affect the structure and function of the cell.

Carbon tetrachloride intoxication in rats is widely used to study necrosis and steatosis of the liver.[32] The initial event of CCl_4-induced free radical generation is a carbon–halogen bond cleavage, probably through one-electron reduction of CCl_4 aided by a particular ferrous cytochrome P450. Chloride ion and trichloromethyl free radical $\cdot CCl_3$ are the major initial products. CCl_3 is converted into $\cdot CCl_3O_2$ through its reaction with molecular oxygen. Like the above-mentioned free radicals, lipid peroxidation is initiated by the interaction of this reactive free radical CCl_3O_2 with polyunsaturated fatty acids (PUFA) of the membrane lipids. The excessive lipid peroxidation caused by these free radicals leads to a condition of oxidative stress, which results in the accumulation of malondialdehyde (MDA). MDA is one of the end-products of the lipid peroxidation process and oxidative stress.[33]

Ethanol is well known as an agent that produces extensive changes in the liver. Liver, which can metabolise ethanol, shows a profound alteration in intermediary metabolism when subject to high doses or with lengthy exposure. The adverse effects are ultimately understandable as consequences of the rate of NADH production and the reduction of the cytoplasmic free $[NAD^+]/[NADH]$ ratio by the reactions of alcohol dehydrogenase and aldehyde dehydrogenase.[34] Activation of lipid peroxidation is one of the manifestations of the action of ethanol, which then causes membrane damage.

Cytochrome P450 II EI serves as a catalyst in the production of ROS due to ethanol metabolism. The free radicals formed by paracetamol, CCl_4 and ethanol damage the liver cell, causing lipid peroxidation, which leads to membrane lesions. During liver injury, the transport function of the hepatocytes is disturbed, resulting in the leakage of plasma membrane,[35] thereby causing an increased enzyme level in the serum. During oxidative stress, the antioxidant enzymes help to overcome cellular damage by scavenging the free radicals and reducing their levels in tissue injury.

Results in this study clearly indicate that lutein mitigates the hepatotoxicity produced by paracetamol, CCl_4 and ethanol in rats. Lutein reduces the liver enzymes in serum and increases antioxidant enzyme and glutathione levels in liver tissue, effectively demonstrating its protective effect.[36]

The antioxidative activity of carotenoid molecules in biological systems has been investigated recently and exploited to prevent macular degeneration. Increased intake of carotenoids like lutein and zeaxanthin reduces the risk of cardiovascular and ocular diseases. In chronic disease conditions, like AMD, the lutein supplement was 10 mg for 12 months; in cataracts there was a supplemented 15 mg daily dose given for 2 years,[37] and in retinitis pigmentosa, 20 mg per day was given for 6 months.[38]

Carotenoids may reduce lung carcinogenesis because of their antioxidant properties. However, few studies have found a direct relationship between intakes of β-caroteinoids and lung cancer risk.[39] Similarly, genotoxic carotenoids and their breakdown products have also been reported.[40]

Lutein is one of the ten phytochemicals identified by the US Food and Drug Adminstration as GRAS (generally regarded as safe) for nutritional supplements. Earlier studies in our laboratory have clearly established that lutein is non-toxic.[41,42] The data presented in this study support the hypothesis that lutein may protect liver from various other toxic substances by effectively preventing the oxidative stress[43] induced during liver damage, something that is seen from the decreased levels of enzymes and oxidative markers. Our preliminary studies indicate that lutein also may be inhibiting the activity of the cytochrome P450 enzyme and this may be a major mechanism of action of lutein in preventing hepatic damage induced by chemicals. Many antioxidant enzymes are under the transcriptional regulation of the Nrf2–keap1 signalling pathway.[44] As lutein is non-toxic, use of this agent to reduce hepatotoxicity, as well as in the treatment of other degenerative diseases, needs further evaluation.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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